Graduate School Form 30
(Revised 08/14)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Shijun Zhang

Entitled

HYDRODYNAMIC DELIVERY FOR PREVENTION OF ACUTE KIDNEY INJURY

For the degree of Master of Science

Is approved by the final examining committee:

Simon Atkinson

Robert Bacallao

Bonnie Blazer-Yost

To the best of my knowledge and as understood by the student in the Thesis/Dissertation Agreement, Publication Delay, and Certification/Disclaimer (Graduate School Form 32), this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Simon Atkinson

Approved by Major Professor(s): _____

_____

Approved by: Simon Atkinson 01/26/2015

Head of the Department Graduate Program Date

HYDRODYNAMIC DELIVERY FOR PREVENTION OF ACUTE KIDNEY INJURY

A Thesis

Submitted to the Faculty

of

Purdue University

by

Shijun Zhang

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

May 2015

Purdue University

Indianapolis, Indiana

I dedicate this thesis to my family for nursing me with affections and love and their dedicated partnership for success in my life.

ACKNOWLEDGEMENTS

I would like to express the deepest appreciation to my mentor, Dr. Simon Atkinson for the continuous support of my master study and research, for his patience, motivation, enthusiasm, and immense knowledge. Without his guidance and persistent help this dissertation would not have been possible.

I would like to thank the rest of my committee, Dr. Robert Bacallao and Bonnie Blazer-Yost. Dr. Bacallao was always available for my questions and he was positive and gave generously of his time and vast knowledge. He always knew where to look for the answers to obstacles while leading me to the right source, theory, and perspective. Dr. Blazer-Yost, thank you for your encouragement, brilliant comments and suggestions. I would also like to thank Dr. David Patrick Basile, whose contribution in stimulating suggestions and encouragement helped me to coordinate my project.

My sincere thanks also goes to George Rhodes for training me in animal surgery and enormous help for my research. I would like to thank Dr. Peter Corridon, who helped me to adapt to the new research environment and gave selflessly advices to my experiment. I would like to thank Dr. Mark Hallett for always listening and lending advice in the past two years. I would like to thank Weimin Xu, who trained me to be a bench researcher with great patience.

Furthermore I thank my fellow labmates in kidney juicy group: Michelle Lu and Alexander Kolb, for the stimulation discussions, for your unlimited support, and for all the fun we have had.

Last but not the least, I would like to thank my family for their endless support.

TABLE OF CONTENTS

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Zhang, Shijun. M.S., Purdue University, May 2015. Hydrodynamic Delivery for Prevention of Acute Kidney Injury. Major Professor: Simon Atkinson.

The young field of gene therapy offers the promises of significant progress towards the treatment of many different types of human diseases. Gene therapy has been proposed as an innovative way to treat Acute Kidney Injury (AKI). Through proteomic analysis, the upregulation of two enzymes, IDH2 and SULT1C2, within the mitochondrial fraction has been identified following ischemic preconditioning, a treatment by which rat kidneys are protected from ischemia. Using the hydrodynamic fluid gene delivery technique, we were able to upregulate the expression of IDH2 and SULT1C2 in the kidney. We found that the delivery of IDH2 plasmid through hydrodynamic fluid delivery to the kidney resulted in increased mitochondrial oxygen respiration compared with injured kidneys without gene delivery. We also found that renal ischemic preconditioning altered the membrane fluidity of mitochondria. In conclusion, our study supports the idea that upregulated expression of IDH2 in mitochondria can protect the kidney against AKI, while the protective function of upregulated SULT1C2 needs to be further studied.

CHAPTER 1. INTRODUCTION

1.1 Acute Kidney Injury

1.1.1 AKI: A Common and Important Clinical of Problem

Acute kidney injury is characterized by rapid loss of kidney function. The major manifestation decreased urine output resulting from a rapid decrease in Glomerular Filtration Rate (GFR). An increase in serum creatinine is commonly used to diagnose the onset of injury [1]. Acute kidney injury has been characterized by various definitions in the past decades. Many of the definitions are complex. The lack of a consensus and accurate definition of AKI made it difficult to determine the etiology and outcomes of AKI, and has been a major barrier to research on new, more effective treatments. The organization named Acute Dialysis Quality Initiative (ADQI) was formed in early 2000. The members of ADQI, including international nephrologist and intensive care specialists, established the RIFLE (risk, injury, failure, loss, end-stage kidney disease) definition and staging system for AKI [2].

In 2007, the Acute Kidney Injury Network (AKIN) [3, 4], established by many of the original members of the ADQI group, released a modified RIFLE staging system in an attempt to increase the sensitivity and specificity of AKI diagnosis (Table 1).

In 2012 the Kidney Disease: Improving Global Outcomes (KDIGO) workgroup published their clinical practice guideline for AKI, which combines the definitions and staging systems defined by both ADQI and AKIN (Table 2). It is foreseeable that these guidelines will be widely adopted and the common set of parameters will help synthesize the information from different future clinical studies.

Acute renal failure has been a great healthcare concern in the U.S. because of its high prevalence in hospitals and high treatment cost. In 2011 AKI cost nearly $4.7 billion for approximately 498,000 hospital stays [5]. AKI accounts for 2%-7% of hospitalized patients per year. Moreover, the ICU population is at greater risk [6, 7], resulting from multi-organ disease and sepsis, the incident of AKI is steadily growing overall [8, 9]. Although prevention strategies and support measures are advancing, AKI still maintains a high morbidity and mortality, especially in the ICU population, where hospital mortality may exceed 50%, especially when there is other organ involvement. In addition to the 30%-70% mortality, the patients that survive their acute illness are at greater risk for chronic consequences, such as chronic kidney disease (CKD) and end-stage renal disease (ESRD) [10-12]. AKI can lead to a variety of complications that lead to poor long term prognosis, including metabolic acidosis, high potassium levels, uremia, and changes in fluid balance [13].

Table 1. RIFLE Classification System for Acute Kidney Injury. From [14].

Stage	GFR[a] Criteria	UO[b] Criteria
Risk	SCr[c] increased 1.5-2 times baseline or GFR decreased > 25%	UO < 0.5 mL/kg/h < 6h
Injury	SCr increased 2-3 times baseline or GFR decreased > 50%	UO < 0.5 mL/kg/h > 12h
Failure	SCr increased >3 times baseline or GFR decreased > 75% or SCr ≥ 4 mg/dL; acute rise ≥ 0.5 mg/dL	UO < 0.3 mL/kg/h 24h(oliguria) or anuria 12h
Loss of function	Persistent acute renal failure: complete loss of kidney function > 4 weeks	
ESRD[d]	Complete loss of kidney function > 3 moths	

a GFR = glomerular filtration rate. b UO = urine output.
c SCr = serum creatinine. d ESRD = end-stage renal disease.

Table 2. KDIGO Staging for AKI Severity. From [15]

Stage	Serum Creatinine	Urine Output
1	1.5-1.9 times baseline or ≥ 0.3 mg/dL increase	< 0.5 mL/kg/h for 6h
2	2-2.9 tunes baseline	< 0.5 mL/kg/h for 12h
3	3 times baseline or increasing serum creatinine to ≥ 4 mg/dL or initiation of renal replacement therapy	< 0.3 mL/kg/h for 24h

1.1.2 Signs and Symptoms of AKI

The symptoms of acute kidney injury are caused by the metabolic disturbances that result from decreased kidney function. Some of the symptoms are caused by the accumulation of urea and other nitrogenous waste products that cannot be properly excreted. These include fatigue, loss of appetite, headache, and nausea. There may also be significant increase in serum potassium that can cause severe disturbances in cardiac function [16]. The inability of the impaired kidney to excrete sufficient fluid from the body can affect the fluid balance, causing pulmonary edema and the accumulation of fluid, particularly in the distal regions of the limbs [17, 18].

AKI is usually diagnosed on the basis of characteristic laboratory findings, such as an increase in blood urea nitrogen and serum creatinine, or inability of the kidneys to produce sufficient amounts of urine (oliguria). However, these clinical parameters do not change significantly until relatively late in the injury process, so the last decade has seen an effort to identify new biomarkers of AKI that could allow the injury to be detected much earlier [19].

1.1.3 Classification of AKI

AKI can be caused by disease [20], crush injury [21, 22], contrast agents [23-25], some antibiotics [26], and more. How these drugs or events cause acute kidney injury remains controversial. From the stand point of pathogenesis, the causes of acute kidney injury are commonly categorized into prerenal, intrinsic, and postrenal.

Prerenal AKI is the most common in developed countries, in hospital inpatients, and particularly in critically ill patients. Prerenal AKI is triggered by a deficiency of blood flow to the kidney, otherwise known as renal ischemia. The systemic causes of prerenal AKI include low blood volume, low blood pressure, heart failure, liver cirrhosis, exposure to substances harmful to the kidney, inflammatory process in the kidney. Other potential causes of prerenal AKI include: renal artery stenosis and renal vein thrombosis by decreasing blood supply to the kidney [27].

Intrinsic AKI is caused by injury mechanisms that are intrinsic to the kidney itself. These types of injury can be subdivided based on the structures affected, such as glomeruli, tubules and renal interstitium. This internal damage may result in glomerulonephritis, acute tubular necrosis (ATN), and acute interstitial nephritis [28].

