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Mitochondria-Targeted Hydrogen Sulphide Donors Protect Renal Cells From Hypoxia Re-Oxygenation Injury

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

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(Integrated Article)

by

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Abstract

Introduction: Hypoxia re-oxygenation in kidney transplantation affects the outcome. Hydrogen sulphide (H_2S), (the newest Gasotransmitter), showed significant protective effect on renal transplantation induced IRI. Our objective was to determine if new mitochondria targeted H_2S donor molecule (AP39) would be more efficacious in protecting renal cells against IRI compared to the non-specific H_2S donor molecule GYY4137. We hypothesized that AP39 would be more potent.

Methods: in vitro porcine kidney tubular epithelial cells (LCC-PK1) were exposed to warm hypoxia, without treatment (Control), with AP39 or GYY4137 followed by re-oxygenation.

Results: 200nM of AP39 protected the cells and maintained a high viability. AP39 was superior to GYY4137. Significant reduction of Apoptosis and ROS were noted in AP39 samples when compared to control. Both BCL2 and BID genes did not show any significant changes, compared to the control and GYY4137 samples.

Conclusion: AP39 is protective and superior to GYY4137 in renal IRI.

Keywords

Hydrogen sulphide, Gasotransmitters, Mitochondria targeted donors, AP39, GYY4137, Ischemia reperfusion injury, Kidney transplantation, Reactive oxygen species, Hypoxia re-oxygenation, Mitochondria, Apoptosis, Renal tubular epithelial cells, Apoptosis related genes.

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1. Introduction

1.1.End Stage Renal Disease

Chronic kidney disease (CKD) - defined as a kidney disease affecting its function and lasting more than 3 months- is a major public health problem. Around 7.8 per 1000 patient years in the USA develop chronic kidney disease (Kurella & Chertow, 2005). The prevalence of chronic kidney disease in Canada, USA and Europe is nearly about the same being around 10 to 11%. Late stages of CKD increase the risk of dying from a cardiovascular disease to 4 times the average risk. The late stage of chronic kidney disease is known as end stage renal disease (ESRD). Such stage of kidney disease requires renal replacement therapy (RRT) as dialysis or if possible kidney transplantation.

The risk of cardiovascular related death in ESRD goes up to 100 times the average risk of the general population (Baigent, Burbury & Wheeler, 2000). Patients with ESRD have many symptoms that affect their quality of life and increase their lifelong morbidity. Around 50 to 90 % suffer from fatigue, pruritis, anorexia, pain or constipation. While around 25% to 45 % suffer from sleep problems, anxiety, dyspnea, restless leg syndrome, dyspnea or depression (Murtagh, et al., 2007).

Many studies compared renal transplantation to dialysis and the vast majority showed significant advantages of transplantation over dialysis (Tonelli, et al., 2011). Transplantation is favorable in terms of mortality (Sezer, et al., 2004; Chauveau, et al., 2009), morbidity, hospitalization, infections, cardiovascular events (Brunkhorst, et al., 2003; Ward, 2000) and overall quality of life (Bajardi, et al., 2003). Transplantation also is the most cost-effective treatment for ESRD (Glanton, et al., 2003). The costs of treating patients living on a transplant are indeed by one-third to one-quarter lower than those spent on dialysis patients (Bruno, et al., 2003).

Not to mention hypotension during dialysis, the dialysis access (catheter) site complications, the electrolyte imbalance and the fatigue after dialysis sessions. Also missing a session or two of dialysis has serious consequences that might be life threatening, like fluid overload leading to pulmonary edema, electrolyte imbalance (hyperkalemia) leading to arrhythmias or uremic encephalopathy with coma.

Despite being a survival necessity in these patients, dialysis does not prevent or decrease the other complications of ESRD like chronic anemia, bleeding tendency, metabolic bone disease (vitamin D deficiency), and immune suppression. All these draw backs and failure of dialysis made kidney transplant surgery the only chance for cure and the best treatment option.

1.2.Kidney Transplantation

Kidney transplantation as discussed previously offers the best survival, disease free survival and the most cost effective treatment for patients compared to dialysis (Tonelli et al., 2011). However the surgical procedure of kidney transplantation is considered a high risk surgery and this risk limits its validity for some patients who have many comorbidities. A long list of surgical complications with a significant number of which being a serious complication with a considerable mortality or morbidity risks. Also the need for immunosuppressing medications that has a long list of side effects including risk of cancers, infections and coronary artery disease adds to the limitations. One of the most disappointing truths about kidney transplantation is its limited durability, especially in the scenario were the transplanted kidney is from a deceased donor, which accounts for nearly seventy percent of the kidney transplants in the United States and Canada in the current practice.

Many factors affecting the graft (transplanted kidney) survival were studied with many efforts made to prolong graft survival. Among these factors are some uncontrollable variables like donors' characteristics and some of the recipients' demographics.

However, among the factors that are adjustable are the ischemia time of the kidney before being transplanted in the recipient. Limiting the time of ischemia limits the injury induced by ischemia and the injury induced by reperfusion after a long period of ischemia. However, due the shortage of organs and the long waiting lists of donors the need for more donors had led to the acceptance of donors after cardiac death with long ischemia times.

Deceased donors are now constituting the majority of kidney donors for transplantation in many countries. According to the organ procurement and transplantation network (OPTN) data, deceased donors were the source of 8594 kidneys compared to 5818 from living donors in 2014. The available types of donors for kidney transplantation are shown in Figure 1.

Donors who meet the criteria for brain death are considered donors after brain death (DBD). Donation after cardiac death (DCD) are donors who suffer untreatable brain injuries but do not fulfill the criteria for brain death who arrest after withdrawal of ventilator and blood pressure support.

As the control of ischemia time in the deceased cardiac donors is limited by the donors condition, organs from such donors showed a higher incidence of the injury known as ischemia reperfusion injury (IRI) which eventually can affect the short and long term graft survival. (Ponticelli, 2014)(Tojimbara et al., 2007)

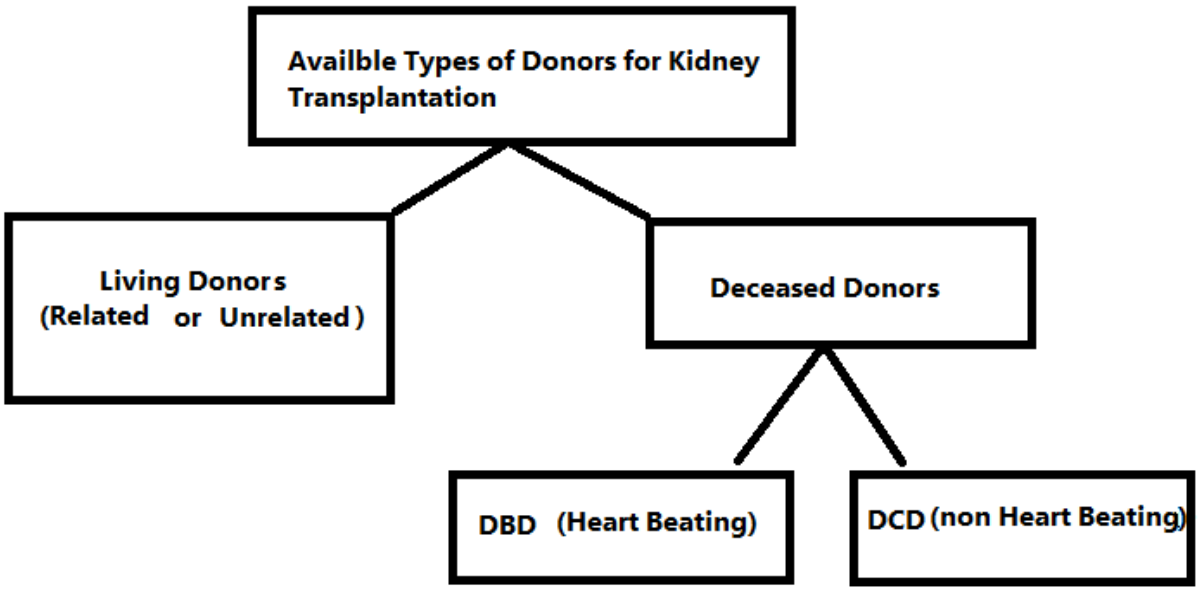

Figure 1

The available types of donors for kidney transplant. DBD: donation after brain death. DCD: donation after cardiac death.

1.3.Ischemia reperfusion Injury and Delayed Graft Function (DGF)

1.3.1.Ischemia Reperfusion Injury

One of the earliest descriptions of ischemia reperfusion injury (IRI) in whole organ systems was in 1975 by Cerra et al. (1975) who demonstrated its importance in canine model in myocardial pedicles. They examined the extent of reperfusion injury and found that increased ischemia times were associated with increased sub endothelial hemorrhagic necrosis. IRI is a pathophysiological process that is inevitable in kidney transplantation, and as will be discussed later, is an important contributor to peritransplant renal injury.

It is not only limited to transplantation, as it also occurs in a wide variety of disease processes like myocardial ischemia (Yellon & Hausenloy, 2007) pre renal-renal injury, ischemic cerebrovascular accidents, and vaso-occlusive crises of sickle cell anemia to name a few (Wallace & Linden, 2010). Ischemia of the transplant organ is quite unique as it has components of both warm and cold ischemia, depending upon the organ donor type and can also be quite variable in terms of the amount of time that a graft may be exposed to the specific injury (Eltzschig & Eckle, 2011). Prolonged IRI can have post-transplant squale, with the primary effects being delayed graft function (DGF), which can be deleterious to the graft in the long term.

1.3.2.Delayed Graft Function (DGF)

DGF is the most common complication in the early in-hospital post-transplant period with an incidence of 2% to 50% following kidney transplantation (Gjertson, 2000; Ojo, et al., 1997; Perico, et al., 2004, Koning, et al., 1995). The exact definition for DGF has been debated by experts for some time. According to one review, there were at least 18 different definitions used in the literature from 1984 to 2007 in 65 published studies (Yarlagadda, et al., 2008).

Most common definition used and most accepted is the need for dialysis in the first week after transplantation. Other definitions used are ATN proven on biopsy (Preidler, et al., 1996; Sadeghi, et al., 2006) failure of serum creatinine to drop 10% or more a day for three consecutive days, serum creatinine decreasing by <1.1mg/dl in the first 5 days post-transplant(El-Maghraby et al.,

2002; Boom, et al., 2000) or a serum creatinine level of 2.5mg/dl or more for up to day 7 post-transplant (Turkowski-Duhem et al., 2005).

Kidneys which do not seem to immediately function optimally but still not dysfunctional enough to meet one of the criteria above for DGF are labeled as slow graft functioning (SGF) (Humar et al., 2002).(Le Dinh et al., 2012). Although some literature require the exclusion of other causes of dysfunction in the definition of DGF (other than rejection or IRI), the development of DGF may be multifactorial. Rejection, anastomotic complications, vascular thrombosis, fulminant disease recurrence and drug nephrotoxicity are examples of these other causes of DGF. The typical and most common cause for DGF, however, is IRI which leads to acute tubular necrosis (ATN) as the typical histological finding (Lechevallier, 1998; Huraib, 2002; Yarlagadda, 2008). Additional evidence suggests that the greater the ischemic time before cold preservation (warm ischemia) the kidney goes through, the higher the rate of irreversible cell damage which eventually reduces graft survival (Siedlecki, Irish & Brennan, 2011). For that reason all efforts should be made to decrease IRI, being the leading cause for DGF. The pathophysiology behind IRI causing DGF involves the activation of the immune system which will be discussed in the sections below. This early immune system activation may also be the early instigator for acute and even chronic rejection process.

1.4.IRI Pathophysiology

Ischemia reperfusion injury is a result of multiple connected cascades initiated by ischemia and directly related to the time of ischemia leading to decreased oxygen tension in the tissue (hypoxia). The shortage of oxygen in ischemia is sensed by the polyl hydroxelase (PHD) enzyme, as they require O_2 as a co factor. The shortage of O_2 leads to inhibition of PHD. Indirectly the inhibition of PHD leads to the activation of the transcriptional factor, hypoxia-induced factors (HIF) and nuclear factor kB (NF-kB) (Eltzschig, 2011).

The activation of HIF during hypoxia stimulates the production of glycolysis enzymes leading to the switch to glycolysis as a source of energy instead of the amino acid oxidation energy source. This leads to the utilization of the cytosolic glycogen, releasing less amounts of ATP as well as the formation of lactic acid as a result of the anaerobic respiration (Eltzschig, 2011).

The subsequent depletion of ATP leads to the inhibition of Na/K ATP dependent channels leading to the accumulation of sodium within the intracellular space. Excess amounts of sodium inside the cell leads to the influx of water inside the cell leading to cell swelling and with more ischemia time more swelling involving the cell organelles, which ultimately lead to cell membrane rupture and cell death (necrosis).

Apoptosis is another manner in which cells die as a result of ischemic injury. The leak of pro-apoptotic molecules from the mitochondria activates cascades of events leading to apoptosis. Opposite to necrosis, apoptosis is characterized by cell shrinkage, cell membrane blebbing, nuclear fragmentation and loss of mitochondrial membrane potential (Hotchkiss, et al., 2009).

The cell injury from ischemia might be reversible as long as it does not exceed a limit of intensity and duration. Once it exceeds that limit, restoration of blood (reperfusion) surprisingly instead of reversing the damage, induces more injury. The reperfusion injury is the results of many events, most of which were preconditioned during the ischemic phase and showed its potency mainly during the reperfusion phase (Eltzschig & Eckle, 2011). These events are mainly:

a. The "No reflow phenomena": This phenomena point to the fact of non-restoration or blockage of the blood flow to variable parts of the ischemic organ (Cheadle, et al., 2011; Leaf, 1973; Summers & Jamison, 1971). During hypoxia, adenylate cyclase activity and intracellular cyclic Adenosine monophosphate (cAMP) level drop significantly. This results in what is called the endothelial dysfunction syndrome (EDS) (Brodsky, 2002). This syndrome is characterized by endothelial cell (EC) swelling, expression of adhesion molecules and impaired EC barrier function increasing the vascular permeability (Ogawa, et al., 1992; Ogawa, 1990). The resulting leaky vessels then allow fluid and cells to infiltrate the surrounding tissues (Flores, et al, 1972; Kelly, et al., 1994; Ogawa, et al., 1992). The endothelial syndrome is the major contributing pathophysiology leading to the no reflow phenomena: The swelling of the ECs together with the tissue edema (caused by the leakage of fluids and cells through the over-permeable vessel walls) causes a luminal and extra luminal narrowing of the affected vessels respectively. This narrowing in the end arterioles is enough to block the perfusion to the tissue area supplied by that arteriole. The expression of adhesion molecules (as part of the EDS) promotes the adhesion of

platelets and inflammatory leukocytes to the endothelium causing micro thrombi, again blocking the terminal feeding vessels.

b. Sterile inflammation and the immune system: Despite the fact that IRI typically occurs in a sterile environment, activation of innate and adaptive immune system contributes to a significant part of the injury.
   Cell death occurring during ischemia and during reperfusion releases multiple cell components and intracellular products into the extracellular space. These are potent chemokines and cytokines that initiate multiple cascades leading to a destructive immune response presented by a sterile type of inflammation. Early in reperfusion, once the circulation reaches parts of the ischemic organ, these chemokines and cytokines diffuse through the circulation recruiting inflammatory cells and activating more of the pro-inflammatory cascades. Among the early components of the immune system activated are the pattern-recognition receptors known as toll-like receptors (TLRs) (Thurman, 2003). The TLRs once activated, they initiate a signaling cascade of cytokines expression that facilitates the bridging of the innate and adaptive immune systems (Carroll & Holers, 2005; Chen & Nunez, 2010a). The over expression of adhesion molecules like E-selectin and intercellular adhesion molecule-1 (ICAM-1) on ECs (as part of the EDS during the ischemic phase) facilitates the adhesion of the inflammatory cells brought up by reperfusion. These adhered inflammatory cells find its way infiltrating the tissue to cause inflammation.

c. The cellular cytotoxic effects of reperfusion: At the cellular level, reperfusion restores the pH of the cells which was found to be cause more injury through different mechanisms: 1- Restoration of pH leads to re-opening of the mitochondrial permeability transition pores (MPTPs). The MPTPs close in ischemia induced acidosis (low pH) as a protective mechanism. Once the pH is restored in reperfusion, it facilitates the injurious uncontrolled influx of previously accumulating cytosolic Ca^{++} and ROS into the mitochondria, causing an irreversible mitochondrial damage. 2- Activates Na/ Ca++ exchange, leading to more accumulation of intracellular Ca++ and cell death. 3- Phospholipases and other destructive enzymes released during ischemia are inactivated in acidotic environments and reactivated once acidosis is resolved

1.5.IRI as a sterile inflammation and the immune system

The recipient immune system is a constant threat to graft survival despite the recent advances in pre transplant immune workup, preparation, prevention and treatment of the sterile type of inflammation seems to play a significant role in limiting IRI. Interestingly, IRI induced inflammation and infection induced inflammation share some similar mechanisms (Chen & Nunez, 2010b). Similar to ligands binding to Toll like receptors (TLR) in initiating the inflammatory response when it detects a microorganism, there are special damage associated molecular patterns (DAMPs) that act as ligands in cases of the sterile inflammation in IRI that also bind to and activate TLR and their downstream effector pathways. Some examples of endogenous DAMPs which can activate TLR include high-mobility group box 1 (HMGB1), Heat shock protein (HSP) fibronectin, hyaluronan, and biglycan are examples of DAMPs that can activate TLR (Wu, et al., 2007).

These endogenous DAMPs are released from the dead or dying cells into the extracellular space (Iyer, et al., 2009; McDonald, et al., 2010). Activation of TLR leads to the release of cytokines, chemokines and other inflammatory mediators which recruit more inflammatory cells (Wu et al., 2007). Recruited inflammatory cells release more ROS leading t further tissue inflammation and subsequent damage. While TLR3 was activated by RNA released from dead cells (Cavassani et al., 2008), both TLR2 and TLR4 TLR2 was proven to be over expressed in response to hypoxia (Kuhlicke, et al., 2007) (oxidative stress) as well as enhance the response of cells of the innate immune system. In a TLR4 -/- chimeric mice, IRI was proven to be less in terms of serum creatinine and histological findings (Wu, et al., 2007).

Neutrophils - representing the humeral part of the innate immune system - are the first to present in the inflammation site and to modulate the inflammation following transplantation and most if not all inflammatory reactions. As part of the adaptive immune system, both T cells and B cells have been shown to play a role in IRI. Trials evaluating the role of T cells and B cells in IRI demonstrated that either the absence of one or both may be protective against IRI (Mehrabi, 2007).

The role of T-cell activation is important in IRI. In fact with respect to CD4 T-cells, the Th 1 pathway was found to participate in IRI while Th2 pathway was found to be protective (Burne, et al., 2001; Fiorina, et al., 2006; Yang, et al., 2006). Unfortunately, the exact mechanism behind its activation is not well understood. T-cells are activated via an antigen dependent or non-antigen dependent pathway. (Satpute, et al., 2009; Shen, et al., 2009)

Cytokines, chemokines, ROS as well as complement can all activate T cells. This becomes increasingly important when we consider the degree of inflammation with resides especially in DCD donation (Boros & Bromberg, 2006; Friedewald & Rabb, 2004). Once the T-cell is activated in IRI it can further lead to enhanced neutrophil recruitment and releases IFNɣ which is an important cytokine of inflammation (Shigematsu, Wolf & Granger, 2002; Yang, et al., 2006).

1.6.Methods of Limiting Transplant-induced IRI

The field of solid organ transplantation is still lacking in methods, medications and or products that can prevent or limit IRI. Current therapeutic measures at reducing IRI include the use of cold storage (Jochmans, 2010; Belzer & Southard, 1988), pulsatile mechanical perfusion (Deng, et al., 2013), the use of various preservation solutions (Groenewoud & Thorogood, 1992) and strong immunosuppressive medication (Mourad, et al., 2012; Mehrabi, et al., 2007; Faure, et al., 2004; Warnecke, et al., 2012). Most recently, the use of endogenously derived gasotransmitters have been shown to provide some relief for tissue IRI (Strüber, et al., 1999).

1.7.Hydrogen Sulphide as a Gasotransmitter

There are currently three known, endogenous produced gaseous molecules with physiological and pathophysiological properties—these molecules have been termed “gasotransmitters”. Gasotransmitters are lipid soluble, endogenously produced, and freely permeate the plasma membrane of a cell to pass the message directly to an intracellular target (Wang, 2002; Wang, 2003b). If a gas molecule, has significant physiological effects, controlled production by endogenous enzymatic reactions, specific inactivation mechanisms and specific cellular targets, it is considered to meet the criteria created by Wang and later on modified by Linden et al for gasotransmeters (Linden, et al., 2010).

Gasotransmitters do not bind to plasma membrane receptors, they actually diffuse into adjacent cells and start downstream function once they reach their multitude of targets. Also instead of being stored in vesicular structures, gasotransmitters must be resynthesized as needed. The three members of the gasotransmitter family are nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S).

NO was the first gasotransmitter to be recognized as a signaling molecule. That was initiated after the discovery of its potent smooth muscle relaxation through the actions of acetylcholine (FURCHGOTT, & ZAWADZKI, 1980). H_2S is the newest addition to the gasotransmiter family (Wang, 2002). Hydrogen sulfide is studied less than the other members of gasotransmitters (Bucci et al., 2012) with much more to be discovered about this molecule.

All three of the gasotransmitters have been found to share effects including vasodilatory, anti-inflammatory, anti-oxidant properties (Mustafa, et al., 2009). The way these gas transmitters interact or link is still not well understood. For example it was found that NO induces the production of H_2S in vascular tissue by increasing both the expression and activity of one of the enzymes that produces H2S (Zhao, Zhang, Lu, & Wang, 2001b).

All three gas transmitters were studied in the field of ischemia reperfusion injury extensively and the fact that these agents have many potentially cytoprotective, anti-inflammatory and perfusion improving effects, (features that perfectly suited the field of transplant) made these molecules attractive to the field of transplant. Perhaps the feature of hibernation and metabolism slowing effects of H_2S made this molecule even more interesting in such a field -organ preservation for transplant- where metabolism slowing methods already showed significant benefits (Belzer & Southard, 1988).

1.8.Hydrogen Sulphide (H2S)

1.8.1.History of H_2S

It is believed that H_2S was described as one of the gases of putrefaction by Johann Baptista van Helmont (1579–1644) who extensivly studied gases and earned the honor of being known as 'the real father of pneumatic chemistry (Leicester & Klickstein, 1952). The Italian physician Bernardino Ramazzini in 1713 described a disease of cleaners of Privies and Cesspits related to an unknown acid that is produced in that working environment causing eye inflammation.

In 1750 Carl Wilhelm Scheele treated ferrous sulfide with a mineral acid, and he noted the resulting stinking odor of H_2S which he called sulfur air, and for that he was the 1st to produce it in the lab. Later in 1777 reported cases in sewers of Paris with eye inflammation and others with asphyxia related death were also related to the same acid bringing its significance and awareness to a higher level. When Warenycia and Goodwin were studying the toxic effects of H_2S in rat and human brain in 1989, they found that detectable amounts of H_2S were produced endogenously (Warenycia et al., 1989). In 1996, Abe et al (Abe & Kimura, 1996) suggested that H_2S was an endogenous neuromodulator, as they showed that physiological concentrations of H_2S enhanced NMDA receptor-mediated responses and aided in the induction of hippocampal long-term potentiation.

Shortly after, Hosoki et al (1997a) reported that an enzyme, which produces H_2S, is present in the ileum, portal vein, and thoracic aorta and proposed that H_2S may be an endogenous smooth muscle relaxant. The recent interest in H_2S research mostly started after the finding that H_2S dilates rat blood vessels both in vitro and in vivo (Zhong, et al., 2003; Du, Yan & Tang, 2003).

1.8.2.Chemical features, toxicity and Sources of H_2S

Hydrogen sulfide H_2S is a colorless gas with a rotten egg smell. Liquid in very cold temperatures or very high pressure with a melting temperature of -85^o C and a boiling temperature of -60.7^o C (http://www.ccohs.ca). It is flammable at a concentration range of 4-46% with an autignition temperature of 260^{o}C-290^{o}C. Its structural formula is H-S-H (www.ccohs.ca). Cold dry air prolongs the half-life of H_2S while high temperatures increase the solubility of H_2S.

H_2S can be detectable by its odor at levels as low as 00.47 ppm. Eye irritation and damage can occur with levels as low as (10-20ppm) and (50-100) respectively. Higher doses of 200- 500 ppm leads to pulmonary edema, nervous system hyper stimulation, respiratory failure and death in 4-6hr. A dose of 800ppm can kill 50% of humans in 5min period and this is known as the lethal concentration (LC50). The longer the exposure and the higher concentration the more sever and irreversible is the injury. A level of 1000ppm can lead to immediate collapse after a single breath (www.newworldencyclopedia.org). Thus the threshold limit value for its presence in the workplace according to the American Conference of Industrial Hygienists was set not to exceed

10 parts per million (ppm) of hydrogen sulfide in air for 8hr/day for 5 days/week (www.acgih.org).

The mechanism behind H_2S toxic effects is not fully understood but the main cellular effect of H_2S in toxicity is by inhibiting cytochrome C, inhibiting oxygen consumption by mitochondrial cytochrome oxidase (Cooper & Brown, 2008) and uncouples the oxidative phosphorylation and by that inhibits the mitochondrial respiration mechanism and ATP production. (Dorman et al., 2002; Guo et al., 2012a) Another related mechanism is the through depletion of Glutathione (GSH) which is a potent cytoprotective antioxidant (Sparatore, et al., 2011; Shan, et al., 1993).

In nature H_2S is found in gases of Volcanos, Salt mines, water swamps, undersea vents, lakes, stagnant sewage (Kresse, et al., 2007; Mitchell, et al., 2001). It also can be produced by the breakdown of organic matter and human/ animal wastes (like in sewage). H_2S may collect in many areas in the surrounding city environments especially lower leveled, poorly ventilated, closed areas such as basements, manholes, sewer lines and underground telephone and electrical vaults (Stellman, 1998).

1.8.3.H_2S in our body

As mentioned above in the section of H_2S history it was found to be produced endogenously late in the 1980's. The H_2S in our body (in normal physiological status with a temperature of 37o C and a pH of 7.4) is mainly (80%) in the form of HS- and < 20% is in the dissociated form H_2S (Dorman et al., 2002; Dombkowski, et al., 2004). Zhao et al reported that H2S concentration in humans plasma ranges between 45-300uM (Zhao, et al., 2001b; Qingyou, et al., 2004).

Others did report that concentrations of H_2S in vertebrate blood varied between 30-100uM, while in the brain showed higher concentrations ranging between 50-160uM (Abe & Kimura, 1996; Dello Russo, et al., 2000; WANG, 2002). However, there have been many arguments demonstrating that these concentrations cannot be correct (Olson, 2009; Olson, 2011a; Olson, 2011b).

Olsen (2012) discussed and summarized this debatable issue in his review "A Practical Look at the Chemistry and Biology of Hydrogen Sulfide" (Olsen, 2012). He pointed that the previously reported concentrations showed huge differences when measured using different H_2S measurement methods. Also some of these high concentrations theoretically can be only

acquired by the exposure to toxic levels of H_2S. The need to determine the real physiological concentrations of H_2S in blood and tissues -if any- still yet to be determined. This is largely depends on identifying the best method for measuring H_2S in different biological tissues and fluids.

1.8.4.Sources and Production of H2S in Humans and Most Mammals

H_2S is produced in our bodies through enzymatic and non-enzymatic pathways. The enzymatic pathway produces H_2S from the amino acids cysteine, cystathionine and homocysteine. The 3 enzymes capable of producing H_2S in humans are: 1) cystathionine gamma lyase (CSE), 2) Cystathionine beta synthase and 3) 3 Mercaptopyruvate sulfartransferes (3MST). Cysteine is an essential precursor for all of these enzymatic pathways.

CBS and CSE are considered the major ones with more extensive studies with the cofactor being active vitamin B6 (pyridoxal 5' -phosphate (PLP)). Both CBS and CSE are mainly localized to the cytosol while 3MST is more found in the mitochondria. CBS catalyze the transsulferation of homocysteine to cystathionine. Then both CBS and CSE then metabolize cystathionine in the production of H_2S.

1.8.4.1.Production of H_2S

The non-enzymatic pathway releases a small amount of endogenous H_2S in humans. Sulfur is reduced by the reducing equivalents from glucose oxidation to produce H_2S. The source of the reducible sulfur is mainly elemental (Benavides et al., 2007).

The enzymatic pathway, the major pathway of H_2S production in our body and majority of mammals.

Three enzymes are known to be responsible for the majority of H_2S production with different concentrations in different tissues. These are :Cystathionine β-synthase (CBS), Cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfotransferase (3MST). (Hosoki, Matsuki, & Kimura, 1997)(Abe & Kimura, 1996)

a. CBS:

In humans CBS is 63- kDa subunits Homotetramor, with each subunit consist of 551 amino acids (Kery, et al., 1994). CBS utilize cysteine or homocysteine to produce H2S. Mainly available in

the cytoplasm CBS is the dominant enzyme producing H_2S in the brain, equal to CSE in the skeletal muscles, gastrointestinal and penile smooth muscles (d'Emmanuele di Villa Bianca et al., 2009; Fiorucci et al., 2005), while CSE dominates in the cardiovascular system (Awata, Nakayama, Suzuki, Sugahara, & Kodama, 1995; Lowicka & Beltowski, 2007).
Without CBS homocysteine accumulates in the tissue as it cannot be catalyzed through the transsulfuration pathway (Jhee & Kruger, 2005). Genetic deletion of CBS results in markedly elevated levels of homocysteine "Homocystinuria". This disease is marked with significant cardiovascular impairment. CBS gene is located on chromosome 21 in humans. More than 150 gene mutations of CBS has been identified in the disease of homocystinuria. (Kruger et al., 2000)
CBS heme has a very strong affinity to CO, which physiologically inhibits CBS. This inhibition might explain the cerebral vasodilation effect of CO.

b. CSE

Cystathionine ɣ-lyase with either (CGL) or more common (CSE). Measuring about 45 kDa. Located on human chromosome 1.
CSE like CBS, produces H_2S from cysteine and homocysteine.
The deficiency of CSE leads to accumulation of cystathionine (hypercystathionenemia) and the excess of excretion in the urine (cystathioninuria) (Renga, 2011). CSE is now believed to be the principole enzyme responsible for H_2S production in the peripheral tissue and of our interest in the kidney (Paul & Snyder, 2012).

c. 3MST

3 mercaptopyruvate sulfotransferase together with Cysteine amino transferase (CAT) can produce H_2S from cysteine when α-ketoglutarate is present.
Both 3MST and CAT are found in the Mitochondria and cytcole. In order to reach maximum activity, 3-MST needs very high pH levels 3MST utilizes the alpha ketogluterate produced by CAT to produce H2S. The H_2S produced by 3-MST is mainly in the form of sulfane sulfur, which is then stored rather than used (Hu, et al., 2011).
In the kidney H2S is produced by all three enzymes with CSE playing the major role (Stipanuk and Beck, 1982). CBS, CSE and 3-MST are primarily present in proximal tubules within the

renal cortex (House, et al, 1997; Nagahara, et al, 1998; Yamomoto, et al, 2013). CBS was established to primarily be localized in the proximal convoluted tubule in the outer cortex whereas CSE to be localized mostly in the proximal straight tubule in the inner cortex and outer medulla (Ishii, et al, 2004; Li, et al, 2006).

1.8.4.2.Fate of H_2S

Concentrations of H_2S in many studies showed to be rapidly balancing. Even after the administration of exogenous H2S sufficient to cause recognizable effects, the levels rapidly equalized back to its normal levels. This stability of H_2S levels is expected with all the potent pathways that work on controlling its levels. This balance also is meant to avoid the toxic effects of H_2S accumulation which could be detrimental to life. There are several pathways involved in H_2S breakdown and elimination (Bhatia, et al., 2012).

Oxidation: Oxidation is the main method of H_2S metabolism. Intracellular, mitochondria plays a major role in controlling H_2S metabolism. As significant amount of H_2S is rapidly oxidized by the mitochondria into sulfate (SO4 2−) and sulfite ($SO_3$2) (Li, Rose, & Moore, 2011).

Methylation: Another less important metabolism pathway is the methylation that takes place in the cytosol. Methylation of H_2S by thiol-S-methytransferase to yield methanethiol and dimethyl sulfide represents another less important mechanism of H_2S degradation, and therefore accounts for a smaller amount of H_2S (Levitt, et al., 1999).

Other: other known pathways for the metabolism of H_2S is by reacting with disulfide containing proteins or metalloproteins like cytochrome C.(Guo et al., 2012a)

Also being a potent reducing agent, significant amount of H_2S is utilized in scavenging and reaction with the produced ROS (Li et al., 2011). Scavengers of H_2S like Methemoglobin or oxidized glutathione also play a role in the consumption of H_2S (Fiorucci et al., 2007). Methemoglobinemia is sometimes induced by 3% sodium nitrite for the treatment of H_2S toxicity to form sulfhaemoglobin.

Majority of H_2S oxidation takes place in the liver to form sulfate which is then excreted in the urine as free or conjugated sulfate (Beauchamp, et al., 1984). A small percentage of the H_2S produced by the fecal bacteria in the colon is excreted in feces according to the study on rats (Levitt, Springfield, Furne, Koenig, & Suarez, 2002).

1.9.H2S protective mechanisms in ischemia–reperfusion

In vivo and Ex-vivo studies proved H2S protection against IRI in the lung (Z. Fu, Liu, Geng, Fang, & Tang, 2008), the liver (Jha, Calvert, Duranski, Ramachandran, & Lefer, 2008), the heart (Elrod et al., 2007a; Sodha et al., 2008) and – most relevant to this thesis- the kidney (Tripatara et al., 2008).

1.9.1.Antioxidant Effects of H_2S

Oxidants or cytotoxic Reactive Oxygen species (ROS) are free radicles and reactive molecules that are generated by molecular oxygen. Molecular Oxygen itself is a stable biradicle while oxygen atom is highly unstable. The Oxygen atom has two unpaired electrons in separate orbits making it vulnerable to release ROS. These ROS are generated during the mitochondrial transport chain of aerobic respiration. ROS are either radicals or non-radicals. Radicles include superoxide radical anion ($•O_2$-) hydroxyl radical (HO•) and peroxyl radicals (ROO•) while non-radical derivatives are like hydrogen peroxide (H_2O_2), peroxynitrite (ONNO-) and hypochlorous acid (HOCL).

Important physiological functions of ROS were recently identified. These physiological roles serve in vascular tone modulation, endothelial function and Induction of host defense (Droge, 2002; Mueller, Laude, McNally, & Harrison, 2005; Pawlak, Naumnik, Brzosko, Pawlak, & Mysliwiec, 2004; Touyz & Schiffrin, 2004). The Excess of ROS is where its pathophysiological role overcomes its physiological function. ROS has the potential to destruct the cell membrane, cell proteins and other cellular structures. ROS in addition to its direct destructive potential, ROS induce damage by activating redox- sensitive signaling pathways (Ushio-Fukai, Alexander, Akers, & Griendling, 1998).

Among these activated signaling pathways are mitogen-activated protein kinases (MAPK) (X. Wang et al., 2011) and transcription factors (NFκB and HIF-1) (Chandel et al., 2000; Narayanan et al., 2014). ROS also increase intracellular-free Ca_2^+ concentration and upregulate proto-oncogene, profibrotic and pro-inflammatory gene express (Griendling, Sorescu, Lassegue, & Ushio-Fukai, 2000; Hernandez-Fonseca, Cardenas-Rodriguez, Pedraza-Chaverri, & Massieu, 2008; Law et al., 2013). In the physiologic status the cells manage to detoxify ROS by the

detoxifying mechanisms. During hypoxia the excess production of ROS leads to cell destruction and signals cascades leading to cell necrosis.