Urinary tract obstruction may cause postrenal AKI. This can be a consequence of prostate problems, kidney stones, obstructed urinary catheter, or tumor masses in the bladder or urethra.

1.1.4 Cellular Mechanisms of AKI

The mechanisms leading to acute kidney injury (AKI) are complicated and its causes vary. One of the most common cause of AKI, renal ischemia/reperfusion injury (IRI) [29-30] is triggered by decreased delivery of oxygen and nutrients to the cells of the kidney, combined with a failure to remove waste products [31]. The kidney is extremely vulnerable to ischemia, due to the high level of metabolic activity required for the

normal function of the organ and the sensitivity of its cells to toxins. In addition to direct injury to the epithelial cells lining the tubule, ischemia also leads to vasoconstriction, endothelial damage, and activation of inflammatory processes.

The epithelium plays an important role in the process of kidney injury. In response to ischemia, epithelial cells lose normal cytoskeletal organization and cell polarity. The epithelial cells in the proximal tubule are the first to respond to the renal injury. The epithelial cells can lose their brush border membrane and microvilli and cell polarity with the dislocation of the adhesion molecules, Na^+, K^+ ATPase and other membrane proteins on the epithelial cells [32]. Severe injury may lead to cell death by either necrosis or apoptosis [33]. In addition, the dislocation of adhesion molecules leads to viable epithelial cells lifting off the basement membrane. In some cases these cells can be shed into the lumen of the nephron and may be excreted in the urine, but some can interact with luminal proteins to obstruct the tubule lumen [34]. In response to renal injury, the epithelium generates inflammatory cytokines and vasoactive mediators to worsen vasoconstriction and inflammation and recruit a variety of inflammatory cells.

Endothelial cells may contribute to AKI in many ways. First, damaged endothelial cells diminish the blood flow of peritubular capillary, which adds to the effect of ischemia on the epithelial cells. The damaged endothelial cells contribute to vasoconstriction by decreasing production of nitric oxide and other vasodilators. The enhanced vasoconstriction is further compounded by small vessel occlusion due to the coagulation triggered by the immune response and the recruitment of white cells, and may lead to regional ischemia [35].

Third, damage to the endothelium can increase the permeability of the microvascular barrier and also reduce the number of microvessels in the outer medulla, which results in chronic hypoxia even when the acute injury is resolved [36, 37]. Chronic hypoxia is a condition in which tissues in the body are progressively deprived of oxygen. It results in a progressive increase fibrosis, further altering the structure of the kidney, which may lead in turn to less delivery of oxygen and nutrients, further epithelial cell injury, interfered regeneration progress, and a vicious cycle of further fibrosis [38, 39].

Both innate and adaptive immune responses play important role in ischemic injury. The innate immune response takes effect in the early response to injury in a non-antigen-specific mode [40]. With ischemic injury, Toll-like receptors (TLRs) expressed by tubular epithelium are up-regulated by endogenous ligands which are released by damaged tissue, which can activate, mature dendritic cells (DCs) [41, 42]. Then DCs activate T lymphocytes, triggering an adaptive immune response. Besides TLRs, injured tubular epithelial cells express adhesion molecules [43], and other ligands for T cells [44], which activate these immune cells, leading to a further increase in inflammation. Proinflammatory cytokines and chemokines can be released by the epithelium to recruit immune cells [45]. Various cell types, including monocytes/ macrophages, DCs, and T cells, release factors leading to increasing vascular permeability and impairing tubular epithelial and endothelial cells [46]. This further adds to kidney injury [47].

The cellular mechanism of kidney injury and repair is still not fully understood and better understanding is needed to improve therapies to prevent renal injury, accelerate the recovery of the organ, and minimize chronic kidney disease.

1.1.5 Management of AKI

Improved treatment and management of AKI will only be possible with better understanding of its underlying cause(s). The current treatment of AKI is mainly supportive in nature. The principles of management of established AKI are to treat or remove the cause (for example, treating sepsis or artery stenosis) and to use supportive measures to try to maintain homoeostasis while recovery of normal kidney function takes place.

Unfortunately, there are currently no specifically targeted therapies that have been developed which can lessen acute kidney injury or hasten recovery; thus, supportive treatment if the only option. This includes fluid, electrolyte and acid-based balance management [48]. Kidney transplant is the option if patients suffer from severe acute kidney injury.

1.2 Ischemic Preconditioning

Ischemic preconditioning (IP) describes a commonly observed phenomenon, in which an organ can be made more tolerant to ischemia/reperfusion injury by preconditioning this organ with a previous, sub-lethal episode of ischemia. Murry and colleagues described the effect of ischemic preconditioning on dog heart in 1986 [49].

Soon after, the protection caused by ischemic preconditioning was shown in other animal models, besides dogs, including rabbits, rats, and guinea pigs [50, 51]. A number of studies have now shown that ischemic preconditioning using short periods of

ischemia is able to decrease the severity of a subsequent ischemic/reperfusion injury in various organs including the heart [51], liver [52], kidney [53], and lung [54].

Though the phenomenon of ischemic preconditioning was first described in the heart and is also extensively studied in myocardial infarction, there is now a substantial literature in the kidney that supports the finding that ischemic preconditioning can protect the kidney against a subsequent episode of ischemia [55].

The kidney is a vital organ in which preconditioning can have a protective effect. Given their demands for high energy and their complex vascular network, kidneys are extremely susceptible to ischemia/reperfusion [56, 57]. Animal experiments have confirmed that both local and distant preconditioning are effective in protecting the kidneys [58, 59].

Meta-analysis indicated that IP can reduce parameter of injury, including serum creatinine, blood urea nitrogen and structural alterations in the kidney observed by histology after kidney I/R injury as compared to controls, suggesting that IP effectively alleviates renal damage after kidney I/R injury. However, the mechanism behind the renal ischemia preconditioning phenomenon has yet to be elucidated.

Understanding the mechanisms responsible for ischemic preconditioning is important for formulating therapeutic strategies aimed at mimicking protective mechanisms – since preconditioning itself is not a plausible clinical approach. Currently, there is evidence for two types of intracellular mechanisms mediates the protective effect of ischemic preconditioning in other organs.

One is the mitochondrial ATP-sensitive potassium (K^+-ATP) channels and the other is a specific form of protein kinase C (PKC) [60]. The protective effect of ischemic preconditioning is believed to be more critically due to the opening of mitochondrial ATP-sensitive potassium channel or mitoKATP [61, 62].

In a cellular energy crisis it is proposed that, mitoKATP channels' open and close to help to restore proper membrane potential. This includes restoring control over internal Ca^{2+} concentration and the degree of membrane swelling. This serves to maintain the proton gradient for mitochondria ATP synthesis [63].

PKC is an important regulator of the opening of mitoKATP channel [64]. Several pathways via which PKC regulates mitoKATP channel have been explored. Possible mechanisms include controlling release of oxygen radicals and controlling levels of Bcl-2 proteins, which typically function as major regulators of apoptosis [65].

1.3 The Role of Mitochondria in AKI

1.3.1 Function and Structure of Mitochondria

Mitochondria are intracellular organelles whose primary function is metabolic energy transduction and ATP synthesis. They also play vital roles in intermediate molecule metabolism, metal ion (calcium and iron) homeostasis, programmed cell death and are major sources and regulators of reactive oxygen species (ROS) [66].

A mitochondrion consists of an outer mitochondrial membrane and an inner mitochondrial membrane. The permeability of the outer mitochondrial membrane differs from that of inner mitochondrial membrane. The protein-based pores on the outer membrane allow proteins and ions to pass through relatively freely. Similar to the plasma membrane of a cell, the inner membrane is less permeable to ions and smaller proteins. The inner membrane is embedded with a number of proteins involved in electron transport and ATP synthesis. The mitochondrial matrix, surrounded by the inner membrane is a gel-like material, containing water and high concentrations of the enzymes involved in the citric acid cycle [67].

Adenosine triphosphate (ATP) used by all the energy-consuming activities of the cell, is predominantly generated in mitochondria through oxidative phosphorylation. Oxidative phosphorylation is achieved by the movement of electrons along electron transport chain (ETC). The generation of the electrochemical gradient requires oxygen consumption by the ETC, and is used to power ADP phosphorylation by complex V (the ATP synthase) [68]. Successive protein complexes, Complex I-IV, embedded in the inner mitochondrial membrane, form the ETC and are responsible for generation of the electrochemical gradient across the inner membrane. The role of these complexes is to transport electrons and pump protons across the mitochondrial inner membrane, and the fifth one called ATP synthase actually manufactures ATP from ADP and inorganic phosphate. Electrons, generated from the citric acid cycle, are carried to complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) by reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2)

respectively. Subsequently, electrons are passed to complex III (cytochrome bc1) through coenzyme Q (CoQ). Then cytochrome c carries the electrons from complex III to complex VI. Finally electrons are transferred to the electron acceptor oxygen to form water. The energy generated from the electron flow is utilized to drive protons (H^+) from matrix to intermembrane space at the level of complexes I, III, and VI to form an electrochemical gradient. This process is called chemiosmosis [69]. The gradient is used to reintroduce protons through the proton channel of complex V (ATP synthase). The proton flow drives the condensation of adenosine diphosphate (ADP) and inorganic phosphate to form ATP (Figure 1).

The mitochondrial membrane is composed of unique phospholipids and proteins. The interdependence of phospholipids and proteins provide a dynamic and fluid environment, which is crucial for mitochondrial function. For example, the membrane impermeability to protons enables the formation of proton gradient [70]. Proper membrane fluidity can organize the ETC complexes [71]. And changes in mitochondrial membrane fluidity can alter bioenergetics [72]. When the optimal membrane fluidity changes, mitochondrial functions are negatively impacted, e.g., membrane-associated enzymes function less efficiently as do membrane receptor-mediated signal transduction processes [73].

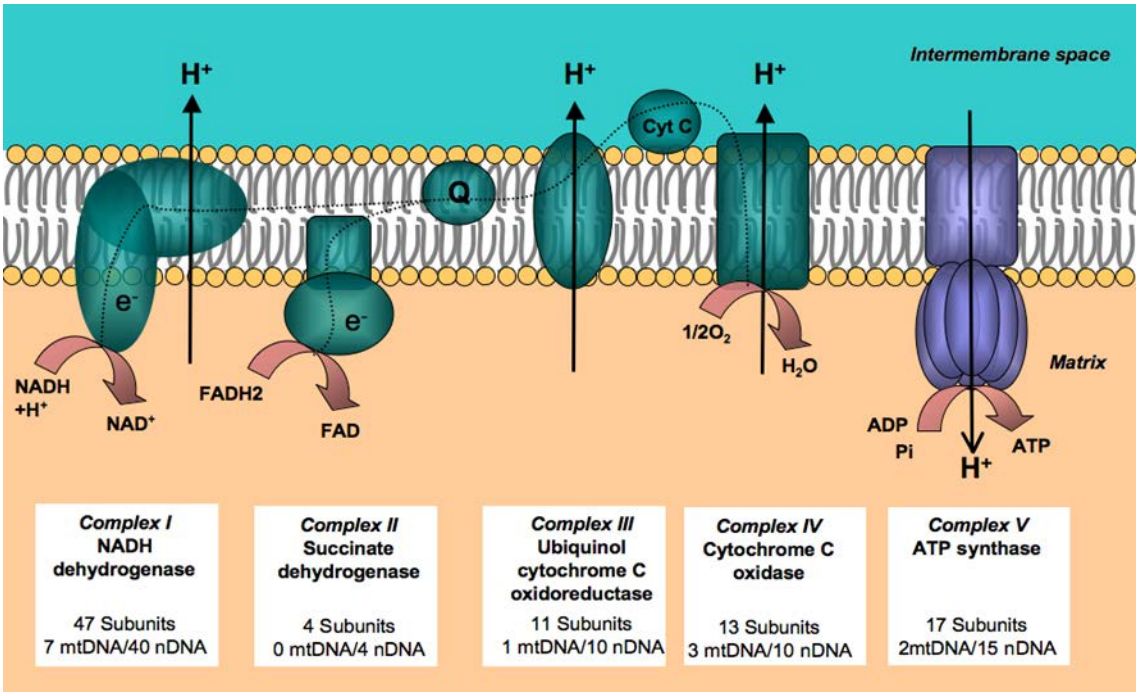


Figure 1. Mitochondrial Electron Transport Chain. Copyright © 2001 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc. ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; CoQ10, Coenzyme Q10; Cyt, Cytochrome; FAD, Flavin adenosine dinucleotide; Fe-S, Iron–sulfur cluster; FMN, Flavin mononucleotide; NAD+, Nicotinamide adenine dinucleotide; NADH, Reduced nicotinamide adenine dinucleotide.

1.3.2 Ischemic Reperfusion Injury

Mitochondrial damage is a key contributor to AKI. Ischemia impairs the mitochondrial ETC by decreasing the activity of complexes and decreases the protection afforded by the antioxidant system. Short times of ischemia may increase the electronegativity of the ETC complexes and leakage of electrons, but may not be sufficient to alter the antioxidant system in mitochondria. During the short episodes of ischemia, the increased reactive oxygen species (ROS) produced following reperfusion (reintroduction of oxygen) can be reduced. Long ischemic period can trigger more profound changes within the mitochondria, such as decreased activity of the complexes

of the electron transport chain [74] and increased membrane permeability [75]. Decreased activity of ETC complexes causes low ATP production following reperfusion.

During ischemic injury, ATP is broken down to ADP, adenosine monophosphate (AMP) and inorganic phosphate. This break down is triggered by anaerobic metabolism. In addition to ATP breakdown, there is also an increase in resting cytosolic free (Ca^{2+}) during ischemia which leads to mitochondrial Ca^{2+} overload. The accumulation of Ca^{2+}, inorganic phosphate and ROS induces changes in permeability [76-78].

The increased mitochondrial inner membrane permeability, called the mitochondrial permeability transition, is considered to drive apoptosis [79]. The mitochondrial permeability transition occurs by the formation of mitochondrial permeability transition pores (MPTP) located in the inner membrane. The opening of MPTP can not only diminish ATP production by causing proton leakage, but also allow small molecules and water to pass, which may result in matrix swelling and rupture of the mitochondrial outer membrane [80]. Among the released substances, activating factors, such as apoptosis inducing factor (AIF), cytochrome c, and Smac/DIABLO, are released into the cytosol to induce apoptosis [81].

1.4 Gene Therapy: An Alternative Management of AKI

1.4.1 Gene Therapy

Gene therapy is the introduction of exogenous or modified genes into a patient's cells and tissues to treat inherited or acquired diseases. Gene therapy is a rapidly growing field of medicine that may hold the cure for many of the diseases and disorders of humankind. The completion of Human Genome Project accelerated the efforts to develop gene therapy as a viable treatment option, initially.

For disorders that result from single genetic aberrations, such as a mutation, truncation or deletion. Subsequently various efforts were launched to extend this treatment method to other monogenic disorders and a wider platform of ailments.

1.4.2 Promises and Problems of Gene Therapy

The potential advantage of the technique, is that it holds the promise of eliminating and preventing hereditary diseases. It is very effective when the gene is delivered to tissue correctly and can fix the problem at the source. Drug side effects can also be avoided. Gene therapies are expected to be developed to treat genetic diseases, tumors, viral infections and neurodegenerative diseases.

In preclinical studies, gene therapy demonstrated robust improvements in animal models which carried mutations leading to different diseases. For example, in 2014, gene therapy was shown to improve muscle strength of a fatal congenital childhood

disease in large-animal models—dogs carrying a naturally occurring MIM1 gene mutation [82]. Currently, over 70% of gene therapy clinical trials are focused on cancer treatment. Most of the clinical trials are in Phase I or II, with less than 1% in Phase III [83]. The vast majority of these trials have focused primarily on the issue of safety.

Various genetic diseases are successfully improved by gene therapy, but the short-lived nature of most current gene therapy approaches means the effect of most treatments are temporary. For example, in 1990, a retroviral vector adenosine deaminase (ADA) gene was transferred into the T cells of two children with severe combined immunodeficiency (ADA− SCID). After 2 years' gene treatment, the number of circulating T cells normalized as did many cellular and humoral immune responses. But the effects only lasted for 4 years [84].

So scientists must find better ways to deliver genes efficiently and achieve sustained expression. There are two categories of delivery vehicles ('vector'). One kind is non-viral vectors. Non-viral vectors can deliver DNA by direct injection, or mixing with polylysine or cationic lipids that allow the gene to cross the cell membrane. The potential advantage of non-viral methods is that the concerns about safety are not as high as when viral vectors are used. But most of these approaches are of poor efficiency of delivery and only give transient expression of the gene [85].

Viral vectors are currently widely used in gene therapy approaches. Viruses have specialized molecular mechanisms that have been refined by evolution to deliver DNA to cells and enable the persistent expression of transgene. However, there are pitfalls to viral vectors which may cause potential problems to the patient—toxicity, immune and

inflammatory responses, and gene control and targeting issues. In addition, the patient would be at risk that the viral vector may recover its ability to cause disease [86]. Insertional mutagenesis by retroviral vectors has the potential to activate oncogenes.

1.4.3 Vital Aspects to Ensure Advancements in Renal Genetic Medicine

1.4.3.1 Exogenous Transgene Vectors

The first requirement for attempting gene therapy is to find capable technologies to transfer genes to various types of cells, tissues, and organs. The development of safe and effective vectors to deliver genes to cells is one of the key aspects to apply widespread genetic treatment. The vector must have the properties to target the specific cells for the disease, achieve sustained gene expression, and avoid pathogenic or adverse effects.

There are five classes of viral vectors derived from retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, and herpes simplex-1 viruses. The five classes of viral vectors can be categorized in two groups, integrating vectors and non-integrating vectors. Both retroviruses and lentiviruses are integrating vectors which can integrate their genomes into host chromatin. Integrating vectors transduce dividing cells. Because the genomes of integrating vectors can be gradually silenced over time, long term expression of transgene in diving host cells still cannot be promised. The

others are non-integration vectors whose genomes persist in the cell nucleus as extrachromosomal episomes. Non-integrating vectors can mediate sustained transgene expression in non-proliferating cells [87].