Excess ROS accumulation intracellular also prolongs the activation of c-Jun-N-terminal kinase gene which promotes apoptosis (Nakano et al., 2006). For that the cell has many mechanisms to detoxify ROS. These are through a variety of enzymes like Glutathione peroxidase and Superoxide Dismutase (SOD) (McCord & Fridovich, 1968) and non-enzymatic molecules: like Glutathione (GSH), Vitamin C, vitamin E and others. Glutathione is considered among the most important defense mechanisms against ROS. Depletion of intracellular GSH leads to accumulation of ROS and cell damage (Wang, 2012).

ROS plays a major role in the injury induced by ischemia reperfusion. Controlling the production of ROS has been an interest in hope to counteract cell damage in a variety of pathological processes. In many studies H_2S was proven as a potent anti-oxidant. H_2S anti-oxidant effects are both direct and in direct. Direct effects of H_2S on ROS are explained by acting as a strong reducing agent. It directly scavenge a number of known potent ROS like peroxinitrite, 1-methyl-4-phenylpyridinium (MPP+) and NO (Schreier et al., 2010; Whiteman et al., 2006) (Whiteman et al., 2004a).

Whiteman et al. using an in vitro model of human neuroepithelioma cell line showed that H_2S inhibits the cytotoxic effects of hydroxychloric acid (HOCl). H_2S also reduced the HOCl induced protein oxidation and lipid peroxidation (Whiteman et al., 2005). In the same in vitro model of human neuroepithelioma H_2S also blocked the cytotoxic effects of peroxinitrite and its intracellular protein nitration and oxidation (Whiteman et al., 2004b), lipid hydro peroxides (LOOH) are non-radical intermediates, and once catalyzed by single electron reduction it triggers the free radical chain mechanism of lipid peroxidation. H_2S treatment was able to destroy 50% of LOOH in oxidized Lipodensity lipoprotein (oxLDL). (Muellner et al., 2009)

H_2S is a strong reducing agent, being able to react with multiple oxidant stressors including superoxide radical anion (Mitsuhashi et al., 2005), hydrogen peroxide, (Geng et al., 2004) and peroxynitrite (Whiteman et al., 2004a). The indirect anti-oxidant effects of H_2S is by up regulating and increasing the level of Glutathione (GSH) and Superoxide dismutase (SOD) (Kimura, Dargusch, Schubert, & Kimura, 2006).

1.9.2.Anti-apoptotic effects of H_2S

Xu et al. studied the effect of H_2S on apoptosis during oxidative stress of hemorrhagic shock in a rat model. They concluded that H_2S administration protected rat lungs against inflammation by suppressing oxidative stress and the Fas/FasL apoptotic signaling pathway as well as attenuating the expression of pro-apoptotic proteins FADD, active-caspase 3, active-caspase 8, Bax, and increasing the expression of Bcl-2. Sodium hydrosulfide alleviates lung inflammation and cell apoptosis following resuscitated hemorrhagic shock in rats.

Another rat model of IRI by Sivarajah A1et al. demonstrated that H_2S attenuates both the decrease in Bcl2 expression as well as the increase in caspase 9 activity in the myocardium (Sivarajah, et al., 2009). Many of the pro-apoptotic Bcl-2 proteins like Bid, Bax and Bak play a major role in apoptosis. Bid binds to the external membrane of the mitochondria, activating the other proapoptotic proteins Bax and Bak (Crow, Mani, Nam, & Kitsis, 2004). Release of cytochrome c from the mitochondria into the cytoplasm (Kluck, Bossy-Wetzel, Green, & Newmeyer, 1997; X. Liu, Kim, Yang, Jemmerson, & Wang, 1996) is mainly triggered by both Bax and Bak activation. Cytochrome c after that initiates various caspases activations powerful enough to initiate apoptosis (Du, et al., 2000; Verhagen, et al., 2000).

Apoptosis inducing factor (AIF) a caspase independent pro-apoptotic protein. Is located in the space between the inner and outer mitochondrial membranes. It is released into the cytoplasm and into the nucleus. It condensates the chromatin within the nucleus and fragments the DNA (Daugas et al., 2000). Another important cytosol protein important in IRI induced apoptosis and affected by H2S is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This transcriptional factor once activated by different stimuli is capable of rapidly binding to the DNA and inducing various gene transcriptions responsible for many cellular events including apoptosis.

In an IRI model, Biermann et al showed that H_2S inhibits apoptosis by inhibiting NF-kB and limiting its binding to DNA. That inhibiting effect of H_2S on NF-KB was only encountered after IRI (Biermann, et al., 2011). In other studies H_2S enhanced the binding of NF-κB to the DNA but still that resulted in decreased apoptosis. This anti-apoptotic influence of NF-κB was significantly diminished in CSE deleted mice (Sen, et al., 2012). As the mitochondria are believed to be the key in apoptosis, controlling it seems to enhance or decrease apoptosis (Wang, 2012). Since H_2S protects the Mitochondria and prevent its destruction through many

mechanisms -will be discussed later in this chapter- It should be true that H_2S must play some role in protecting against apoptosis.

1.9.3.Vasorelaxant effects of H_2S

As described in the mechanism of injury of IRI previously, blood vessels play an important role in IRI. In fact most of the IRI models ischemia is induced by the severe constriction or blockage of the vessels (Vincent, et al., 2011). During reperfusion a balanced vascular tone to maintain enough blood pressure but still relaxed enough to allow sufficient amount of blood flow supplying nutrients and oxygen to the tissue is essential.(Eltzschig & Eckle, 2011)

The involvement of gasotransmitters, in the regulation of vascular tone was first noted with the discovery of NO physiological roles. Recently H_2S and NO were found to act in a way of synergism in inducing vasorelaxation in vascular smooth muscle cells (VSMCs) (Hosoki et al, 1997). In 1997 Hosoki et al demonstrated that exogenous H_2S relaxed rat aortic tissues in vitro (Hosoki, Matsiki, & Kimura, 1997b). When exogenous H2S proved to induce hypotension, it raised the hypothesis that endogenous H_2S must play a role in regulating the tone of vessels in the human body.

The main source of H_2S in the vascular walls is the enzymatic pathway of CSE. That was demonstrated clearly when Yang et al. CSE knockout mice showed low levels of H_2S in their vascular tissue and as a result of low H_2S levels, the mice developed significant hypertension (Yang et al., 2008). Majority of studies on the vascular system showed H_2S to produce vasorelaxation (Wang, 2009). Other considers H_2S function on vascular smooth muscles to be biphasic. As it causes vasorelaxation in high doses (100µM- 1,600 µM) while low concentrations (10-100 µM) causes' vasoconstriction (Ali et al., 2006; Lim, Liu, Khin, & Bian, 2008).

Mechanism by which H_2S induces this response of vasodilation is not fully understood but many mechanisms were proven to induce it. Trying to define the mechanisms behind the vasorelaxant effects of H_2S, the activation K+ channels seems to play the major role. The fact that H_2S plays a role in regulating the vascular tone is one of the evidences to explain H_2S potency in preventing IRI. Like the other gasotransmitters NO and CO, H_2S showed potent vasorelaxant effects. When considering site of release, target tissues and mechanism of action, H_2S is significantly different than the other gasotransmitters.

NO and CO mediate vasorelaxation by increasing the cellular cGMP activity and/or stimulating KCa channels in vascular smooth muscle cells (SMCs) (Zhao et al., 2001b). Zhao et al. studied the effects of H_2S in vivo and in vitro while looking for the effects of H_2S on the cardiovascular system. Intravenous injection of H_2S provoked a transient but significant decrease in mean arterial blood pressure. They concluded that the drop in blood pressure was mainly due to the vascular tone since heart rate was not affected. Similar effect was induced when the KATP channel opener pinacidil was used. While on the other side the effect of H_2S was blocked when the KATP channel blocker Glibenclamide was used. This last also showed that H_2S in physiological concentration rate is believed to play a role in vasorelaxation. (Zhao, et al., 2001a) H_2S opens the KATP channels causing hyperpolarization of the SMCs and impairing the voltage dependent Ca^{++} channels (Wang, 2009). When Nelson et al. used blockers for different K channels (KATP, KCa^{++} and Kv channels) trying to identify the role of each, all showed to play a role in the relaxation of the vessels walls. In a step towards identifying the relation between NO and H_2S similar vasorelaxation effect.

NO was found to enhance the concentration of H_2S in the vascular tissue via increasing the activity of CSE either directly or by increasing the activity of cGMP- dependent protein kinase and up regulating the expression of CSE with a mechanism that is still unclear (Zhao et al., 2001b). In the other hand, H_2S is believed to facilitate the release of NO and other EDHF (Tang et al., 2013). Unlike NO and CO, H2S vasorelaxant effect showed to be independent of the activation of cGMP (Tang et al., 2013).

H_2S also is unique as it is synthesized in both endothelial cells (EC) and smooth muscle cells (SMC). CSE being the major enzyme responsible for H_2S production in SMCs while 3MST/CAT enzymatic pathway produces H_2S in the ECs (Shibuya, et al., 2009). H_2S released from both SMCs and ECs and opens the K ATP channels, causing hyperpolarization of the SMCs of the vessels wall and impairing the voltage dependent Ca^{++} channels (Wang, 2009).

This by itself qualifies H_2S as an endothelial derived hyperpolarizing factor (EDHF). H2S also releases other EDHF from the ECs. While NO is an EDHF in large arteries, H_2S is believed to be an EDHF of small resistance vessels that affects blood pressure. (Mustafa et al., 2011; Nagao, Illiano, & Vanhoutte, 1992; Urakami-Harasawa, et al., 1997; Wang, 2003b). It is suggested that the targets of H_2S on SMCs and ECs are different. In ECs H_2S acts on Kca^{++} channels which hyperpolarizes the endothelial cell itself and releases other vasoactive substances (Qiu &

Quilley, 2001), while H_2S targets ATP dependent K^+ channels on SMCs, hyperpolarizing the SMCs themselves (Wang, 2012). New data suggest that H_2S is an endothelial derived hyperpolarizing factor (EDHF). CSE being the major enzyme responsible for H_2S production in SMCs while 3MST/CAT enzymatic pathway produces H_2S in the ECs (Shibuya, et al., 2009). As NO is an EDHF in large arteries, H_2S is believed to be an EDHF of small resistance arteries (Wang, 2003a; Wang, 2009).

It is hard to tell wither the SMCs H_2S or the ECs H_2S plays the major role in the vasorelaxant effect, but it is suggested that the targets of H_2S on SMCs and ECs are different. And that the target of H_2S on Endothelial cells is KCa^{++} channeles which hyperpolarizes the endothelial cell itself and releases other vasoactive substances (Qiu & Quilley, 2001; Wang, 2012). While H_2S targets ATP dependent K^+ channels on SMCs, hyperpolarizing the same cells –SMCs- (Wang, 2012). Taking into consideration that ATP dependent K channels is the major contributor to H_2S vasorelaxant effect, SMCs role is much more significant than the endothelial cells as an end target of H_2S vasorelaxation.

Another suggested mechanism of H_2S vasorelaxation is by part related to its anti oxidant effects. H_2S reduces the availability of H2O2 (Meng et al., 2007) which is a potent ROS. H_2O_2 was proposed to be an EDHF itself (Shimokawa & Matoba, 2004; You, et al., 2005). Alternative mechanisms, that might contribute to its vasorelxant effects is its direct inhibition of ECs angiotension-converting enzyme.

1.9.4.Anti inflammatory effects of H_2S

The increased production of H_2S during inflammation reflects that it must have a role. Controversy in the protective versus harmful effects of H_2S in many fields exists, and it is most conflicting in the field of inflammation. The general principle understood and agreed upon widely states that H_2S effects are dose dependent, as high concentrations induces toxic effects (Cheung, Peng, Chen, Moore, & Whiteman, 2007) while low doses can be protective most of the time (Elrod et al., 2007b; Jha et al., 2008; Sodha et al., 2008).

The effects of H_2S are also - to some degree - cell specific and dependent on the experiment's cell conditioning (Rose et al., 2005). As many trials proved that H_2S functions as an anti-inflammatory, almost as many proved that it promotes inflammation. Here we will discuss the

anti-inflammatory role as it is most relevant to the subject of this thesis. Many mechanisms were supported by experimental evidence on the protective effects of H_2S as an anti-inflammatory agent. Tissue edema, one of the hallmarks of inflammation that results from leaky vessels and cellular tissue infiltration. In IRI it seems to play an important role in the No reflow phenomena as mentioned in the IRI mechanism section above.

Exogenous H_2S decreased edema, probably due to inhibition of plasma and inflammatory cells leakage, while inhibitors of H_2S enzymatic production increased the development of edema in inflammatory conditions (Zanardo et al., 2006). H_2S induced some anti proliferative effects on T-cells (Valitutti, Castellino, & Musiani, 1990) which for some extent limits the inflammatory response. Another anti-inflammatory effect of H_2S that affects the leukocytes is its ability to induce apoptosis in poly morphonuclear (PMN) cells (Mariggio et al., 1998).

Also H_2S is known as a modulator for leukocyte adherence to the endothelium of the vessels. The adherence of leukocytes to the walls of the vessels facilitates its infiltration into the tissues to induce inflammation. (J. Wallace, 2007) H_2S donors decreased leukocyte adhesion induced by both Asetylsalysilic Acid-induced and Formyl-Methionyl-Leucyl-Phenylalanine (fMLP), while inhibitors of H_2S production promote leukocyte adhesion (Wallace, 2007).

Also the leukocyte rolling, which is an essential step proceeding the leukocyte tissue infiltration, was inhibited by H_2S during IRI (Yusof, et al., 2009). Inhibition of H_2S production was proven to increase the rate adherence of the inflammatory cells to the mesenteric vascular endothelium of rats (Zanardo et al., 2006). The mechanisms behind H_2S modulation of leukocytes adhesion are not fully understood. Evidence supports that H_2S suppresses intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and leukocyte function-associated antigen-1 (LFA-1) on the leukocytes. Both ICAM-1 and LFA-1 are important for the adhesion of leukocytes on the endothelial cells. (Fiorucci et al., 2005)

Studies also showed that H_2S decreased expression of the pro-inflammatory markers: TLR 4, TNF α, C-C chemokine receptor type 5, INF γ, IL 2,(Zhu et al., 2012), IL-1B, IL6 and IL 8 (Mariggio et al., 1998; C. Yang et al., 2011)In the same time it increased IL-10 with its potent anti-inflammatory features(Esechie et al., 2008) and scavenged and reduced the production of the tissue destructing ROS (Yang et al., 2011) The activation of NF-κB a key step in many inflammatory responses. Once activated, NF-κB initiates the cascade of pro inflammatory gene up-regulation for synthesis of many inflammatory cytokines and chemokines. H_2S was proven to

inhibit or suppress the activation of NF-κB in many experiments (using different H_2S donors) and hence decrease the inflammation induced by lipo polysaccharide induced oxidative stress (Li, Whiteman, Guan, Neo, Cheng, Lee, Zhao, Baskar, Tan, & Moore, 2008b; Li et al., 2007a). Interestingly that in other experiments H_2S was noted to enhance the inflammatory reaction through NF-κB dependent and independent pathways to act as a pro-inflammatory agent (Ang, Moochhala, MacAry, & Bhatia, 2011; Stuhlmeier, Broll, & Iliev, 2009). It might be the balance in H_2S levels that determines the line between being pro-inflammatory, neutral or anti-inflammatory. This balance is between the level of H_2S and the events the tissue is going through.

1.9.5.Mitochondria Protection Effects of H_2S

Mitochondria is not only the main factory of energy in cells, it is also the pace maker of cell death (Foo, Mani, & Kitsis, 2005; Kroemer, Galluzzi, & Brenner, 2007). Cytochrome c oxidase, an important enzyme in the respiratory electron transport chain of the cell. As the respiratory chain occurs within the mitochondria cytochrome C oxidase also is localized in the mitochondrial membrane. Cytochrome C oxidase is one of the intracellular targets for H_2S actions. Its inhibition by H_2S was found to be the key step in H_2S lethal toxicity and also the main step in inducing what is called the suspended animation state in some animals (Cooper & Brown, 2008; Hill et al., 1984).

During the oxidative stress, Ca^{++} and ROS accumulate within the cell. Once the mitochondrial permeability transition pore (MPTP) allows the Ca^{++} to enter the mitochondria, this large amount of Ca^{++} leads to destruction of the mitochondria. Rather than synthesize of ATP the MPTP causes mitochondria to break down leading later on to cell death. Many pathways have been proposed to prove and explain the protective effect of H_2S on mitochondria. H_2S improves the mitochondrial respiration recovery rate, increases complex I and complex II efficiency and reduces the mitochondrial swelling during IRI (Elrod et al., 2007b).

When Elrod et al (2007) isolated mitochondria from murin hearts and exposed it to in vitro hypoxia assay for 30 min. The group treated with 10uM H_2S showed a 67% recovery of respiration rate while the vehicle one showed only 36% recovery (Elrod et al., 2007b). He also (Elrod et al., 2007b) assessed the mitochondrial function in vivo mice model of IRI in which a

group was treated with H_2S 50uM/kg at the start of reperfusion. The isolated mitochondria of the H_2S treated group significantly showed higher rate Oxygen consumption when analyzed for both complex I and complex II efficiency. Also electron microscopy showed less swelling in the mitochondria of H_2S treated group.

Another important mechanism of H_2S protection to the mitochondria is by blocking the mitochondrial permeability transition pore (MPTP). Shanmuganathan et al. studied the role of the MPTP as a target of cardio protection. Using cyclosporine A, (a known inhibitor of MPTP) at the onset of re-oxygenation did protect human myocardium against lethal hypoxia–re-oxygenation injury. That proved that protecting the mitochondria protects the myocardium from IRI and makes it an interesting target for treating much cardiovascular disease.

Other important role for H_2S within the mitochondria during IRI is the up regulation of ROS scavengers SOD and GSH, and decreasing levels of reactive oxygen species (ROS). ATP is a major source of cell energy. The hydrolysis of the phosphate bonds results in energy release from the ATP molecule. Majority of ATP is produced in the mitochondria with the remaining amount produced in the cytosol via glycolysis and phosphorylation. H_2S also act as a supply for ATP during hypoxia, as it was found to improve mitochondrial ATP production during hypoxia in SMCs (M. Fu, Zhang, Wu, Yang, Li, & Wang, 2012a).

For that H2S does not only reduce metabolic demand significantly, it also act as an energy substrate to maintain ATP production in hypoxic conditions (Guo et al., 2012b). Mitochondrial ATP production showed to improve with H_2S in SMCs with Hypoxia induced impaired ATP production (Guo et al., 2012a). It has been demonstrated that H_2S metabolism in the Mitochondria slows the metabolic rate and during hypoxia it improves ATP production (M. Fu, Zhang, Wu, Yang, Li, & Wang, 2012b).

1.10.H2S Donor molecules

The use of H_2S donor molecules has enabled the study of H_2S on different species, tissues, cells or reactions. Unfortunately the use of H_2S as a gas is limited by the hazardous potential of its toxicity. The inability to sustain a precisely controlled concentration was also a limiting feature. These H_2S releasing compounds (H2S Donors) had some draw backs and limitations that pushed

scientists to invent some new compounds to meet the experiments' need. H_2S donors can be subdivided according to the following categories which will be discussed in detail further:

a. Natural:

Inorganic or Organic (Plant or organo sulphur compounds)

b. Synesthetic

Agonists of H_2S -synthesized enzymes.

Cysteine activated donors

H_2S -releasing drug hybrids

Slow releasing donors

1. Non-Mitochondrial
2. Mitochondria-targeted Donors.

1.10.1.Natural

a. Inorganic donors

The two most often used salts NaHS and Na2S, are among the simplest donors still used in H_2S research. The release of H_2S from these molecules is immediate. As these salts rapidly release large amounts of short lasting H_2S. This pulse release most of the time does not mimic the endogenous small amount and prolonged slow release of H_2S. These types of donors might expose some tissues to a rapid increase in H_2S to induce some adverse response instead of benefit. This might be part of the explanation behind the controversies in exogenous H_2S divergent results in some studies.

Other important inorganic donors are the precursors used in endogenous H_2S synthesis, N-acetyl cysteine, L-cysteine and D-cysteine are considered and used in research as a source of H_2S introduction. These precursors when enhanced can significantly increase H_2S to with the catalysis of CSE and CBS. One of the use of theses precursors is that their increased production in our bodies has minimal side effects.

b. Organic: Organo Sulfar Compounds (OSC)

Garlic has been used as a natural medicine since long time in many cultures. Garlic derived OSC were found responsible for most of the therapeutic potentials of garlic.

These compounds including diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), S-propargyl-cysteine & S-allylcysteine were studied extensively in the last few years due to its anti-inflammatory and anti-cancer potential.

1.10.2.Synesthetic H2S Donors

Recently many H_2S donors have been invented in order to study H_2S effects with more utilization of its function and more control of its release and concentration.

a. Cysteine activated H2S donors

Other synesthetic H_2S donors are the cysteine activated Donors. These enzymes are based on the N- (benzoylthio) benzamide template. Their H_2S release is controlled by cysteine, which its availability in different tissues is variable. As recommended by the inventors, the use of these cysteine dependent donors might need the addition of cysteine in some tissues that are deficient in cysteine (Zhao, 2011).

b. H_2S-Releasing Drug Hybrids

Just like when NO gained its popularity, scientists were tempted by the cytoprotective properties of H2S and successfully invented some H_2S releasing drug hybrids. These are different from NO releasing drug hybrids in terms of better efficacy and safety profile (About-Mohamed, et al., 2004).

The main reason found behind the less toxicity induced by the H_2S hybrids (compared to the NO hybrids) were related to the ability of H_2S to inactivate superoxide by the formation of GSH. The use of dithiolethione moiety was involved in the synthesis of the majority of these H_2S hybrids which releases H_2S much slower than most of the known H_2S donors. And hence not affecting the mitochondrial respiration (Li, et al., 2007; Giustarini, et al., 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs) are potent medications used to subside inflammation and pain associated with many diseases. The use of NSAIDs is still limited by its significant side effects, such as gastric ulcers, platelet dysfunction, asthma exacerbation, acute renal failure and heart failure. These compounds share the magnified anti-inflammatory of NSAIDs as well as H_2S while adding the advantage of H_2S other cytoprotective mechanisms. The invention of these derivatives was based on the evidence behind the H_2S potent effect in preventing leukocyte adherence to the endothelium opposing the adverse effect of cyclooxygenase COX inhibitors (Grander, 1992). The leukocyte adherence to the vessels endothelium was found to play a major role in the NSAIDs induced gastric mucosal injury (Wallace, et al., 1993; Wallace, 2000). These H_2S releasing derivatives like when used on rats did not show gastric damage unlike the case with the NSAIDs alone (Wallace, et al., 2007). In fact these derivatives showed to have more potent anti-inflammatory effects than the currently used NSAIDs (Fiorucci et al., 2007; Li et al., 2007b). And as most of the other H_2S donors, the effect of using these NSAID derivatives donors showed protective role against IRI (Rossoni et al., 2008). Mesalamine derived ATB 429, Naproxen derived ATB 346 and Diclofenac derived ATB 337 are some of the known hybrids that showed the success of this combination. Other hybrids are being studied in different fields of medicine like, H_2S -donating sildenafil (ACS 6), H_2S -donating latanoprost (ACS 67) for glaucoma treatment and H2S-donating levodopa (ACS 84) for Parkinson's disease.

c. Slow releasing H_2S Donors

1. Non Mitochondria-targeted H_2S Donors

The release of endogenous H_2S from cells is likely to occur in a slower rate of release with less amounts than that from sulfide salts, and therefore sulfide salts may not mimic the biological effects of naturally produced H_2S (Li, Whiteman, Guan, Neo, Cheng, Lee, Zhao, Baskar, Tan, & Moore, 2008a; Whiteman et al., 2010). The synthesis of slow releasing H2S donors served this interest of mimicking the real biological release of H2S. Also serves the fact that slow release might prevent or atleast limit the toxic effects of H2S on mitochondrial respiration (Li et al., 2007a).

GYY 4137 Also known as morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate. This Lawesson's derivative compound achieved the purpose of its invention, sustaining a prolonged slow release of H_2S (Lee, et al., 2011). This synthetic molecules is capable of releasing H_2S over extended periods of time, in small amounts, more like physiological endogenous H_2S release. The H_2S released from GYY4137 was reported to peak at 6 to 10 minutes and to remain raised for over 180 min. (Li et al., 2008)

GYY4137 proved its efficacy as a H_2S slow donor in many experiments as antihypertensive with effects on the vessels walls and blocking the k channels, as an anti-inflammatory (Burguera, Vela-Anero, Magalhaes, Meijide-Failde, & Blanco, 2014), as an anti-cancer agent. GYY4137, a hydrogen sulfide (H_2S) donor, shows potent anti-hepatocellular carcinoma activity through blocking the STAT3 pathway as an anti-atherosclerosis agent (Liu et al., 2013). More relevant to this thesis GYY4137 was proven to protect the mitochondria and limit the cytotoxicity induced by oxidative stress. In IRI GYY4137 proved in Ex vivo kidney and heart preservation to be protective (Li et al., 2008a).

2. Mitochondria Targeted H_2S Donors

Being a key intracellular organelle with major control on many critical cellular functions, the mitochondria have attracted researchers in efforts to gain some control over its function. Recently a novel H_2S donor was created, with the advantage of targeting the mitochondria and releasing H_2S in a slow and steady manner.

This novel invention was based on using mitochondria targeting molecules like Triphenylphosphonium (TPP+) linked together with H_2S moiety dithiolethione or thiohydroxybenzamide. TPP is one of the lipophilic cations with highly positive charges attracted to the negative membrane potential inside the mitochondria and taken up comprehensively and rapidly by the mitochondria in vivo, by that it can carry its attached molecules (selective small molecules) through the mitochondrial membrane.

The H_2S moiety dithiolethione or thiohydroxybenzamide are derivatives of non-steroidal anti-inflammatory drugs used in these novel H_2S donors are characterized by their slow and steady release of H_2S. Dithiolethione have been shown to produce H_2S in vivo and provide potent anti-inflammatory results (Kashfi, 2013; Giustarini, et al., 2010). Being able to ‘target’ mitochondria

and limit the oxidative stress mediated by the mitochondria have given these molecules a considerable potential advantage.

In the current project we used AP39 (10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy) decyl) triphenylphosphonium bromide) which was generously given to us by Dr. Matthew Whiteman from University of Exeter Medical School, United Kingdom (Le Trionnaire, et al., 2014) produces an overall yield of 73% using Phosphonium salt prepared from 10-bromodecanoic acid and triphenylphosphine under reflux conditions in acetonitrile 33 and coupled, using standard carbodiimide conditions, to 5-(4-hydroxyphenyl)- 3H-1,2-dithiole-3-thione 2). Final purification of AP39 was achieved using flash chromatography on silica gel (eluting with methanol), the product forming a crisp foam (which could be handled as a solid) on removal of the solvent.

The novel H_2S donor AP39 generates H_2S at physiological pH more rapidly and more efficiently than GYY4137 and more important is its location of release within the mitochondria. What makes mitochondrial targeted donors interesting is that they target the power horse of the cell (the mitochondria). When the inventors of these mitochondrial targeted H_2S donors compared it to the non-mitochondrial slow releasing donors GYY4137 and AP72, they found that the mitochondrial donors were more potent in preventing oxidative stress injury in human brain micro vascular endothelial cells (HMEC).

The effect of AP39 was tested in some recent studies and proved to produce cytoprotective and antioxidant effects during oxidative stress. Pretreatment with AP39 of murine microvascular endothelial cells exposed to oxidative stress, attenuated the production of superoxide by the mitochondria, damage to the mitochondrial DNA and bioenergetics dysfunction. Worth mentioning is that AP39 stimulated the mitochondrial electron transport and cellular function at low concentration and had an inhibitory effect at the higher concentrations (more than 300nM) (Szczesny et al., 2014).

Mitochondrial targeted donors AP39 and AP123 were compared to non targeted donors GYY4137, AP67 and AP72 in an assessment of their cytoprotective role against oxidative stress toxicity (Le Trionnaire, et al., 2013). The trial used Human brain micro vascular endothelial cells (HMEC) and exposed it to a mixture of oxidants including H_2O_2. ROS generation, mitochondrial membrane potential ($\Delta\Psi m$), mitochondrial ATP synthesis and caspase3/7 activity were all assessed in different samples. Cell viability was significantly preserved by GYY4137 and AP72

(100-500μM) after treatment with oxidative stress agents. However, the potency was substantially increased by targeting mitochondria with AP39 and AP123 (<100nM). The collapse of ΔΨm normally observed in oxidative stress conditions was inhibited by all SRHDs. Overall "ROS" generation was markedly decreased after treatment with SRHDs. Only AP72 was able to inhibit the activation of caspase 3 after treatment with staurosporine/etoposide, but all compounds inhibited caspase-3/7 activity. Finally, pre-treatment of HMEC with SRHDs preserved ATP generation after treatment with rotenone. These data suggest that SRHDs can inhibit/reverse oxidative stress-mediated cellular injury, and highlight the increased potency of the mitochondria- targeting H2S donors AP39 and AP123 compared to GYY4137 and AP72. Strategies increasing H2S bioavailability, in particular targeting mitochondria, may represent a new therapeutic opportunity to limit mitochondrial dysfunction.

1.11.Rationale, Objectives and Hypothesis

1.11.1.Rationale

The mitochondria is the cellular organelle that controls metabolism, energy production (ATP) and cell death, and hence, the introduction of H_2S -with its protective effects against hypoxia re-oxygenation- within the mitochondria will theoretically increase the cell resistance to death or injury. With this theory in mind mitochondria targeted H_2S (AP39) should be more potent than the other widely used H_2S donor GYY4137.

Recently the slow releasing H_2S donors gained interest -being more physiological- (both AP39 and GYY4137 are slow releasing donors). While AP39 as a mitochondria targeted donor,

showed protective results in hypoxia re-oxygenation in endothelial cells in it was never studied in renal cells.
Renal tubular epithelial cells are the most vulnerable renal cells to hypoxia, and hence protecting them from IRI may impact kidney injury limitation.

1.11.2.Objectives

1- Identify the effect of AP39 on IRI in renal tubular epithelial cells.

2- Compare the effect of AP39 to those of GYY4137 (most used H2S donor lately)

3- Identify the mechanism behind the protective effects of AP39 on cellular levels.

1.11.3.Hypothesis

"We hypothesized that AP39 will have significant protective effects against hypoxia re-oxygenation when added to the porcine renal tubular epithelial cells (LLC PK1) in-vitro. Also AP39 will result in superior cell protective effects to GYY4137 after 24 hours of hypoxia followed by 24 hours re-oxygenation".

Chapter 2

2.Methodology

2.1.Experimental Design

2.1.1.Cell Culturing:

All of the steps of work in preparing the cells or the experiment plates were done in the cell culturing chamber under aseptic technique. Porcine Kidney Epithelial cells PK1(ATCC Georgetown University Washington DC USA) was the cell line used for all the in vitro experiments due to their availability, affordability, ease of culture and most importantly that they represents one of the most important cells affected in the kidney tissue by IRI. Cells were grown in a size 75 cm3 flasks with 15-20 ml of Media 199 (GIBCO@lifetechnologies.IncCarlsbad, Calif., north of San Diego. USA) supplemented by 10% Fetal Bovine Solution (FBS) and 1% Penicillin/streptomycin solution (Modified Media 199).

Flasks were incubates in a cell incubator with physiological pH, 37˚ C Temperature, 95% air, 5% CO_2 and Ultra violet (UV) sterilization. Culturing media was changed every 48hrs to allow for nutrient supply and removal of metabolic waste. When confluence of the cells reached 80 - 90%, cells were split and used to prepare wells for experiments.

2.1.2.Cell preparation for experiments:

When cells became 70-90 % confluent, they were washed with 10ml phosphate buffer solution (PBS). PBS is aspirated and then 700uL of 0.25% trypsin is added, and flask was tilted to each side so that trypsin to cover the bottom of the cell. Flasks incubated for 3-5 minutes then 10 ml of media is added to the flasks to mix with the trypsin and the detached cells then aspirated into a 15 ml test tube and centrifuged for 5 min in a speed of 500 /m.

Trypsenized media is aspirated away and cells pellet is re-suspended in Media 199. Amount of media added is according to the desired number of wells to be prepared and number of cells desired for each well. Cells were plated in 12 well plates with surface area of $3.7cm^2$ and volume capacity of 6.76ml with 50-80 x 10^4 cell/ml, in each well we put 1ml of cells in the modified media 199 (Media 199 with 10 % Fetal Bovine Serum FBS and 1% of penicillin streptomycin mix).

Minimum of 2 plates were prepared, one to be used as "Normoxia samples" and another one exposed to IR "Hypoxia samples". Plates were stored in the cell incubator for a period of 1-2 days until the cells were confluent. Experiments are started when wells were 70-80% confluent (see Experiments section below).

Both Normoxia samples and Hypoxia samples shared the same number of wells and both shared the same number of control samples (media alone) as well as treatment samples (media + H2S donor). Control samples in Normoxia samples were used as the standard to define toxic doses of the treatment, while control samples in Hypoxia samples used to define the extent of hypoxia on the cells and in comparison with treatment samples used to define the benefit or harm of the treatment dose used.

2.1.3.Ischemia Reperfusion in Vitro model

a. Hypoxia (IRI)

Culturing media (modified media 199) was aspirated from the wells (when plates are confluent) leaving the adherent cells ready for new media. DMEM (glucose and FBS free media) 1 ml was used for each well in the Hypoxia samples plates with or without treatment. Cells were placed in the Hypoxia chamber device (HypOxystation H85, info@hypoxygen.us,) which allowed us to control temperature and concentration of Oxygen (O2), Carbon Dioxide (CO_2) and Nitrogen (N). The Hypoxia chamber was set to a temperature of 37˚C, O_2 concentration of 1%, CO_2 of 5% and N of 94% with a compact gas control system to maintain gases concentration as set all the time. The Hypoxia samples plates were placed in the hypoxia chamber when it reached the preset temperature and oxygen concentration and was kept there for 24 hours. Being in Hypoxia as well as nutrients free media simulated the ischemic condition whereas the subsequent washing of cells and reconstituting in modified medium 199 1ml was added to each well with then plates then placed in the cell culturing incubator for 24 hours at 37˚C, room air, simulating Reperfusion/re-oxygenation).

The reason why 24 hours was used as the target time of hypoxia was based on the observation that 20 hours did not seem to be efficient enough to reduce cell viability in the Control samples to less than 50% (inadequate cell injury) while 28 hours and more showed a reduction of viability down to less than 10% (too much cell injury).

b. Normoxia

Plates Modified Media 199 1 ml was used for each well with or without treatment. The Normoxia samples plates are placed in the cell culturing incubator (air concentrations of O2 and CO2) for 24 hours then 1ml of modified Media 199 was added to each well and plates were returned to cell culturing incubator to complete the 48 hours of the experiment.
The treatment samples were prepared by using the desired concentration of the treatment (AP39 or GYY4137) mixed in the media (modified Media 199 in Normoxia samples and DMEM in Hypoxia samples) to form a solution for each desired concentration, then 1 ml of the prepared solution was added to the corresponding labeled well in either Normoxia or Hypoxia samples.

AP39: This compound was generously donated by Dr. Matthew Whiteman for the purposes of these experiments. (Peninsula Medical School, Exeter University, Genetics of complex Traits, Institute of Biomedical and Clinical science, St Luke's Campus, Magdalen Rd, Exeter,). Figure 2. The solid form (small crystals) (Molecular weight: 721.7692) was diluted in Dimethyl sulfoxide (DMSO) in a concentration of 20uM as a stock solution and stored immediately in -80˚C for one time use without refreezing. AP39 stock solution was then used depending on how many samples (number of wells or milliliters) and desired concentration (desired dose).
Example: When 4 samples (4 ml) of 200 nM of AP39 is needed the formula C1 V1 = C2 V2 was used 200nM x 4000 uL = 20000nM (stock solution concentration) x V2

V2= 200 x 4000 / 20000 = 40uL in 4 mL of media

GYY 4137: (Cayman Chemical Company, Ann Arbor, Michigan. USA) solid form (powder) (MW: 311.14) was diluted in Dimethyl sulfoxide (DMSO) in a concentration of 4mM as a stock solution and stored immediately in -80˚C for one time use without refreezing. Figure 3.