Non-viral vectors, unlike viral vectors, require additional methods to ensure that efficient transfection is achieved. They can be delivered by injection, liposomes (cationic lipids mixed with nucleic acids), nanoparticles, and other methods. Compared to viral vectors, non-viral methods are safer for repeat administration [88], and easier for mass production. And most importantly, non-viral vectors have lower immunogenicity.

1.4.3.2 The Construction of Non-viral Vectors

Distinct from the cell's chromosomal DNA, plasmids are circular, double-stranded DNA (dsDNA) molecules. These extrachromosomal DNAs occur naturally in bacteria, yeast, and some higher eukaryotic cells. The most extensively used non-viral vectors are E. coli plasmids. There are three essential elements that vectors must have: (1) an origin of replication to allow the bacterial cells to replicate the plasmid DNA, (2) a drug-resistance gene working as selectable marker, and (3) a region for DNA insertion. But if the plasmid containing antibiotic resistant gene is delivered to the patient, the patient could obtain antibiotic resistance. Expression vectors require sequences that encode for: (1) A polyadenylation tail: to protect the mRNA from exonucleases and ensure translational termination and stabilize mRNA. (2) Kozak sequence: is necessary for ribosome assembly. (3)Promoter: an essential element to initiate transcription of the

vector's transgene. (4)Reporter genes: can be used to identify that the plasmid contains inserted DNA sequence. (5)Targeting sequence: can target the expressed protein to specific locations. (6)Protein purification tags: can be used for purifying the expressed protein.

1.4.3.3 Efficient Renal Gene Delivery Techniques

Unlike viral vectors which have efficient molecular mechanism to infect hosts, non-viral vectors cannot pass through cellular barriers to access host DNA [89]. In order to enable the expression of transgenes, methods must facilitate the non-viral vectors to pass through the cell membrane, access the nucleus and get into the host chromatin.

In 1990, a direct injection of naked DNA into murine models was first demonstrated in vivo. The effects of this protein expression vectors was long lasting, but localized [90]. Today a number of other non-viral methods have been developed to deliver naked DNA and each has advantages and disadvantages.

Another promising non-viral route is to package plasmid DNA with liposomes and administer the mixture by intravenous injection. Like other non-viral routes these have low toxicity and low immunogenicity. Cationic lipids can bring plasmid DNA in the cytoplasm by interacting with the cell membrane. Once cationic lipids are absorbed by cytoplasm, plasmid are released from endosomal compartments [91]. Besides liposomes, biocompatible, biodegradable polymers can be used as plasmid carriers in a

similar way. These non-viral routs are utilized to treat cancer and cystic fibrosis, however, the transfection efficiency needs to be improved [92].

Once the genetic materials enter the nuclei, it works to either enable or inhibit the expression of the gene product of interest in transformed cells and their progeny.

Due to the complex renal vascular structures and various vascular permeability, gene transfer in the kidney faces the problem of low efficiency. Proximal tubule epithelial cells have the potential to act as a transgene target, because they have great capacity to endocytose exogenous materials [93, 94]. But the glomerular filtration barrier blocks the transgene's accessibility to the apical domain of epithelial cells, which limits the uptake of transgene [95]. It is possible that gene vectors could access the proximal tubule epithelial cells through peritubular capillaries. But the efficiency of the accessibility is unknown.

Many studies have delivered adenovirus vectors to the kidney via different methods and observed different levels of gene expression. The adenovirus was delivered through arterial injections in normal [96] and cystic rats; pelvic catheter infusions in normal rats [97]; and tail vein [98] and cortical micropuncture [99] injections in uninjured animals. Due to the complex anatomy of the kidney, the gene expression levels differed in renal cell types and anatomic regions in the kidney. The variable results appear to depend on transgene infusion site, volume and rate, as well as the organ temperature and the use of vasodilators.

In contrast, hydrodynamic fluid delivery has been developed to address these challenges by increasing vascular permeability to efficiently deliver exogenous substances throughout the kidney.

In sum, there are several challenges to renal transgene delivery. The techniques presented have provided varied levels of success and are capable of inducing harmful side effects. Therefore, there is a critical need for the development of safe and efficacious transgene delivery options. Such techniques would assist the delivery of clinically relevant genes that can induce transient genetic modifications with minimal physiological interference or damage, and help realize the promise of gene therapy.

1.5 A Novel Renal Gene Delivery Technique: Hydrodynamic Fluid Delivery

1.5.1 Hydrodynamic Fluid Delivery

Hydrodynamic delivery, developed in the late 1990s, was utilized to deliver plasmid DNA in whole animals via intravascular injection. The pressure in capillaries generated by hydrodynamic injection enhanced endothelial and parenchymal cell permeability [100-102].

This new non-viral gene delivery approach is remarkably efficient, safe, and convenient. In some cases, hydrodynamic delivery enables long-lasting transgene expression in transfected animals and is shown to be long-lasting and reach therapeutic levels [103-110].

Early studies of hydrodynamic gene delivery involved the delivery of genes to the liver, and established that this is a potentially effective method for targeted delivery to a particular organ. Now more hydrodynamic delivery procedures have been developed to adapt to other gene delivery applications and for use in other organs [111-115].

1.5.2 Mechanism of Hydrodynamic Fluid Delivery

Hydrodynamic fluid delivery is aimed at generating increased fluid pressures within thin, and stretchable capillaries. It is believed that enhanced fluid flow generated from pressurized injections generates transient pores on the capillary endothelium [116] and epithelium [117]. The fluid flow and disruption of the physical barriers separating the target cells from the circulation enables DNA or other macromolecules to pass through the plasma membranes of the surrounding parenchyma cells. With time, the exogenous molecules are trapped inside the parenchyma cells when the membrane pores close [118-120]. Much of this proposed mechanism is speculative.

1.5.3 Hydrodynamic Fluid Retrograde Renal Vein Delivery

In less than a decade, hydrodynamic gene delivery has been widely accepted as a tool for gene therapy studies. Several laboratories are focusing on adapting the hydrodynamic delivery method for studies in the kidney. Renal artery, renal vein, and ureter may be ideal delivery paths for hydrodynamic renal gene delivery [121].

Hydrodynamic fluid delivery via renal vein injection has been shown to achieve widespread transfection of tubular epithelial cells using plasmid and viral vectors [122]. The renal vein is easy to access and it also provides a viable injection site for hydrodynamic delivery. Compared to other procedures, hydrodynamic fluid renal vein delivery is relatively easy for surgeons to perform, achieves wide, and long lasting expression of the exogenous gene, and produces minimal injury to the kidney.

1.6 Kidney Protective Genes Encoding IDH2 and SULT1C2

1.6.1 Mitochondrial Modifiers: IDH2 and SULT1C2

1.6.1.1 Overview of IDH2

Isocitrate dehydrogenase 2 (IDH2) is a member of Isocitrate dehydrogenases (IDHs), which catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate. The catalytic function of IDHs is crucial [123]. All eukaryote genomes contain three IDH genes, IDH1, IDH2 and IDH3. IDH3 is a NAD^+-dependent enzyme located in the mitochondrial matrix and acts in the Krebs cycle [124]. The other two isocitrate dehydrogenases, IDH1 and IDH2, are NADPH-dependent enzymes and share similar structure [125]. IDH3 irreversibly decarboxylates isocitrate to yield 2-oxoglutarate while reducing NAD+ to NADH, whereas IDH1 and IDH2 catalyze reversible reactions, either

decarboxylating isocitrate to 2-oxoglutarate while reducing NADP+ to NADPH or acting in the reductive carboxylation (reverse) reaction to convert 2-oxoglutarate to isocitrate while oxidizing NADPH to NADP+.

IDH1 is located in the cytoplasm and peroxisomes, and highly expressed in the liver. Besides IDH1 having the capacity to catalyze the conversion of isocitrate to α-ketoglutarate, it has been shown that IDH1 is involved in cellular metabolic processes such as lipid and glucose metabolism [126, 127], and cellular defense against reactive oxygen species and radiation [128-130].

IDH2 is localized to the mitochondrial matrix, and highly expressed in heart, muscle, and activated lymphocytes [131]. IDH2 not only regulates the citric acid cycle, but also protects cells against oxidative stress [132, 133].

IDH1 and IDH2 caught the interests of scientists, because their mutations could contribute to tumorigenesis and cancer progression through indirect effect that increase the overall mutation rate in cancer cells. Cancers characterized by mutations in either IDH1 or IDH2 include gliomas and acute myeloid leukemia [134].

1.6.1.2 SULT1C2

SUL1C2 is located in the cytoplasm and is a member of the sulfotransferase family. Sulfotransferase can catalyze the transfer of the sulfonate moiety from PAPS (3'-phosphoadenosine 5'-phosphosulfate) to many substrates. Sulfate conjugation is an important pathway in the biotransformation of many neurotransmitters, hormones,

drugs and other xenobiotics. In mammals, sulfotransferase are divided into two subfamilies, SULT1 and SULT2. Members of the SULT1C subfamily, including SULT1C1 and SULT1C2, have been shown to catalyze the sulfate conjugation of thyroid hormones and carcinogenic hydroxyarylamines. The significance of this activity in the context of renal ischemic injury is not obvious.

1.6.2 Discovery of the Protective Function

Ischemic preconditioning (IP) is utilized to protect organs by producing resistance to the injury normally caused by the loss of blood supply, and thus oxygen. If the blood supply to the kidney of the rat is impaired for a short time then restored, the kidney becomes robustly protected from a subsequent ischemic insult for an extended period of time [135].