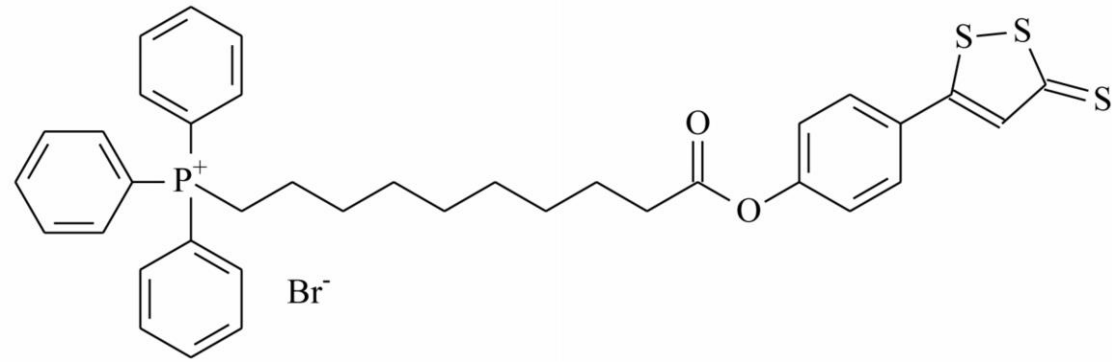

Figure 2: Structure of AP39

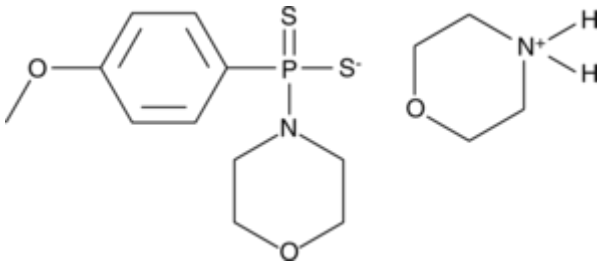

Figure 3: Structure of GYY4137

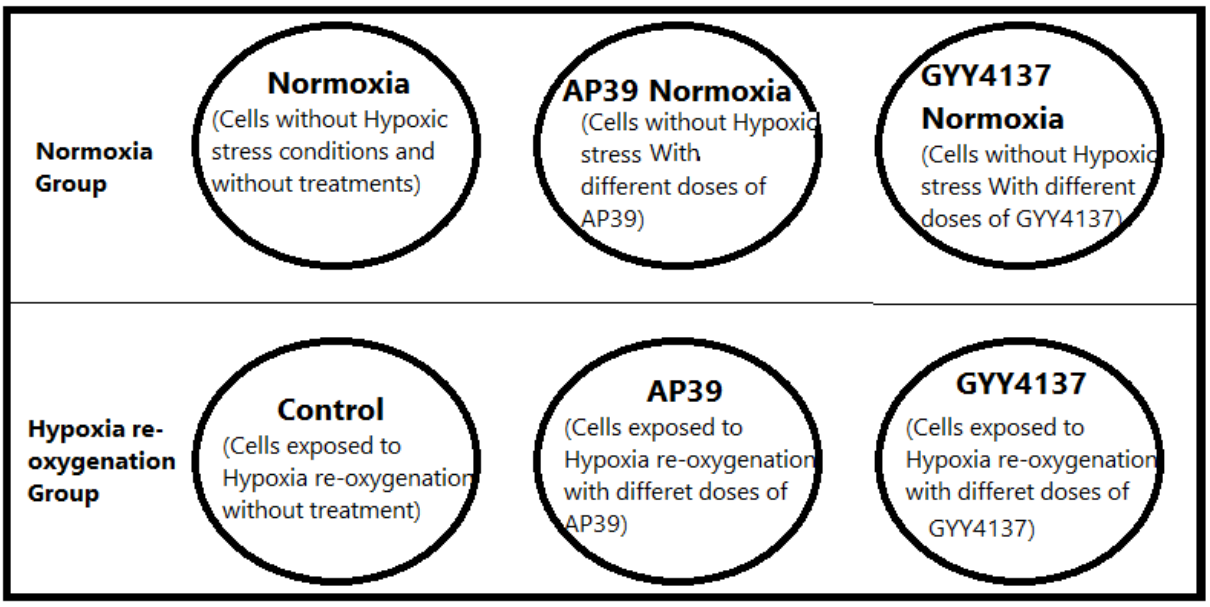

Figure 4 Different groups of cells or samples of cells.

2.1.4.Viability Assay Reactive Oxygen Species Assay

To detect the percentage of viable, Necrotic, Apoptotic and Late apoptosis early necrosis cells as well as ROS, Flow cytometry was used after staining the samples with 7-ADD (7-AAD; Bio Legend, San Diego, CA), Annexin V (Annexin V; Bio Legend, San Diego, CA) and DHR123 as detailed in sections below.

2.1.5.Cell preparation for Flow cytometer

Contents of each well were transferred into flow cytometer tubes labeled and grouped in correspondence to the wells. Each well was washed with PBS, then 200 uL of 0.25% trypsin was added to each well and allowed to act on detaching the cells off the wells for 3-5 min in the incubator. 1ml of modified Media 199 was added to each well then each well content was transferred into its corresponding flow cytometer tube.

Flow cytometer tubes were run in the centrifuging machine for 5 min with a speed of 500x g. The cells pellets formed in the bottom of the tubes were re-suspended with 1 ml of PBS. Then again tubes are run in the centrifuge machine for the same period of time on the same speed. Finally cells were re-suspended by adding 100uL of 1x Annexin V Buffer solution (needed for the Apoptosis stain Annexin V and DHR 123). Then 1 microliter of Annexin V stain and 3 microliters of 7AAD stain were added to each sample but one of the stains in each of the heat killed containing tubes (heat killed cells were prepared from one of the Normoxia samples by keeping the cells in a heat block for 10min in 60˚C) labeled as control for each 7-AAD and Annexin V.

Flow Tubes were stored in the dark for 10 min then 400uL of 1x Annexin V Buffer solution was added (to reach the minimum 500uL sample volume needed for Flow cytometer) before running the tubes in the flow machine.

Same cell preparation steps for flow cytometry detailed in the section above were followed, Instead of staining the samples with viability stains, 25uM of DHR 123 was used to stain all samples except for the unstained sample (used for voltage setting). Flow tubes were stored in the dark for 15 min before adding the remaining 400uL to each tube before running it in the flow cytometer.

2.1.6.Flow cytometer

After washing the cells and staining it as described in the preparation step, samples were analyzed by flow cytometry using the Beckman Coulter FC 500 flow cytometer (Beckman Coulter Canada LP, Mississauga, ON). Using the two laser excitation wavelengths, 488 and 633. FL2-572 BP filter was used for PE Annexin V as well as DHR 123 (in a separate run and different samples) while FL4- 675 filter was used for 7AAD. Number of events setting was set for maximum of 5000 and voltage and compensation settings were set according to the unstained samples.

For viability tests unstained samples, heat killed cells stained with Annexin V or 7AAD (positive control) were used to set the compensation and voltage to determine viabile cells in the experiment samples.

2.1.7.Quantitative RT-PCR analysis

a. RNA isolation:

In preparation for quantitative RT- PCR, RNA isolation from the plates was done using Ribozol (Ribozol ; AMRESCO, LLC. 6681 Cochran Road Solon, OH 44139 USA) to lyse the cells then stored at -80˚C until later were the Ribozol product protocol was followed to complete the isolation of RNA.

b. DNA synthesis

Following RNA isolation, Nano drop technology was used to evaluate the concentration and the quality of RNA. Super Script II Reverse Transcriptase (Invitrogen, Inc.) was used together with Oligo(dT) $_{12–18}$ primers following the product protocol. The cDNA concentration and quality was measured by the Nano drop technology as well before performing the PCR step. Isolated RNA and cDNA had a 260/280 ratings >1.9.

c. qPCR analysis

Apoptosis represented the most significant cell death pattern that was reduced by the H_2S donors, for that the genes selected were those mainly known to be anti apoptotic or pro-apoptotic to determine if any explains the protective effects of these H_2S donors.

Primer-BLAST software (NCBI) was used to design the primer sequences. Primers used are BCL2-associated X protein (BAX), mitogen-activated protein kinase 1 (MAPK1), BH3 interacting domain death agonist (BID), B-cell CLL/lymphoma 2 (BCL2) and hypoxanthine ribosyltransferase (HPRT-1), Fold-change in gene expression was calculated via the ΔΔCt method, using HPRT-1 as a reference gene. SYBR Green PCR Master Mix (Quanta Biosciences Inc.) was used for all the cDNA samples. Analysis was done using the StepOnePlus Real-Time PCR thermal cycler and software package (Applied Biosystems Inc.). Primers were purchased from Life technologies Inc. Burlington, ON. Canada and are listed in Table 1.

2.2.Statistical analysis

Statistical analysis was performed with (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA) using the ANOVA for independent groups. Statistical significance was accepted at $p< 0.05$. Data are presented according to the mean ± standard deviation (SD) in all figures and tables.

Table 1: Primer Details

MAPK1		
	Forward primer	AAACCTTCCAACCTGCTGCT
	Reverse primer	GACTTGGTGTAGCCCTTGGAAT
Bcl 2		
	Forward primer	AGGATAACGGAGGCTGGGATG
	Reverse primer	TATGGCCCAGATAGGCACCC
BAX		
	Forward primer	CCCTTTTGCTTCAGGGGATGA
	Reverse primer	CCGCCACTCGGAAAAAGACT
BID		
	Forward primer	GGATTCTAAGGTCAGCAACGGT
	Reverse primer	ATCGATTGCCGTCTGTCTGC

Chapter 3

3.Results

The objectives of the current study were to evaluate role of H_2S donors in protecting porcine kidney epithelial cells from the injury induced by hypoxia and re-oxygenation.

In our in vitro model we were able to induce this injury and asses its effects on the viability of LLC PK1 cells. We were also able to assess the percentage of the different forms of cell death that occur due to this injury. Flow cytometry using the known florescent stains (7AAD and Annexin IV) used to assess cell viability, necrosis, apoptosis and late apoptosis early necrosis are interpreted below.

3.1.Cells Viability After Hypoxia re-oxygenation injury

3.1.1.Control (Non Treated Cells) Viability

We exposed LLC PK1 cells to hypoxia for 24hr followed by re-oxygenation for 24hr then examined cell viability using flow cytometry. Cell viability decreased to 27% (SD +/- 13.1) in samples without any treatment (control) when compared to Normoxia cells that was kept for 48hr in normal culturing conditions (Viability of 82 % (SD +/- 6.2). The difference was statistically significant ($P<0.01$)

Figure (5 and 6).

3.1.2.AP39 Protective Effects on Cells Viability

Targeting the cells with AP39 at concentrations of 200nM added 10 minutes before exposing the cells to hypoxia maintained cell viability above 79% (SD +/- 10) after 24hr of hypoxia followed by 24hr of re-oxygenation. This protective effect was statistically significant ($P<0.01$) in comparison to the control samples (without any treatment). When compared to Normoxia samples, AP39 treated cells exposed to hypoxia re-oxygenation showed a viability which is nearly the same and statistically showed no significant difference indicating the potent protective effects of AP39 against hypoxia re-oxygenation. Figures (5, 6 and 7).

3.1.3.GYY4137 Protective Effects on Cells Viability

The non- Mitochondrial donor GYY4137 at concentrations 200-500nM maintained cell viability at an average of 43% (SD +/- 30) after hypoxia re-oxygenation. GYY 4137 showed to improve the viability of the cells in comparison with the control non treated group exposed to hypoxia re-oxygenation and the difference was statistically significant ($P< 0.02$). Figures (5,6 and 7).

3.1.4.Comparison Between AP39 and GYY4137 Protective Effects on Cell viability

AP39 had superior protective results compare to non – mitochondrial H_2S donors. The results showed no statistical significance between the non-treated Normoxia samples and AP39 treated cells (exposed to hypoxia re-oxygenation injury). GYY4137 was not able to achieve this statistical non-significant results achieved in the AP39 group when compared to the Normoxia samples, even with higher doses reaching up to 500mM. The higher doses of GYY4137 showed similar results, but showed toxic effects in non-stress samples (treated by high dose GYY4137 but in Normoxia conditions). Figures (6 and 7).

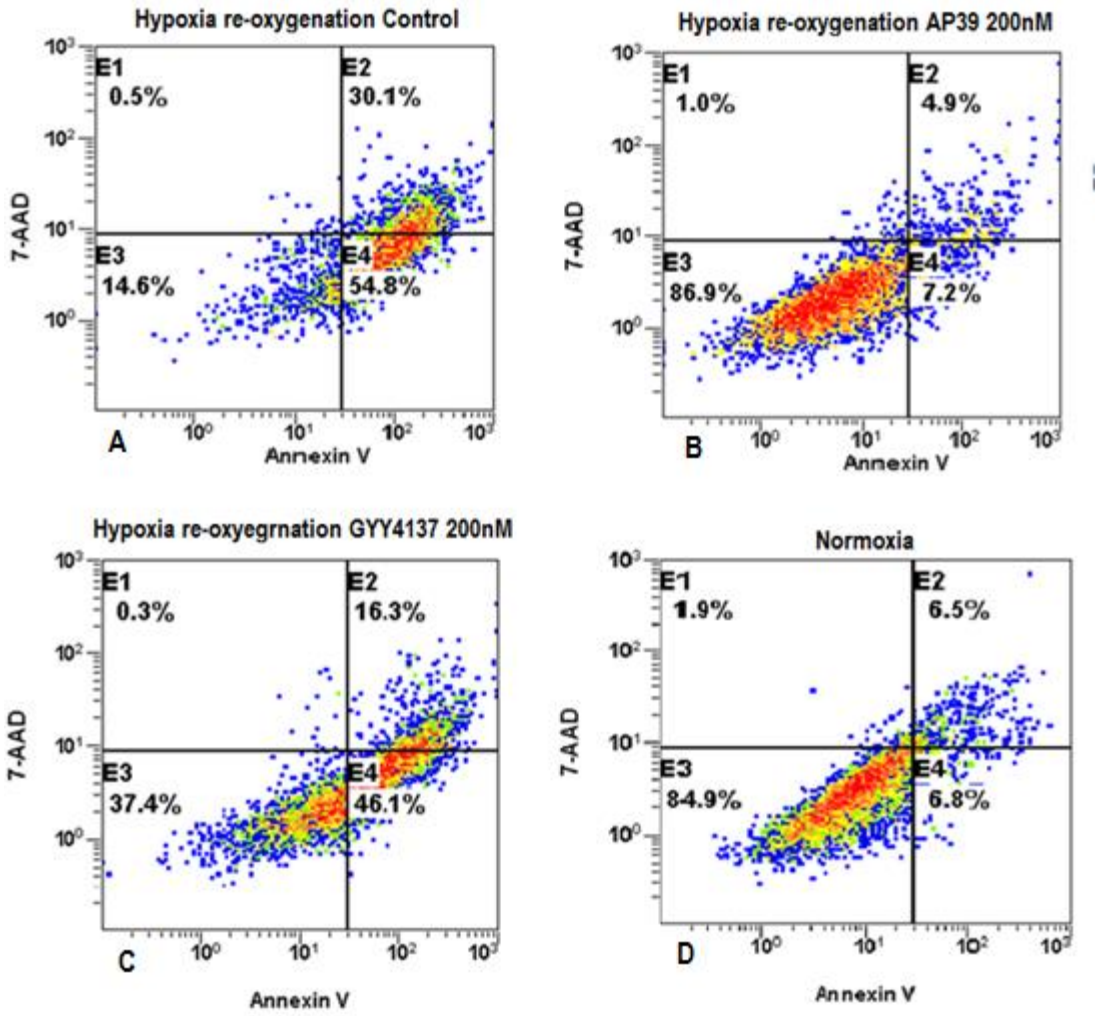

Figure 5: Flow cytometer 2-D Plot analysis of all treatment groups: A) Control samples exposed to hypoxia re-oxygenation, B) AP39 200nM treated samples exposed to hypoxia re-oxygenation, C) GYY4137 200nM treated samples exposed to hypoxia re-oxygenation, D) Normoxia samples Not exposed to hypoxia. The color represent cell population concentration: red represents maximum cell concentration while blue the least. The screen in each flow cytometer figure above is divided to four quarters labeled E1-4: E1 present Necrosis, E2: Late apoptosis early necrosis, E3: Viable tissue and E4: Apoptosis. All based on the detection of the two viability stains 7AAD and Annexin V.

The figure reflects the improvement in cell viability in the H2S treated samples in comparison to control samples. Even after hypoxia re-oxygenation exposure AP39 treated samples showed similar viability to the non-stressed Normoxia samples.

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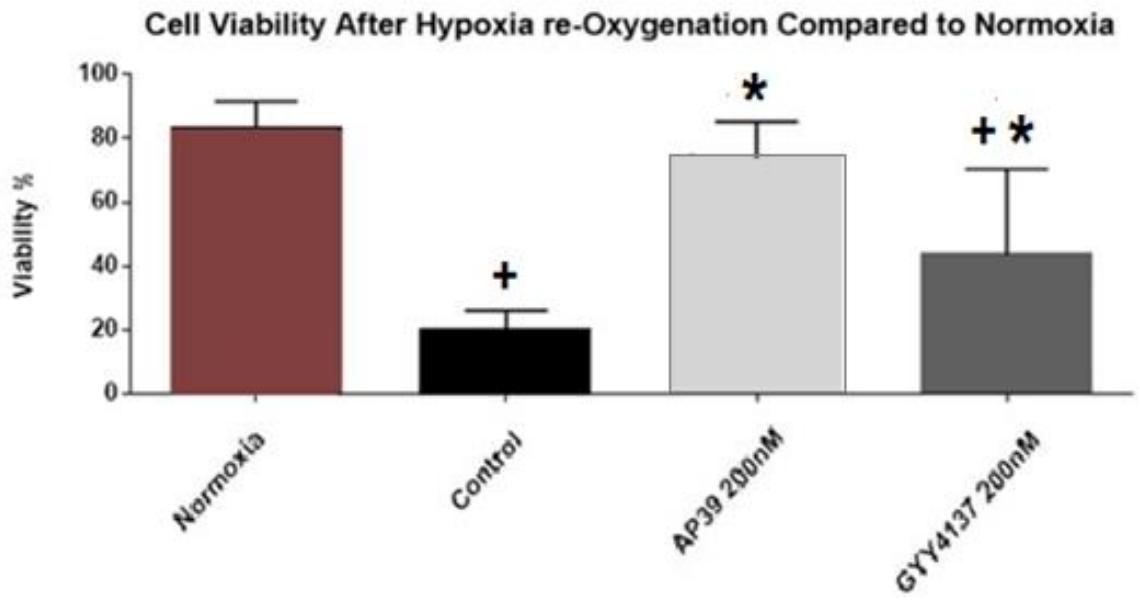

Figure 6: Cell viability after hypoxia re-oxygenation with 200nM AP39 and 200nM GYY4137 compared to control (hypoxia re-oxygenation samples without treatment) and to Normoxia (non-stressed samples without treatment). Multiple comparison of the Standard deviation of groups using one way anova: * Statistical significance exist when compared to the Control samples P<0.01. + Statistical significance exist when compared to the Normoxia samples P<0.01. Each treatment group had an average n= 7. Bars on top of each samples column represent the Standard of Error for the column.

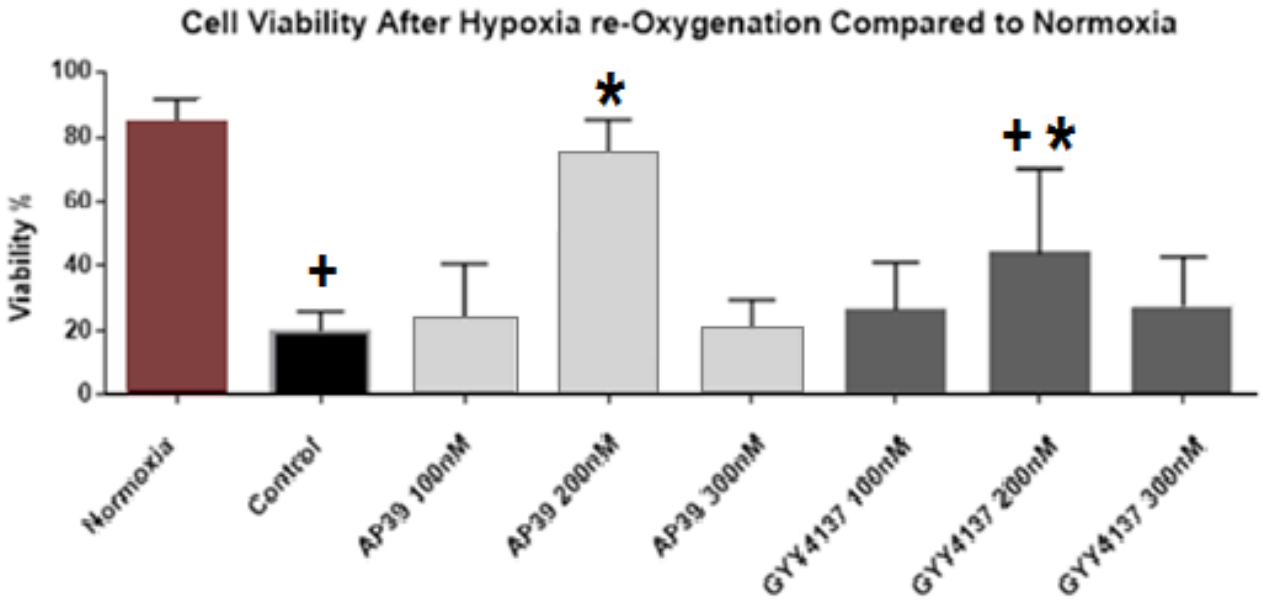

Figure 7: Cell viability after hypoxia re-oxygenation with different doses of AP39 and 200nM GYY4137 compared to control (hypoxia re-oxygenation samples without treatment) and to Normoxia (non-stressed samples without treatment). Multiple comparison of the Standard deviation of groups using one way anova: * Statistical significance exist when compared to the Control samples P<0.01. + Statistical significance exist when compared to the Normoxia samples P<0.01. The analysis above reflect the best dose identified for both AP39 and GYY4137 (200nM). Each treatment group had an average n= 5 based on repeated experements. Bars on top of each samples column represent the Standard of Error for the column.

3.2.Mitochondria-targeted H2S donor reduces Apoptosis, Necrosis and Late Apoptosis early Necrosis

Analysis of the different forms of cell death detected by using flow cytometry with different donors of H2S- showed interesting results. Detailed results discussed below.

3.2.1.Control Cells Death Forms

In the Control cells after hypoxia re-oxygenation, most of the non-viable cells were shifted towards the apoptosis axis (Annexin V marker uptake) indicating an apoptosis of around 48% (SD +/- 6.5) Figure (6). Very few percentage of cells 1.5% (SD +/- 2) where in the necrosis axis (7AAD marker uptake) Figure (8), while the late apoptosis early necrosis axis (uptake of both Annexin V and 7AAD) constituted up to 28% (SD +/- 16) of the non-viable cells, Figure (7). Compared to apoptosis <13% (SD +/- 12) in the Normoxia cells, the increase in apoptosis in control cells after hypoxia re-oxygenation was almost near 4 times the Normoxia, which was statistically significant Figure (6). Both necrosis and late apoptosis early necrosis percentage doubled in the control cells compared to the Normoxia cells Figure (7 and 8).

3.2.2.AP39 Effects on Cell Death Forms

Cells treated with AP39 200nM had a significant drop in apoptosis to as low as 14% (SD +/- 8) which when compared to the control samples and Normoxia cells showed statistical significance and non-significance respectively Figure (6). AP39 dropped cell late apoptosis early necrosis to 6.8% (SD +/- 5) compared to 28.6% (SD +/- 16) in the control group that showed statistical significance with a $P< 0.01$ Figure (9). Necrosis was significantly reduced when cells were treated with AP39 200nM to show a statistical significance when compared to the control samples and non-significance when compared to the Normoxia samples, Figure (8).

3.2.3.GYY4137 Effects on Cell Death Forms

GYY4137 did significantly decrease Necrosis when compared to the control. There was no significant difference when comparing Necrosis percentage in Normoxia and GYY4137 treated samples Figure (10). GYY4137 also significantly decreased the percent of late apoptosis early

necrosis in comparison to the control samples and with no statistical significance when compared to the Normoxia samples Figure (9).

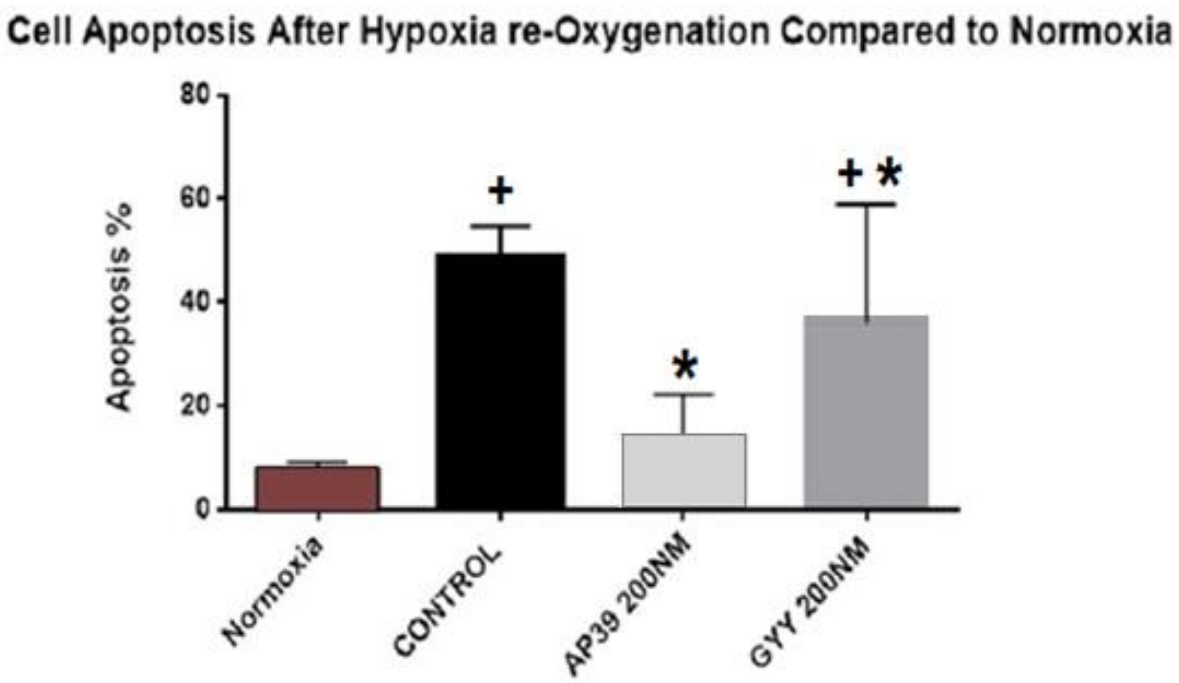

Figure 8: Analysis of percentage of apoptotic cells after hypoxia re-oxygenation with 200nM AP39 and 200nM GYY4137 compared to control (hypoxia re-oxygenation samples without treatment) and to Normoxia (non-stressed samples without treatment). * **Statistical significance exist when compared to the Control samples (P<0.01).** + **Statistical significance exist when compared to the Normoxia samples (P<0.01). Each treatment group had an average n= 5 based on repeated experements. Bars on top of each samples column represent the Standard of Error for the column.**

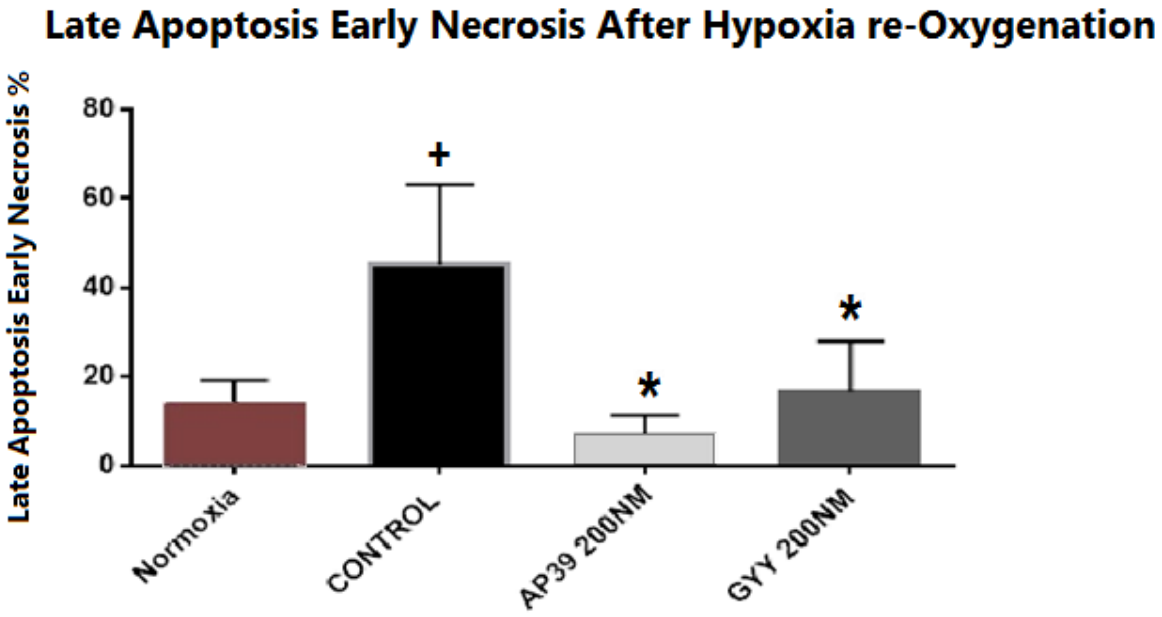

Figure 9: Analysis of percentage of late apoptosis early necrosis cells after hypoxia re-oxygenation with 200nM AP39 and 200nM GYY4137 compared to control (hypoxia re-oxygenation samples without treatment) and to Normoxia (non-stressed samples without treatment). * Statistical significance exist when compared to the Control samples (P<0.01). + Statistical significance exist when compared to the Normoxia samples (P<0.01). Each treatment group had an average n= 5 based on repeated experements. Bars on top of each samples column represent the Standard of Error for the column.

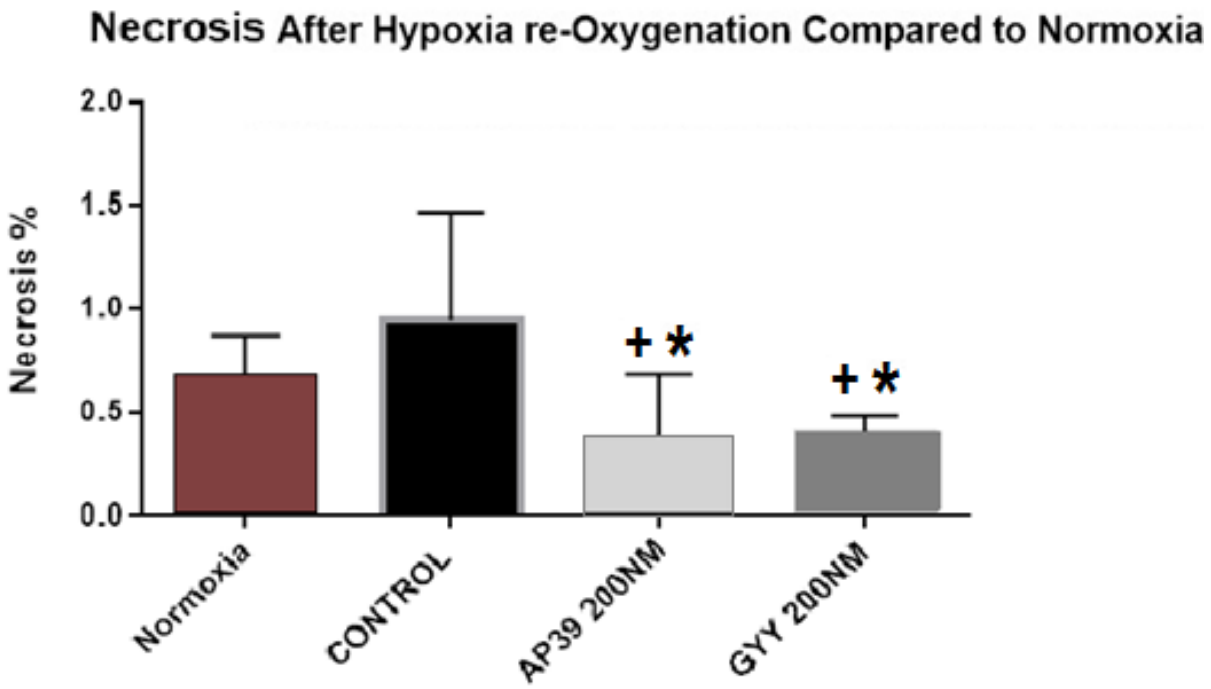

Figure 10: Analysis of percentage of necrotic cells after hypoxia re-oxygenation with 200nM AP39 and 200nM GYY4137 compared to control (hypoxia re-oxygenation samples without treatment) and to Normoxia (non-stressed samples without treatment). * **Statistical significance exist when compared to the Control samples (P<0.01).** + **Statistical significance exist when compared to the Normoxia samples (P<0.01). Each treatment group had an average n= 5 based on repeated experements. Bars on top of each samples column represent the Standard of Error for the column.**

3.3.Mitochondria Targeted H_2S Donor decreased the reactive oxygen species

Part of the work on identifying the important mechanisms by which the mitochondria targeted H2S defend the cells more effectively than the non-targeted donors, amount of ROS produced within the cells were analyzed using the flow cytometer to detect the florescent stain DHR123 which is activated in the presence of ROS.

In the control group ROS was evident up to 78% with a mean of 75 % (SD +/- 3.5) and detected on flow cytometer. While Normoxia samples in culturing environment (non stress conditions) had maximum of 15% (SD +/- 2.6). Difference was statistically significant between the 2samples ($P<0.01$) Figure (10). In the AP39 200nM treated cells, the amount of ROS was 33.5% with a mean of 34.8% (SD +/- 1.25). Results were statistically significant when compared to the control cells ($P<0.01$). GYY4137 dose of 200nM (that was effective in overall viability) did not induce any reduction in ROS and showed similar amount of ROS without any significant difference when compared to the control sample (Figure 11 and Figure 12).