Ischemic preconditioning can protect the kidney against subsequent ischemia /reperfusion injury, but the mechanism is not fully understood. One proposed mechanism is that it results in altered expression of mitochondrial proteins or cytoplasmic proteins associated with mitochondria that affords a resistance to ischemic/reperfusion injury. Proteomic analysis carried out by Dr. Bacallao, Dr. Basile and Dr. Witzmann, showed that several proteins in renal mitochondria were elevated following preconditioning. Liquid chromatography- mass spectroscopy (LC/MS) was used to analyze the proteome of kidney cortical mitochondria isolated following 14 days of recovery from ischemic preconditioning. Two of the upregulated proteins were

selected for further analysis; IDH2 and SULT1C2. Several experiments performed by Dr. Peter Corridon demonstrate that hydrodynamic delivery of IDH2 and SULT1C2 genes can protect the kidney from AKI generated from IRI.

Serum creatinine measurements were carried out by Dr. Corridon. Serum creatinine clearance is a standard biomarker used to gauge renal function. Creatinine is a byproduct of normal muscle metabolism and should be generated by the muscles and excreted by the kidney at a relatively constant rate. When kidney function is impaired, nephron capacity is altered to limit renal clearance. Such an event can eventually reduce the excretion of creatinine, thereby often decreasing urinary creatinine excretions, while increasing serum creatinine levels [136].

On the 7th day after each rat received a hydrodynamic injection containing either saline or a plasmid, they were subjected to moderate ischemia-reperfusion injury (IRI). The serum creatinine levels were monitored for a week after inducing IRI and showed that the hydrodynamic delivery of IDH2 or SULT1C2 genes was sufficient to blunt the effect of IRI in Sprague Dawley rats. Specifically, serum creatinine levels in these rats remained within normal levels despite being subjected to moderate IRI, when compared to rats that received hydrodynamic or tail injections of saline or fluorescent plasmids (Figure 2).

Dr. Corridon also evaluated mitochondrial activity in vivo using 2-photon fluorescence microscopy and the potential-sensitive dye TMRM (tetramethylrhodamine methyl ester). TMRM is a fluorescent probe which binds to the inner and outer leaflets

of the inner mitochondrial membrane. And the fluorescent intensity of TMRM can be used to monitor the membrane potential of mitochondria.

Jugular vein infusions of the mitochondrial membrane potential-dependent dye TMRM were used to gain insight into mitochondria function in various groups of live rats. TMRM fluorescent signal intensities measured in rats that previously received hydrodynamic injections of either IDH2 or SULT1C1 plasmid vectors, as well as those that received ischemic preconditioning, were significantly greater than those measured in rats that received hydrodynamic saline injections or uninjected normal rats. Mitochondrial potential was maintained in animals that received hydrodynamic delivery of either gene relative to controls (Figure 3). These data outline a possible increase in mitochondrial activity related to the upregulation of the mitochondrial enzymes facilitated by hydrodynamic IDH2 and SULT1C1 gene delivery and ischemic preconditioning.

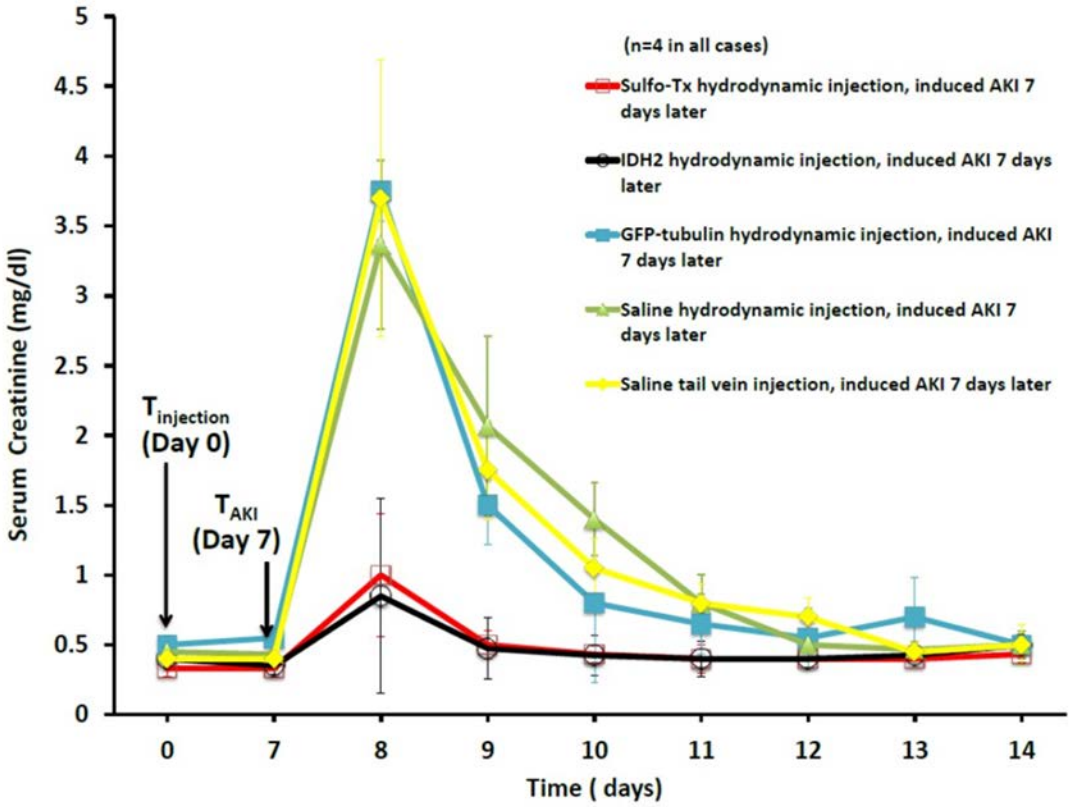


Figure 2. Serum creatinine measurements of hydrodynamically treated rats. Hydrodynamic-based IDH2 and SULT1C2 enzyme upregulation protects the kidney from moderate forms of renal injury. Rats hydrodynamically treated with plasmids encoding mitochondrial proteins seven days earlier, appear less susceptible to moderate ischemia-reperfusion injury.

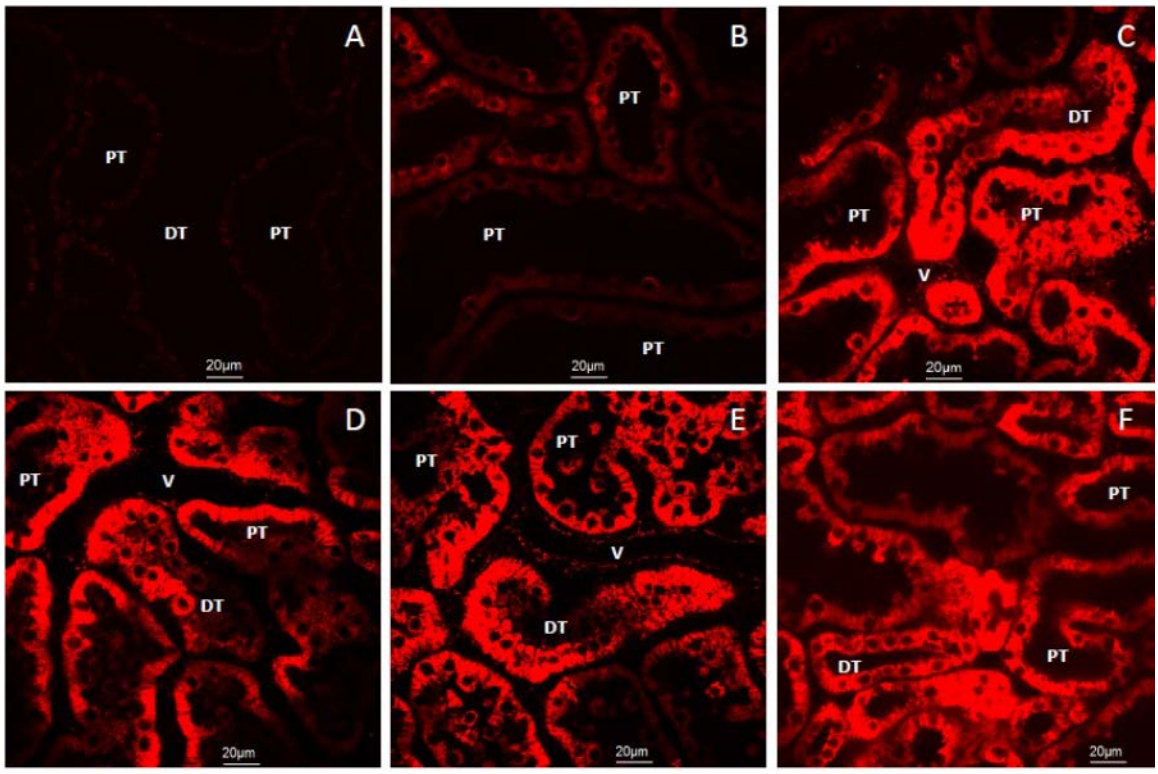


Figure 3. Mitochondria membrane potential activity examined in nephron segments of various live rats. Image (A) represents baseline autofluorescence in a normal rat that did not receive the TMRM dye. In the other cases live rats received hydrodynamic injections of (B) saline; (C) IDH2 plasmids; (D) SULT1C2 plasmids; (E) SULT1C2 plasmids in the uninephrectomized rats. The rat imaged in (E) was subjected to ischemic-preconditioning and did not receive a hydrodynamic injection. Rats (B) through (F) were given TMRM via jugular vein after being exposed to 30 minutes of ischemia one week after receiving hydrodynamic injections or ischemic preconditioning, and were imaged approximately 15-20 minutes after receiving jugular vein infusions of TMRM and renal blood flow was restored.

1.7 In Vitro Assays for Mitochondrial Respiration

In vitro measurements of mitochondrial respiration use manipulations to monitor several different functional “states”. Chance and Williams (1955) defined respiratory states and designed a protocol for mitochondrial activity measurement with isolated mitochondria (Figure 4). The classical titration protocol starts with State 1. In State 1, mitochondria are added to an isosmotic medium containing sufficient phosphate. Respiration is slow in State 1, because mitochondria are under respiratory substrate starvation. In State 2, substrate is added (e.g., succinate, pyruvate, etc.). Mitochondrial respiration is still low in the presence of substrate due to the absence of any added ADP. In State 3, ADP is added and respiration is activated at the presence of substrate and ADP. In this State, respiration is high and can be represented as the slope of oxygen consumption. ADP is gradually depleted by phosphorylation to ATP. As State 3 continues, respiration markedly decreases to a slower rate due to the limited ADP. That state is State 4. Finally, in State 5, depleted oxygen in the closed oxygraph chamber restricts respiration [137]. The mitochondrial respiration can be measured through the consumption rate of oxygen during the procedure in the presence of State 3.

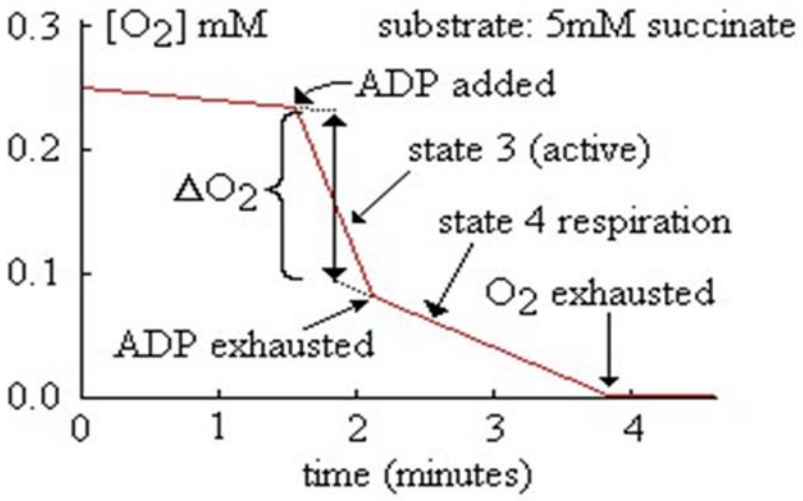


Figure 4. Graph of consumption of O2 (adapted from Curti & Uenura, 1998)

1.8 Mitochondria Membrane Fluidity Measurement: Laurdan

The function of membrane proteins and, particularly, the interactions between them, are strongly influenced by the local environment in the lipid bilayer. This affects the likelihood of protein-protein interactions that are functionally important, such as those between the components of the ETC. In my study, the fluidity of mitochondria membrane was measured by using Laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene). Laurdan is a fluorescent membrane probe synthesized by Gregorio Weber in 1979. It is sensitive to the polarity of its environment in the bilayer. The emission spectrum of laurdan can shift according to the dipolar relaxation of laurdan in the liquid environment, reflecting both the polarity of the environment and the mobility of the probe molecules. Laurdan molecules distribute homogeneously in the lipid bilayer and move freely. In particular, the emission spectrum of laurdan differs when it is incorporated into a lipid bilayer in the liquid crystalline phase as compared to the gel phase, two states that are proposed to significantly affect the activity of membrane proteins and complexes. It has been confirmed that the emission maximum of laurdan is centered at 490 nm when the lipids are in a liquid-crystalline phase, and the emission maximum is centered at 440 nm when the lipids are in a gel-phase. The shift of emission of laurdan can be expressed using Generalized Polarization, GP= (I440-I490)/(I440+I490). GP values above zero reflect a preponderance of the gel phase, while GP values below zero are indicative of more liquid-crystalline area. Because of these properties, laurdan has become a useful tool to detect changes in membrane phase properties [138, 139].

1.9 Hypothesis

Our hypothesis is that hydrodynamic retrograde renal vein fluid delivery of the genes IDH2 and SULT1C2 can modify mitochondria to prevent subsequent acute kidney injury caused by ischemia-reperfusion injury, and will mimic the effect of ischemic preconditioning. IDH2 and SULT1C2 that have been shown by our collaborators to be upregulated in the kidneys of rats that are resistant to moderate ischemia-reperfusion injury. Thus, we simulated this renal regulatory and protective process in kidneys by transferring certain concentrations of these genes via hydrodynamic delivery.

In order to identify the efficiency of transferring plasmids in kidney delivered by hydrodynamic fluid delivery, we used in situ PCR to detect the amplified transgenes and western blotting was used to detect the upregulation expression of these genes.

As IDH2 plays an indispensable role in the TCA cycle, we hypothesized that the upregulation of IDH2 could enhance the mitochondria respiration capability. We expected that when IDH2 plasmid was delivered to rats and they were then subjected to moderate ischemia/reperfusion injury, the mitochondria activity of these kidneys would be improved compared to controls. The role of SULT1C2 in mitochondria function, if any, is unknown. But as a member of sulfotransferase family, it could catalyze the sulfate conjugation to mitochondria membrane. Both sulfate iron and membrane phospholipids have a negative charge, and modification of the overall charge on the membrane or of particular components could alter the fluidity of mitochondria membrane. We hypothesize that SULT1C2 protects the proper function of mitochondria by regulating the fluidity of the mitochondrial membrane.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell Culture and Live Animals

2.1.1 Cell Culture

2.1.1.1 Mouse Kidney Cell Culture

We used the S3 segment of the proximal tubule epithelial cells [140] cultured in a mixture of 500 ml of essential media with, 7.5% of sodium bicarbonate, 7% of fetal bovine serum (FBS), and 1% of streptomycin (Fisher Scientific, Pittsburgh, PA) The cells were grown in a 37°C, 5% CO_2-38% CO_2 humid incubator.

2.1.1.2 Live Rats

Male Sprague Dawley (Harlan Laboratories, Indianapolis, IN), Frömter Munich Wistar (Harlan Laboratories, Indianapolis, IN) with weight ranging from 250gm to 400 gm, were used for these studies. The rats were given free access to standard rat chow and water throughout our studies. Experiments were conducted in accordance with the National

Institutes of Health Guidelines and were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

2.2 Ischemia/Reperfusion Injury

2.2.1 Unilateral Clamp Model

Rats were anesthetized with intraperitoneal injections of 60 mg/kg pentobarbital (Hospira, Inc., Lake Forest, IL and Custom Med Apothecary, Indianapolis, IN), and then placed on a heating pad to maintain normal physiological temperature. Using a standard model to generate renal ischemia-reperfusion injury, unilateral (left) renal pedicle clamps were applied to occlude blood flow for periods of 30-45 minutes, which corresponds to moderate/acute kidney injury. At the end of each injury period, the clamp was removed to reinstate renal blood flow and animals were allowed to recover.

2.2.2 Ischemic Preconditioning

Rats were again anesthetized in the manner described above and subjected to bilateral pedicle clamps to occlude renal blood flow for periods of 30-45 minutes. The incisions were also temporarily closed during ischemia. The animals were then allowed to fully recover for a period of 14 days.

2.3 Plasmid Vectors

We prepared plasmid DNA for gene therapy using Qiagen Maxi Prep systems (Qiagen, Chatsworth, CA, USA). The DNA was suspended in saline for gene delivery. These plasmids encode: enhanced EGFP-occludin (a gift from Dr. Clark Wells, IUSM) and H2B-tdTomato (a gift from Dr. Richard Day, IUSM). We used non-fluorescently labeled plasmid vectors that encode mitochondrial enzymes isocitrate dehydrogenase [NADP], mitochondrial (IDH2) and sulfotransferase (OriGene Technologies, Inc., Rockville, MD). The IDH2 vector was an ORF clone of Homo sapiens isocitrate dehydrogenase 2 (NADP+), mitochondrial (IDH2), nuclear gene encoding mitochondrial protein as transfection-ready DNA. The sulfotransferase vector was an ORF clone of Homo sapiens galactose-3-O-sulfotransferase 2 (GAL3ST2) as transfection-ready DNA. For these hydrodynamic injections, the range of doses we used was 1 to 3 µg of plasmid DNA per gram of body weight.

2.4 Cell Culture Transfection

Immortalized cell cultures were grown in a 35 mm glass bottom, No. 1.5 coverslip dishes, with standard thickness of 0.17 millimeters (Corning Inc., Corning, NY). We followed the Effectene Transfection Reagent protocol provided by Qiagen (Valencia, CA), for plasmid-based transfections. We transfected cells at a multiplicity of infection (MOI) of 10:1, and a 24 hour incubation period using both types of viral vectors.