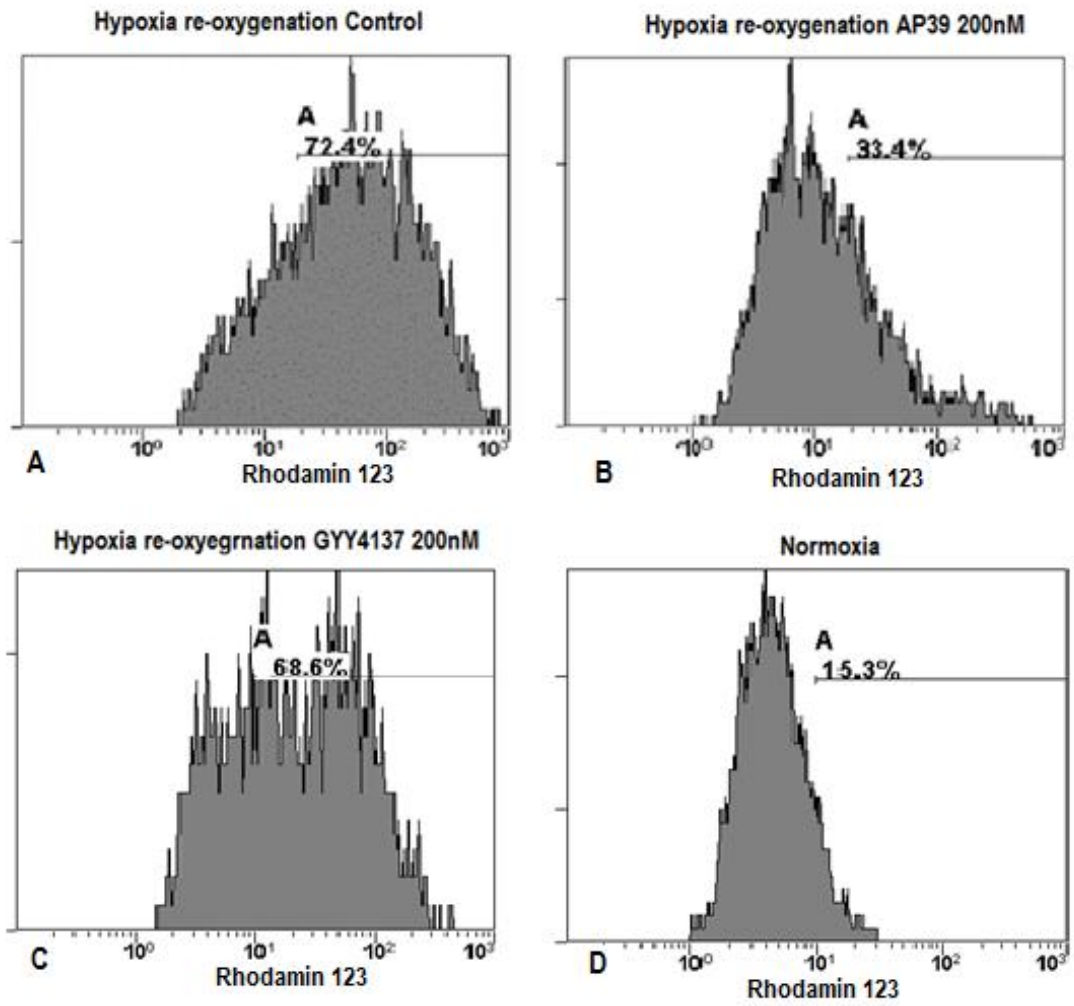

Figure 11: Flow cytometer Histogram Plot analysis for the detection of ROS in different treatment samples: A) Control samples exposed to hypoxia re-oxygenation, B) AP39 200nM treated samples exposed to hypoxia re-oxygenation, C) GYY4137 200nM treated samples exposed to hypoxia re-oxygenation, D) Normoxia samples (Not exposed to hypoxia and without treatment). All based on the detection of Rhodamine 123. The figure reflects the reduction of ROS in the AP39 samples in comparison to control samples but no reduction in GYY4137 samples.

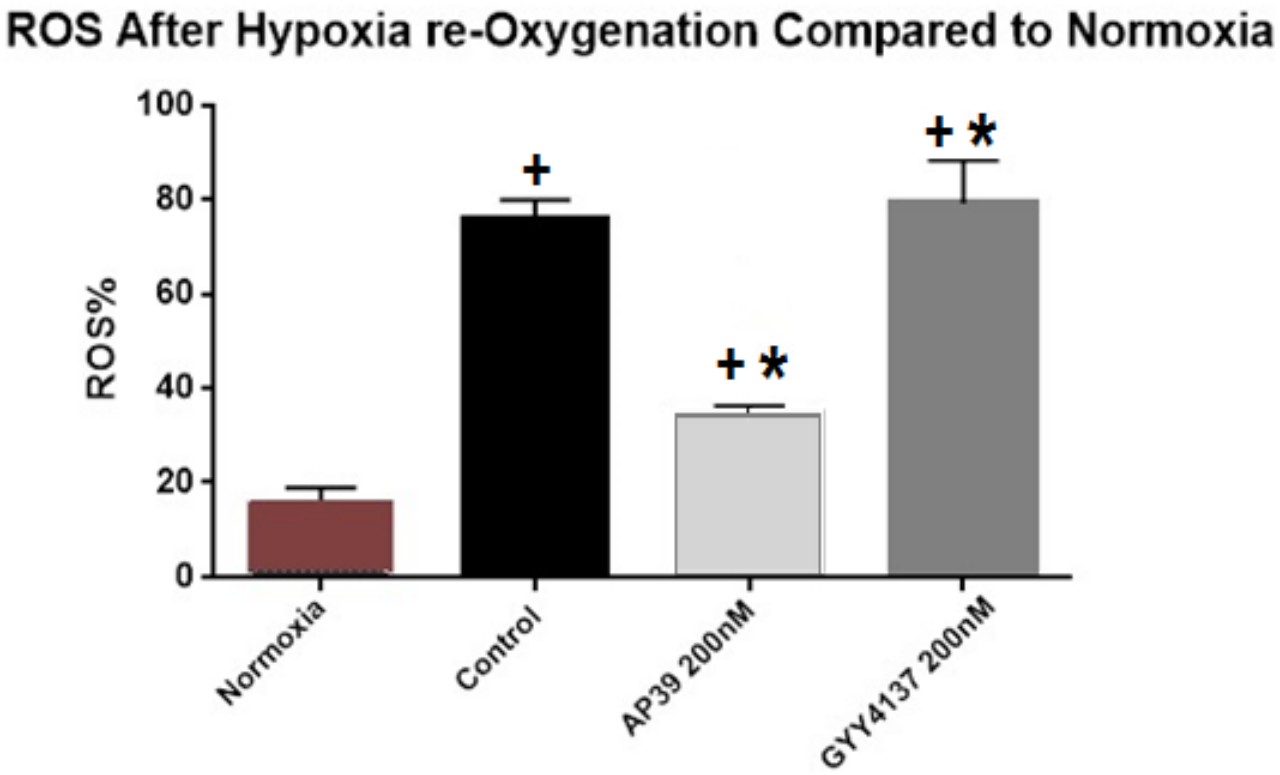

Figure 12: Percent of ROS detected by Flow cytometry after hypoxia re-oxygenation with 200nM AP39 and 200nM GYY4137 compared to control (hypoxia re-oxygenation samples without treatment) and to Normoxia (non-stressed samples without treatment). * Statistical significance exist when compared to the Control samples P<0.01. + Statistical significance exist when compared to the Normoxia samples P<0.01. Each treatment group had an average n= 4 .

Bars on top of each samples column represent the Standard of Error for the column.

3.4.Evaluation of Apoptosis Related Genes

Further work to define the mechanisms behind the difference in potency between AP39 and GYY4137 in protecting LL PK1 cells from hypoxia re-oxygenation injury, we used the real time q PCR analysis. Since apoptosis was most prominiant cell death pathway in hypoxix re-oxygenation injury and most remarkably prevented by H_2S donors we focused on some common genes known to play a role in the control of apoptosis and mitochondria protecting mechanisms. The genes tested were: Bid (BH3-interacting domain death agonist), Bax (Bcl-2-associated X protein), BCL2 (apoptosis regulating gene by controlling the mitochondrial membrane permeability) and MAPK (regulates cell signals in responses to stress).

In the Control samples Both Bid and Bax dropped significantly after the hypoxia re-oxygenation stress compare to the non-stressed Normoxia group. While both MAPK and BCL2 showed marked amplification, the increase in MAPK was more significant Figure (13).

When AP39 treated cells were compared to control and Normoxia samples, AP39 fully prevented the drop in Bax and prevented the increase in MAPK in comparison to the control samples and more in favor towards the Normoxia samples. Changes in Bid and BCL2 were not as obvious as the other tested genes, however the increase in BCL2 was slightly higher than the Normoxia samples but not as high as the control samples.

In the GYY4137 treated samples, It slightly limited the drop in Bax when compared to the control samples but definitely not as much as the treatment with AP39 did, that was also the case in MAPK were it did increase more than the AP39 samples but not as high as the control samples. GYY4137 treatment showed changes that is opposite to the Normoxia group in Bid and more similar to the control in BCL2 (Figure 13).

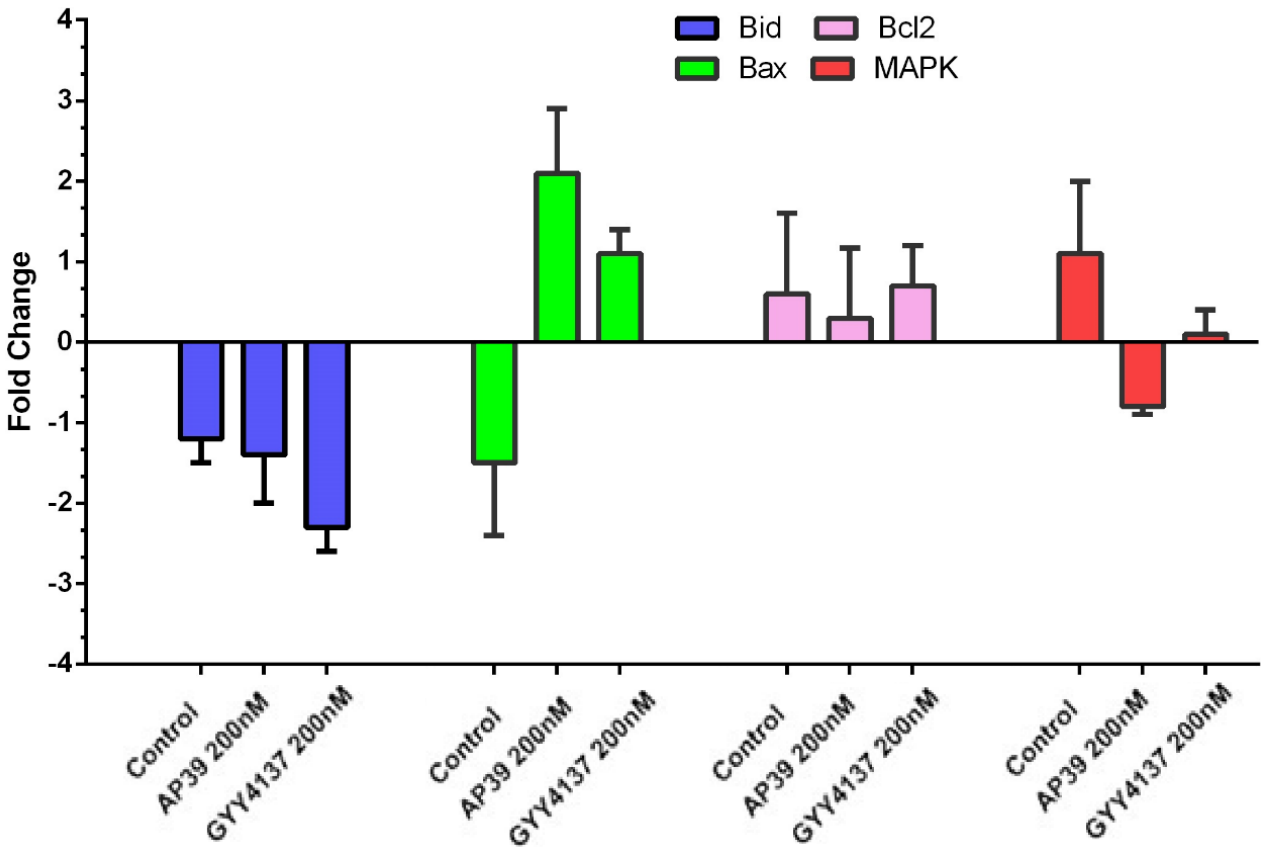

Figure 13: PCR results reflecting the expression of various genes involved in apoptosis. The zero point in the fold change axis represent the level of gene expression in Normoxia samples. Bid gene expression was reduced in both H2S treatment samples but more in GYY4137 samples, Bax and Bcl2 increased in both H2S treatment samples. MAPK decreased in the AP39 but increased slightly in the GYY4137 samples.

All samples had average of n=5.

Chapter 4

4.Discussion

Delayed graft function is a major limiting factor in kidney transplantation which has a significant effect on both the short and long term outcome of the graft. It is commonly accepted that one of the major contributors to DGF is IRI. Several studies have demonstrated the efficacy of H2S in protecting various tissues during IRI including heart, brain and muscle. We have recently shown that H2S not only protects the kidney during cold IRI, but it also has a protective effect against warm IRI associated with transplantation in both short and long term on animal studies (Lobb, et al., 2012; Lobb, et al., 2014).

The importance of mitochondria as an organelle with a major role in energy production and cell death has always received a significant amount of interest from those studying IRI. In fact, H2S has been shown in several studies to have multiple protective roles including its role as a potential donor of electrons in the electron transport chain, and thus contributing to ATP synthesis. Other protective benefits are by reducing ROS induced mitochondrial damage and limiting the mitochondrial membrane permeability.

Recently, mitochondria targeted H2S donors have been created and have shown promising protective effects during oxidative stress in human endothelial cells, however, these donors have not yet been tested on renal cells. Here we compared these mitochondria targeted donors with one of the widely accepted H2S donors GYY4137 (due to its slow releasing mechanism that simulates the physiological production of H2S in the body).

The H_2S donor AP39 (one of few mitochondria-targeted H_2S donors) showed to be protective against IRI in our study. As shown in figure (5) the addition of AP39 was able to improve cell viability following IRI as well as significantly reducing cell apoptosis as shown in figure (8). Previous studies on AP39 showed its initial efficacy in protecting murine brain microvascular endothelial cells from oxidative stress (Szczesny et al., 2014)

Our study showed that introducing H_2S donors targeting the mitochondria during the ischemic phase of IRI protected the renal tubular epithelial cells from the harmful cascades during both ischemia and reperfusion injuries. The potent protective effect of the mitochondria targeting H_2S donor AP39 was superior to that of the non-mitochondria targeting H_2S donor GYY4137. We

believe this study is the 1st study to show the efficacy of AP39 on protecting kidney tubular epithelial cells from hypoxia re-oxygenation.

Our study showed that 200nM of AP39 achieved the best protective effects which is within the same range (50-300nM) used in Szczesny's (Szczesny et al., 2014), however, the dose that showed best results in our study was 200nM. Variability might be due to the use of different cell line and different species with different sensitivity and metabolism of H_2S and the degree and mechanism of the oxidative stress we used.

The ischemia reperfusion model we used has the advantage of being a non-chemical real hypoxia stress using the high efficiency hypoxia chamber, assuring the maintenance of 1% O2 concentration during the entire hypoxia phase of the experiment as well as humidity and sterility. We also noted that previous studies on LLC-PK1 using chemical or enzymatic methods of ischemia showed a disagreement on the time of hypoxia needed to induce sufficient injury in different studies (Wiegele, Brandis, & Zimmerhackl, 1998); (Ueda, Kaushal, Hong, & Shah, 1998)(Hotter, Palacios, & Sola, 2004).

A disadvantage of such a model when compared to the chemical and enzyme induced IRI is the longer duration needed for cell injury in the hypoxia chamber.(Kurian & Pemaih, 2014) This was the only disadvantage reported by Kurian et al. on the same cell line where they tested the effects of NaSH (Rapid releasing H2S donors) on three different models using chemical, enzyme or chambers to induce IRI. However they noted that 12hr ischemia was not enough to induce more than 50% injury in the chamber model.(Kurian & Pemaih, 2014) they stated that the hypoxia chamber type of hypoxia requires more time of exposure to induce significant damage.

In our study we did achieve significant cell injury after 20 - 24hr. When cells were exposed to < 20hr of hypoxia, the injury was not able to induce enough cell death, at least not consistent as the 24hr. Specially with the increased number of passages on cultured cells their ability to tolerate <20hr of hypoxia was higher. When cells were exposed to 28hr or more of hypoxia, some samples showed a near 100% cell death which affects the ability to notice some of the effects of different treatments as well as the chance for reversibility or worsening of the injury during re-oxygenation.

Despite the different available H_2S donors in the field, GYY4137 has the advantage of being a slow releasing H_2S donor which we believe is needed for its effects to last as long as possible in such long periods of warm hypoxia which is also more physiological (Li, Salto-Tellez, Tan,

Whiteman, & Moore, 2009). Previously used H_2S such as NaHS and Na2S were noted to release large amounts of H_2S as fast boluses which may induce toxic, less lasting effect and less physiological.

Ling Li et al showed the benefits of using a slow H_2S releasing donor such as GYY4137 over the inorganic H_2S donors as NaHS. GYY4137 did not show the cytotoxic, cell cycle altering effects or apoptosis that were shown with NaHS on cultured rat vascular smooth muscle cells. Excessive and rapid release of H_2S (when NaHS was used) initiated a cascade of signaling pathways that results in cell death. Concluding that slow build up to low concentrations of H_2S is more physiological and can improve cell viability in vivo which is simulated by the use of slow releasing H_2S donors. While the cytotoxic effects or pro-apoptotic effects occurs when high concentrations of H_2S build up over a short period of time simulated by the use of inorganic rapid releasing H_2S donors.

Interestingly GYY4137 was shown to induce vasorelaxation that can drop the blood pressure and might be of concern if used in humans. It will be of interest to evaluate the effects of AP39 on similar studies to see if its effective dose affects the blood pressure or not. In the current study, we chose to use porcine renal tubular epithelial cells due to their multiple advantages. Other than being easy to culture and affordable, renal tubular epithelial cells are the most sensitive cells in the kidney to ischemia. Due to the function of ion transport they require a high metabolic rate but their ability to function in anaerobic glycolysis is limited.

Using a glucose and serum free media during the hypoxia phase and switching back to glucose and serum rich media during reperfusion simulates the more clinical scenario where cells are deprived of energy sources during hypoxia then regain those during reperfusion. This way of alteration in media to simulate the real ischemia reperfusion situation was previously used and showed efficacy during in vitro studies (Jiang, Liu, Luo, & Dong, 2010). The fact that AP39 targets the mitochondria and releases H_2S there, supports the hypothesis that AP39 protective effects were significantly more potent than GYY4137 when equal doses were used. AP39 was superior in results even when we used higher doses (50 – 500uM) of GYY4137 that was used previously in our lab and in published in-vitro work.