2.5 Cell and Tissue Imaging

2.5.1 Confocal Fluorescence Imaging of Live Cells

All confocal imaging studies were conducted an Olympus FV 1000-MPE Microscope (Olympus, Tokyo, Japan). Cell cultures were grown in 35 mm glass bottom, No. 1.5 coverslip dishes, with standard thickness of 0.17 millimeters (Corning Inc., Corning, NY). The dishes were placed above the objective and the microscope was set to acquire 512×512 blue-, green- and red-pseudo-color images. To minimize both photoxicity and photobleaching, we reduced both the energy level of the excitation light and the duration of excitation. We also used a Warner DH-35 dish and OW objective warmers to maintain culture temperatures as needed for prolonged imaging sessions.

2.5.2 Intravital Two-Photon Fluorescence Microscopy

In anesthetized rats, we shaved the left flank and made vertical incisions to externalize the left kidney. The kidney was then placed inside a glass bottom dish containing saline, which was set above either a 20X or 60X water immersion objective for imaging, with the animal's body acting as weight to stabilize the kidney in this position. A heating pad was then placed over the rat to maintain its core temperature. Fluorescent images were acquired from externalized organ within 800-860 nm excitation wavelength range207. Measurements were made with an Olympus FV 1000-MPE Microscope set with a Spectra Physics MaiTai Deep See laser, tunable from 710-

990 nm, with dispersion compensation for two-photon microscopy (Olympus Corporation, Tokyo, Japan). The system is also equipped with two external detectors for two-photon imaging, and dichroic mirrors available for collecting blue, green and red emissions. Emitted light is collected using a 3-fixed band pass filter system: 420-460 nm (blue channel), 495-540 nm (green channel), and 575-630 nm (red channel). It should be noted that as we investigated EGFP-based expression, we merged the pseudo-green and pseudo-red colors to further differentiate between GFP-based fluorescence and endogenous tissue fluorescence. This was done because renal tubules are known to have high levels of green autofluorescence.

2.6 Hydrodynamic Retrograde Renal Vein Delivery to the Kidney of Live Rat

We adapted the injection process outlined above to incorporate vascular cross-clamping as a means to potentially reduce exogenous substance leakage and renal uptake. For this technique, we first clamped the renal artery, followed by the renal vein, using micro-serrefines. The vein was then elevated with either 3-0 or 4-0 silk loops. At that time either a tissue dye or transgene suspension was infused retrograde into the vein as previously outlined. The needle was removed. And a cotton swab was applied to the injection site to induce hemostasis. The venous clamp was removed first, and then arterial clamp was removed. The total clamping period lasted no more than 3 minutes. After this, the midline incision was closed and the animal was allowed to fully recover.

2.7 Western Blot Analysis

Whole kidneys were extracted from anesthetized rats, the cortex from kidneys was excised and homogenized in RIPA buffer(150mM NaCl, 50mM Tris -Cl PH 8.0, 1%Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS).

The extracts (3 µg protein/lane) were separated through the10% Bis-Tris Mini Gels (Novex® by Life Technologies TM, Cat. # NP0301), along with 7.5ul molecular weight markers (Precision Plus Protein TM Dual Color Standards, BIO-RAD, Cat. # 616-0374). Proteins were electroblotted to nitrocellulose membrane. The membrane was blocked by incubation in TBS with 3% FBS for 12h at 4°C. Membranes were incubated with primary antibody in TBS with 0.3% FBS for 1hour at room temperature. After three washes, the membrane was incubated with appropriate secondary antibody HRP D&R in antibody dilution buffer at room temperature for 1 hour. After three washes, the immunoreactive bands were visualized by chemiluminescence using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Cat. # 34080).

2.8 In Situ PCR

Whole rat kidney sections were washed in cold RNase-free PBS. Tissues were fixed in 4% paraformaldehyde-sucrose at 4°C for 18 h. Fixed tissues were mounted in optimum cutting temperature compound (Polysciences, Warrington, PA), and 5- to 7-mm cryosections were mounted and placed onto RNase-free Probe-On Plus slides. The slides were kept at -80°C until used for direct in situ PCR. Tissue digestion conditions were

standardized by performing digestions in 1 unit/ mL proteinase K for a fixed time. For standardization of digestion conditions, the digestion time of the enzyme, which did not change the histoarchitecture of the renal tubules, was monitored under a light microscope at 2- to 3-min intervals until an optimal digest time was achieved. Enzyme digestions were stopped by incubating the slide for 1 min at 65°C in PBS buffer.

The slides were then immersed at room temperature in 1 unit/ml proteinase K (Invitrogen, P/N 100005393) solution for 1.5 min, 2min, and 3 min, or in ddH2O. After proteinase K treatment, the slides were incubated in 65°C PBS for 1min.

For amplification of the target IDH2/SULT1C2 sequence, PCR was carried out in situ on the sections using an MJR PTC-100 thermal cycler. Slides were kept at 4°C before the start of the PCR reaction. 20µl PCR mix was overlaid on the sections, and the slide was sealed with adhesive coverslips (Sigma, St. Louis, MO). Reactions were performed in the presence of 1X AccuPrime™Pfx Reaction Mix 0.3 mM, 0.3µM 5' forward and 3'reverse primer, and 2.5 units of AcciPrime™Pfx DNA polymerase (Invitrogen, Cat No. 12344-024). The MgSO4 is included in the 10X AccuPrime™Pfx Reaction Mix (Invitrogen, P/N 55013) at a final concentration of 1mM. dNTPs are included in the10X AccuPrime™Pfx Reaction Mix at a final concentration of 0.3mM. The 3' primer (5'-TGA CTG GGC ACA ACA GAC AAT -3'). The 5' primer (5'- GAT CGA CAA GAC CGG CTT -3') was conjugated to Cy5. Primers were synthesized by Integrated DNA Technologies.

Slides were sealed with a colorless nail polish. PCR was carried out for one cycle at 95°C for 2 min, followed by 30 cycles with denaturation at 95°C for 15 s, annealing at 48.2°C for 5 s, and extension at 72°C for 50 s. When the PCR was complete, samples

were kept at 4°C. Coverslips were removed, slides were soaked for 1 min in 1X PBS at room temperature, counterstained with hematoxylin, and rinsed with 1X PBS and double distilled water. Samples were overlaid with Permount (Fisher Scientific) and covered with coverslips.

Samples were imaged with a Zeiss LSM 510 confocal microscope equipped with argon and helium/neon lasers. Samples were excited at 633 nm of light, and images were collected with a 650-nm emission filter in the light path. All images were collected using standardized laser intensities and photomultiplier tube settings for amplification and dark levels.

2.9 Mitochondria Respiration Assay

2.9.1 Homogenization

After the kidney was harvested from the rat, the capsule layer was peeled and the cortex was shaved off. At 4°C the cortex was gently homogenized with Mitochondrial lysis buffer(250mM sucrose, 20mM HEPES, 10mM KCL, 1.5mM, MgCl2,1.0mM EDTA , pH 7.9) using a Wheaton Teflon pestle and 15mL homogenization tube.

2.9.2 Mitochondria Purification

The homogenate was spun at 750×g for 10min at 4°C. The supernatant was transferred to different microfuge tube and spun at 10,000×g for 10min at 4°C. The supernatant was removed, the pellet was resuspended in 1ml of Lysis buffer and was spun at 10,000×g for 10min at 4°C. After a total of 3 washes, the pellet was resuspended in 1ml lysis buffer and kept on ice with caps open.

The concentration of mitochondria fraction was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Tek Instruments, Inc.).

2.9.3 Mitochondria O_2 Consumption Measurement

Before starting each experiment, the O2 electrode of the Oxygen Meter (Model 5300, Yellow Springs Instrument Co.) was calibrated. The electrode was surrounded by a temperature-controlled water-jacketed glass chamber maintaining the temperature at 37°C.

The mitochondrial fraction was diluted to 1-3μg/μL using the Terzic Buffer (110mM KCL, 5mM K2HPO4·3H2O, 10mM MOPS, 10mM Mg·Acetate, 1mM EDTA, 1μM tetrasodium pyrophosphate, 0.1% BSA, pH 7.15) for a total volume of 2.5ml and was then transferred into O2 chamber with stirring.

The electrode was gently inserted into the chamber. The O_2 percentage was recorded when the following substances were added every 1 minute in the following order: Terzic buffer; substrate（ 1M succinate or 1M pyruvate), and ADP. The

substances were injected by Hamilton syringe to give a final concentration of 10mM in the chamber. At 10 minutes, the record of mitochondria respiration was stopped.

2.10 DNA Agarose Gel Electrophoresis

The E-Gel® with SYBR Safe 1.2% agarose gel (Invitrogen, G521801) was inserted into the E-Gel® PowerBase™v.4. Each well of the gel was loaded with 500ng plasmid DNA. The electrophoresis time was set as 30 min.

2.11 Laurdan Mitochondria Fluidity Assay

The mitochondria fraction was extracted in the same way as the mitochondria O_2 consumption assay. When the kidney was harvested from the rat, the capsule layer was peeled and the cortex was shaved off. At 4°C the cortex was gently homogenized with mitochondrial lysis buffer using a Wheaton Teflon pestle and 15mL homogenization tube, and the mitochondria fraction was purified by several rounds of centrifugation at 4°C.