We found that the effects of AP39 on improving cell viability during IRI was superior by at least 20%. Our working hypothesis was that AP39 releases H_2S at the level of the mitochondria where it is utilized as a source of ATP, albeit inefficient, and works to provide a protective effect during

oxidative stress. Unfortunately this action is not yet fully understood. Given that the majority of cell death in vertebrates is thought to be through the mitochondrial pathway of apoptosis (Green & Kroemer, 2004) and these signal pathways are of significant importance in the pathogenesis of many ischemia related disease, (Thompson, 1995) we believe that our data support a novel therapeutic application for H_2S donors, particularly ones that are targeted to the mitochondria. Among renal diseases, the first description of apoptosis was in 1987 in an animal model of hydronephrosis (Gobe & Axelsen, 1987). IRI was noted to significantly increase apoptosis and remarkably more in kidneys with delayed graft function (Oberbauer, Rohrmoser, Regele, Muhlbacher, & Mayer, 1999). These early studies clearly point to the important role of apoptosis in IRI and that controlling it or reducing it may contribute to an improvement in the outcome of kidney as well as other solid organ transplants.

Both H_2S donors in our study successfully improved cell viability by reducing all apoptosis, necrosis and late apoptosis early necrosis, but the reduction in apoptosis by AP39 was exceptional. AP39 reduced apoptotic cells to a level similar to that of Normoxia (not exposed to IRI) samples without any statistical significant difference between the two. Some studies have used both Annexin V and 7AAD to define Late apoptosis early necrosis cells as necroptosis with or without the addition of a RIP1 inhibitor that blocks necroptosis pathway with necrostatin-1(Nec-1) or TUNNEL staining (Pan et al., 2014)(Ch'en, Tsau, Molkentin, Komatsu, & Hedrick, 2011). Whether all such cells were truly reflected as necroptosis or late apoptosis early necrosis or late apoptosis, it is still reduced with both donors significantly.

To further understand the protective mechanism behind AP39's effects against hypoxia re-oxygenation in renal tubular epithelial cells, we evaluated the reduction of ROS. The reduction of ROS was evident by at least 20% and 5% in the AP39 and GYY4137 respectively when compared to the control group. Considering the hazardous effects of these ROS on the mitochondria and the entire cell, explains that even a minor reduction in such molecules can explain part of the protective effect of these donors. The test we used for ROS detection, the DHR123 assay is considered the best effective probe used in human granulocytes as flow cytometric assessment of the oxidative burst (Walrand et al., 2003), and is also known to be highly sensitive to H_2O_2.

Superoxide is formed first in the inner mitochondrial membrane at the electron transport chain (ETC) I and III by a transfer of a free electron to molecular oxygen. The mitochondrial enzymes

then reacts with it to form H_2O_2. In the presence of GSH the enzyme glutathione peroxidase (GSPx) converts H_2O_2 to water as a defense against ROS. (Boveris & Chance, 1973); (Turrens & Boveris, 1980); (Turrens, Freeman, Levitt, & Crapo, 1982)

To some extent H_2O_2 is known to be less reactive than superoxide, while the latest is known to be produced more by the mitochondria (Quinlan, Perevoschikova, Goncalves, Hey-Mogensen, & Brand, 2013). Despite the fact that both H_2S donors were able to protect the cells from hypoxia re-oxygenation, the amount of persistent DHR123 signals on flow was still significant. This reflects the presence of less reactive amounts of ROS (like H_2O_2) that induces a positive signal but insufficient or of less reactivity (not like superoxide) to induce injury.

Whether AP39 and GYY4137 reduced the production of ROS or consumed the ROS produced during this hypoxia was not addressed in our study. When Shen et al. did western blots on human umbilical vein endothelial cells (HUVECs) after an in vitro IRI in both NaHS treated cells and compared it to control, NaHS increased Bcl2 (anti-apoptotic protein) but decreased Bax as well as many other pro-apoptotic proteins like Caspase 3 and 9 (Shen et al., 2013). In a non IRI model where NaHS was added to lung fibroblasts cells, the anti-apoptotic Bcl2 protein was not unregulated, which reflects the different effect of H2S on Bcl2 in normal versus IRI conditions. (Baskar, Li, & Moore, 2007) Many other mechanisms of H2S protection of the mitochondria have previously been shown including modification of the action potential, electron donor, stimulant of electron transport in the mitochondria (Wang, 2012) attenuation of homocysteine induced toxicity (Kamat, Kalani, Tyagi, & Tyagi, 2014) or Inhibition of Ca^{++} induced mitochondrial permeability transition pores.

As our study was focusing on the effect of targeting the mitochondria by this new H2S donor during IRI, selecting members of the family of Bcl-2 proteins was chosen due to its known role in apoptosis. The Bcl-2 is the prototype member of a family of proteins containing at least one Bcl-2 homology (BH) region. Based on the classification of the family of Bcl-2 proteins for classification we selected a major anti-apoptotic protein like Bcl-2 and pro-apoptotic multidomain proteins: Bax and Bad (De Marchi et al., 2004).

Previously some studies showed that Bax expression was associated with DNA fragmentation and induction of apoptosis (Lasso, et al., 2007). However De Marchi et al showed that blocking the Bcl-2 family pro-apoptotic proteins like Bax does not fully protect the mitochondria and that mitochondria can still be permeabilized by inducing a permeability transition PT such as high

intra cellular Ca_2, hexokinase/VDAC-dependent mechanism and other mechanisms. (Majewski et al., 2004)(Kroemer, Galluzzi, & Brenner, 2007) Also in another study Bax did not show any changes in an ex vivo model on rat cerebral cortex after 24 hypoxia neither alone nor after re-oxygenation of hypoxia followed by oxygenation (Hu et al., 2003). The renal tubular cells of human kidneys also did not show any difference in Bax expression in Oberbauer R et.al study. These authors studied biopsy samples from transplanted kidneys from cadaveric donors with evidence of ischemic injury and apoptosis and compared it to biopsies from well-functioning kidneys of living donation with significantly less apoptosis (Oberbauer et al., 1999).

In our study there was an unexpected increase expression of the gene of this pro-apoptotic protein Bax in the AP39 treated samples. Normally Bax and Bid are cytosolic proteins but once apoptosis signals are initiated both of these proteins start to form supramolecular openings or bind to the permeability transition pore complex (PTPC) to release proteins from the inter membrane space (IMS) of the mitochondria leading to cell death (Kroemer et al., 2007). Knowing the role of Bax a reasonable explanation for its rise in the AP39 group might indicate that AP39 does not inhibit the apoptosis initiating signals (hence transcription of Bax is overexpressed), instead it inactivates the cascade that leads to apoptosis by inactivating Bax, This inactivation of Bax might be leading to a persistent transcription of its gene in order to get the usual feedback inhibition (that is accomplished by an active Bax) that shuts down the apoptosis signaling mechanism or perhaps cell death. Also another possible explanation is that a number of other molecules are known to bind to the pro-apoptotic proteins when formed and inhibit their function. For example, 14-3-3e a molecule known to prevent Bad from translocation to the mitochondria and it also sequestrates Bax in the cytosol (Kroemer et al., 2007). Along with the Bcl-2 family, Ca_2 and some chemotherapeutic agents (Brenner & Grimm, 2006) showed to affect the PTPC opening which supports the theory of another pathway other than the pro apoptotic genes tested in LLC-PK1 cells.

IRI is no doubt much more complex than being represented by an in vitro hypoxia re-oxygenation model, but hypoxia re-oxygenation is the most important role player in initiating many cascades responsible for the full picture of IRI. Modulating the hypoxia re-oxygenation factor will continue to be of major research interest. The exact cellular hypoxia re-oxygenation conditions in real IRI in the living tissues are difficult if ever possible to be exactly simulated in an in vitro experiment.

Our study shows clearly that AP39 has a potent protective effect on renal tubular cells against hypoxia re-oxygenation as well as its superiority when compared to GYY4137. Further in vitro work should consider looking into the amount, duration and peak of H_2S released after AP39 and compare it to that released from similar doses of GYY4137. That might add more explanation to the comparison, however, the potent protective effects of AP39 was not challenged with even higher doses of GYY4137.

Our data suggests that AP39 is a viable candidate for ex vivo studies aiming to avoid the cold injury that is applied as a concept of organ storage. However there is no reason why AP39 cannot be of benefit in cold hypoxia re-oxygenation injury trials. Also in vivo experiments on animal models of kidney IRI or transplant will remain an essential step for the transition of the use of such H_2S donor in the clinical practice to assure its safety and efficacy.

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Department of Surgery, Division of Urology, London, Ontario, Canada
Discipline: Kidney Pancreas Transplantation
July 1, 2014 to July 31, 2015

2. Masters of Surgery:

2013 – 2014 **University of Western Ontario**
Department of Surgery, Division of Urology, London, Ontario, Canada
Discipline: Kidney Pancreas Transplantation
August 1, 2013 to May 20, 2015

3. Research Fellowship Training

2013 – 2015 **University of Western Ontario**
2013 – 2014 **University of Western Ontario**
Department of Surgery, Division of Urology, London, Ontario, Canada
Discipline: Kidney Pancreas Transplantation
August 1, 2013 to June 30, 2014

4. Post-Graduate Medical Training

2007 – 2012 **King Abdulaziz Medical City, National Guard Health affairs**
Department of Surgery, Division of General Surgery
Jeddah, Western region, Saudi Arabia
Discipline: Residency in General Surgery (PGY 1-5)
Oct 1, 2007 to Nov 1, 2012

2012 **The Methodist Hospital**
Department of Surgery, Division of Transplant
Houston, Texas, USA
Discipline: Clinical observer in Transplant (2 weeks, PGY: 5)
Supervisor: Osama, Gaber MD, FACS
Feb 6, 2012 to Feb 24, 2012

2011 **King Abdulaziz University Hospital**
Department of Surgery
Jeddah, Western region, Saudi Arabia
Discipline: Clinical elective in General Surgery (6 months, PGY: 4)
Supervisor: Professor A. Makkawi , MD RCSE
January 1, 2011 to Jun 31, 2011
Discipline: Clinical elective in Endoscopy (6 weeks, PGY: 4)
Supervisor: Professor A. Alsibyani , MD SGSB
Aug 1, 2011 to Sep 14, 2011

Cont. ACADEMIC RECORD

5. Internship Training:

2006 – 2007 General Surgery 3 months
Internal Medicine 3 months
Pediatrics 2 months
Obstetrics & Gynecology 2 months
ER 3 weeks
Elective General Surgery 3 weeks

6. Medical School:

2000 – 2006 **King Abdulaziz University, Medical College.**
Faculty of Medicine; Degree: Doctor of Medicine
June 2000 to July 2006

PROFESSIONAL CERTIFICATIONS

United States Medical Licensing Examination
2014 Examination Step II Clinical Knowledge

College of Physician and Surgeon of Ontario
2013 - present Registration Number: 101901
Membership Certificate

English Language Examinations
2013 International English Language Testing System

Saudi Board of General Surgery
2012 General Surgery Board Certificate

Arab Board of General Surgery
2010 General Surgery Board Certification-Part I (September 2007)

Medical Council of Canada
2007 License#:
Medical license Evaluation Examination (September 2007)

Saudi Council for Health Specialties
2006 Medical License Evaluation Examination

PROFESSIONAL SOCIETY MEMBERSHIPS

2014 – present	American Society of Transplantation
2014 – present	American Society of Transplant Surgeons
2013 – Present	College of Physicians & Surgeons of Ontario
2008 – present	Saudi General Surgery Society

EXECUTIVE & COMMITTEE POSITIONS

2012 - 2013	**Member, Trauma Committee** King Abdulaziz Medical City, Jeddah, Saudi Arabia
2012 - 2013	**Co-Chair, Resident Day Organizing Committee** King Abdulaziz Medical City, Jeddah, Saudi Arabia
2010 - 2012	**Member, Resident Day Organizing Committee** King Abdulaziz Medical City, Jeddah, Saudi Arabia

COURSES ATTENDED

2014	**DaVinci Robotic Surgery Training Program** University of Western Ontario. London. ON. Canada
2014	**Excel Advanced course 2014** University of Western Ontario. London. ON. Canada
2014	**Pub Med powers 2014** University of Western Ontario. London. ON. Canada
2013	**Biostatistics 9509A (Auditing) 2013** University of Western Ontario. London. ON. Canada
2013	**Clinical research course 2013** King Abdulaziz Medical City, Jeddah, Saudi Arabia
2013	**Basic Life Support –BLS (renewed)** King Abdulaziz Medical City, Jeddah, Saudi Arabia
2013	**Single port laparoscopy course** King Fahad Armed Forces, Jeddah, Saudi Arabia
2012	**Surgery review course 2012** King Abdulaziz Medical City, Jeddah, Saudi Arabia
2011	**Advanced Trauma Operative Management-ATOM** King Abdulaziz Medical City, Jeddah, Saudi Arabia
2011	**Advanced Trauma and Life Support** King Fahad Armed Forces Hospital, Jeddah, Saudi Arabia
2011	**Bowel and Vascular Anastomosis** King Abdulaziz University Hospital, Jeddah, Saudi Arabia
2010	**Advanced Cardiac Life Support** King Abdulaziz Medical Center, Jeddah, Saudi Arabia

2010 **Basic and advanced Laparoscopy course**
King Faisal Specialist Hospital, Jeddah, Saudi Arabia

2008 **Focused Assessment with Sonography for Trauma –FAST**
King Abdulaziz Medical City, Jeddah, Saudi Arabia

2008 **Basic and advanced Laparoscopy course**
King Abdulaziz Medical City, Jeddah, Saudi Arabia

2008 – 20012 **Surgery Clerkship Orientation**
King Abdulaziz Medical City, Department of Surgery
-Taught basic principles and techniques of suturing and knot tying to medical students and interns

PUBLIC RELATIONS

2004 – 2009 **United Hands Team**: *Formal official representative of the team*.
A charity team consisting of volunteers of male& female medical students working together to assure a good quality health service for poor needy people in Jeddah, Saudi Arabia (www.uhteam.org).

2005 – 2006 **The International Diabetes Day** : Organizing committee

2004 **The Diabetes Awareness Summer Campaign Workshops**: Organizing committee

PRESENTATIONS and INVITED TALKS:

2014 "Graft Thrombosis and VTE post Kidney Transplant"
UWO, Kidney Transplant Journal Club, London, ON, Canada

2014 *" Simultaneous Liver Kidney Transplant"*
UWO, Liver Transplant Rounds, London, ON, Canada

2014 *"The Best Induction Choice in Elderly Renal Transplantation",*
UWO, Kidney Transplant Journal Club, London, ON, Canada

2013 *"Benign Surgical Diseases of The Liver"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2013 *"The Abdominal Trauma Made Easy"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2013 *"Kidney Transplant for The Surgical Boards"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2012 *"Physiological Monitoring and Ventilation in Critical Care"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2012 *"Management of Septic Shock in Surgery"*
King Abdulaziz Medical City Academic Teaching, Jeddah, Saudi Arabia

2011 *" Management of Hernias in Pediatrics"*
King Abdulaziz Medical City Academic Teaching, Jeddah, Saudi Arabia

2011 *"Anatomy of the Vessels and Physiology"*
King Abdulaziz Medical City Academic Teaching, Jeddah, Saudi Arabia

2010 *"The Retroperitoneal Injury Zones"*

King Abdulaziz Medical City Academic Teaching, Jeddah, Saudi Arabia

2010 *"Gallbladder Benign Surgical Disorders"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2010 *"Peritonitis Types and Treatment"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2010 *"Bleeding Disorders and Perioperative Optimization"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2009 *"Nutrition in The Surgical Patient"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2009 *ICU Made Easy*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2009 *"Colonic Polyps and Colon Cancer Screening"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2008 *"Benign Breast Diseases"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

2008 *"The Surgical Patient Metabolism"*
Grand rounds, King Abdulaziz Medical City, Jeddah, Saudi Arabia

AWARDS and DISTINCTIONS

2012 **Resident of the year:**
King Abdulaziz Medical City , Jeddah, Saudi Arabia

2009 – 2013 **Performance Award:** *for acting in resident day Movies*
King Abdulaziz Medical City , Jeddah, Saudi Arabia

ABSTRACTS PRESENTATION:

2015 **Canadian Urology Association:**
Robotic Vs Laparoscopic Donor Nephrectomy Pain and Cosmetic outcome.
To be presented June 2015

2015 **American Congress of Transplant:**
Hydrogen Sulphide Protects Renal Epithelial Cells from Ischemia Reperfusion Injury

To be presented May 2015

RESEARCH TRAINEES SUPERVISED

2014 - 2015 *"Does Heparin supplementation to kidney pancreas transplant recipients diminish graft thrombosis rates?"* Mr. Patrick Anderson (MD Candidate), Medical student research project, UWO Schulich School of Medicine and Dentistry.

2014 - 2015 *"Donor organ morphometrics on transplant outcomes"* Schulich Research Opportunities Program (SROP) project of Mr. Haris Jafre (2nd year medical student). UWO, Schulich School of Medicine.

PEER-REVIEWED PUBLICATIONS

1. Lobb, I., Sonke, E., **Aboalsamh, G.**, Sener, A. Hydrogen sulphide and the kidney: important roles in renal physiology and pathogenesis and treatment of kidney injury and disease. **Nitric Oxide: Biology and Chemistry**, *Submitted September 2014*.

PUBLICATIONS in PREPARATION

1. **Aboalsamh, G**., Anderson, P., Al-Abbassi, A., Yanko, D., McAlister, V., Luke, P.P.W., Sener, A. The Effect of Post-operative Intravenous Heparin Infusion on Simultaneous Kidney-Pancreas Transplant Outcomes. *In preparation for submission to* ***Transplantation.***

2. **Aboalsamh, G**., Grewal, J., Saha, M., Sener, A. Mitochondrial hydrogen sulphide donor molecules may be more effective against hypoxia induced ischemic injury. *In preparation for submission to* **Nitric Oxide: Biology and Chemistry.**

3. Jaffe, H., **Aboalsamh, G**., Archembault, J., Sener, A. Donor organ morphometrics and renal transplant outcomes. *In preparation for submission to* ***Transplantation.***

4. Posada, D., **Aboalsamh, G**., Sener, A. Role of off-clamp partial nephrectomy in patient outcomes: A novel use of the ALTRUS device. *In preparation for initiation of Surgical experiments.*

5. **Aboalsamh, G**., Sener, A. Luke, P. Normothermic Oxygenated Blood Kidney Preservation in comparison to Standard Preservation Methods. *In preparation for initiation of Surgical experiments.*

EXTRACURRICULAR INTERESTS

Soccer, Beach Volley ball, tennis, painting and cooking.