The mitochondrial fraction was mixed with Laurdan in the ratio of 300:1, and the mixture was loaded into 96 wells plate and incubated at 37°C for 5 minutes with the plate covered. The fluorescence emission intensity was acquired at 440nm and 490nm (excitation= 355 nm) at 37°C using the spectrofluorimeter.

Generalized polarization (GP) was calculated from the emission intensities using the following equation adapted from the work of Parasassi et al. : $\mathrm{GP} = \frac{\mathrm{I440}-\mathrm{I490}}{\mathrm{I440}+\mathrm{I490}}$.(I440 and I490 refer to the average emission intensities at those wavelengths.)

2.12 Statistical Analysis

We computed the mean and S.E. for all data sets, and evaluated the statistical significance of our results using the Student's t-test. All statistical analyses were evaluated using 95% confidence threshold.

CHAPTER 3. RESULTS

3.1 Hydrodynamic Fluid Delivery Facilitates Reliable Transgene Expression in Sprague Dawley Rats

3.1.1 Fluorescent Protein Expression in Cultured Cells Using Plasmid Vector

We observed non-viral and viral derived fluorescent protein expression in cultured cells using confocal microscopy (Figure 5). Occludin is an integral plasma-membrane protein located at the tight junctions. In the cells treated with EGFP-occludin (Figure 5B) fluorescent signals were detected on the membrane, which showed anticipated probe localization. Fluorescent H2B-tdTomato protein expression was observed throughout the nuclei of transfected cells (Figure 5C).

3.1.2 Plasmid Expression in Renal Cells with Retrograde Hydrodynamic Renal Vein Delivery

Dr. Corridon performed hydrodynamic injections augmented with vascular cross-clamping. These injections generated widespread fluorescent protein expression lasting over a month after the introduction of non-viral transgenes.

Using plasmids we successfully transfected live rat kidneys with EGFP occludin (Figure 6A), and H2B-tdTomato (Figures 6B and 6C). Fluorescent protein was observed 1) in proximal and distal tubules; 2) within glomeruli; 3) within the peritubular interstitium; 4) within the renal capsule; and 5) within the perirenal fat.

Images obtained from live rats that expressed EGFP-occludin and H2B-tdTomato plasmid-derived fluorescent proteins provided clear signs of the expected probe localization and morphology. EGFP-occludin expressed in proximal tubule cells. EGFP-occludin fluorescence signals ran between adjacent nuclei as punctate fluorescent bands along regions that correspond to tight junctions (Figure 6A). The H2B-tdTomato signals colocalized with Hoechst counterstained nuclei (Figures 6B and 6C).

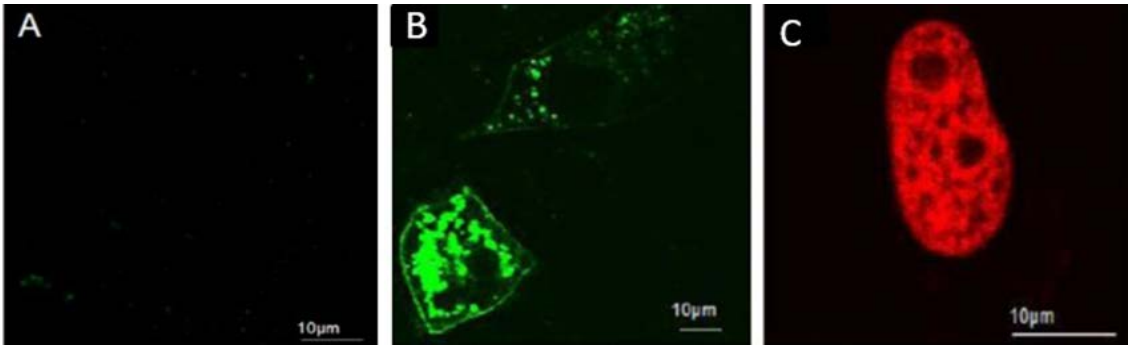


Figure 5. Plasmid-derived fluorescent protein expression in live cells. Confocal laser scanning micrographs obtained from S3 proximal tubule epithelial mouse cell cultures incubated with: (A) Null vector; (B) EFGP-occludin plasmid vectors; and (C) Histone H2B-tdTomato plasmid vectors. These images were taken 1 day after plasmid transformation. Image (B) outlines characteristic plasma membrane-derived tight junction staining and (C) presents typical nuclear and cytoplasmic staining representing histone localization.

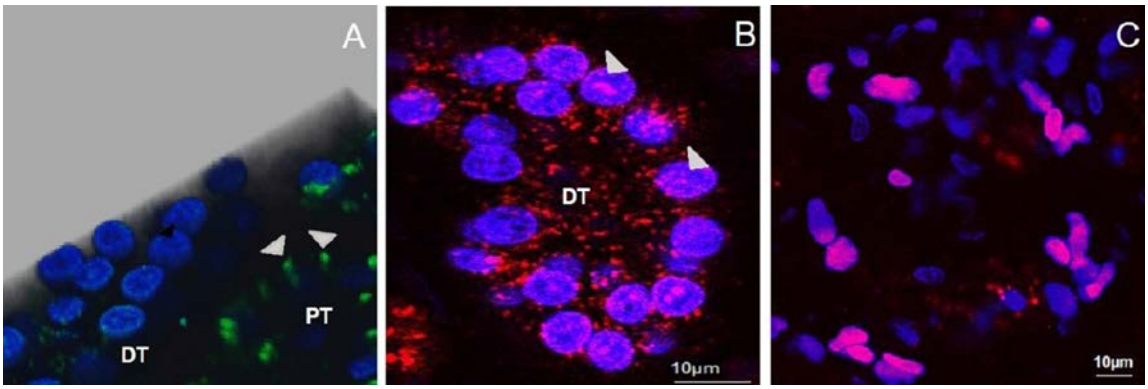


Figure 6. Hydrodynamic delivery facilitates the expression of a variety of plasmid vectors in live rat kidneys. Transgene expression is observed in proximal tubule cells (A), distal tubule cells (B), and within the renal capsule (C). These images were taken from live rats 3 days after receiving hydrodynamic injection of EGFP-occludin (image A), H2B-tdTomato (images B and C). Arrowheads indicate regions of transgene expression.

3.1.3 In Situ PCR Confirmed the Hydrodynamic-Based Transgene Amplification

The primers used for in situ PCR were designed against the Kanamycin resistance gene contained in both SULT1C2 and IDH2 plasmid vectors, and so will uniquely identify the exogenous transgenes. The upper primer was labeled with Cy5. The fluorescent labels permitted direct visualization of plasmid delivery by confocal microscopy.

Before in situ PCR, we optimized the PCR conditions using SULT1C2 plasmid as template. The 375bp size PCR product band was shown in DNA agarose gel (Figure 7).

One group of Sprague Dawley rats were subjected to hydrodynamic SULT1C2 plasmid injection, while the control group received saline injection. The kidney tissue sections were pre-treated with 1 unit/mL Proteinase K solution. Proteinase K can denature the proteins in tissue and inactivate DNases and RNases. Proteinase K is also used to increase the permeability of the kidney tissue to enable the effective PCR reaction. At first, we digested the kidney sections from the rats subjected to SULT1C2 delivery with proteinase K for 3 min, and then performed in situ PCR with primers and Taq polymerase. In the initial experiments, the tubule structures were destroyed by over-digestion with proteinase K and the high temperature cycles of the PCR reaction (Figure 8A). Then we tested the digestion time by treating the sections with proteinase K for 2min (Figure 8B) and 1.5min (Figure 8C). With no proteinase K digestion, the kidney structure was maintained, but the fluorescent signal was weak (Figure 8D). The proximal tubule segments of the sections treated with 1.5 min digestion better retained their structure than those treated with 2 min digestion (Figure 8B and Figure 8C). Though the

tubules structure was still altered with 1.5 min digestion, the fluorescent signals were much stronger than those of the sections without proteinase K digestion. Thus, 1.5 min proteinase K incubation was selected as the optimal condition for the digestion.

With 1.5 min digestion, little fluorescent signals was noted in samples imaged without in situ PCR (Figure 9A and 9D). When the in situ PCR reaction was performed on kidney sections without Taq polymerase, only weak fluorescent signals were detected (Fig 9B and 9E). In contrast, when direct in situ PCR was performed with all necessary primers and Taq polymerase in the tissue section that subjected to SULT1C2 plasmid injection, significant levels of additional fluorescent signals were observed in proximal tubule segments (Fig 9F). There was only weak signal in kidneys from saline-injected, control rats. This data demonstrates that plasmid DNA is being delivery to the cytosolic compartment of renal tubule epithelial cells. Therefore hydrodynamic delivery of plasmid DNA is effective in delivering.

3.1.4 Overexpression of Transgene Expression Confirmed via Western Blot

Four groups of rats received, respectively, hydrodynamic delivery of IDH2 plasmid, SULT1C2 plasmid, or saline alone, or were subjected to ischemic preconditioning. Samples of the kidney cortex were subjected to Western blotting. We observed increased levels of IDH2 or SULT1C2 expression in rats that received hydrodynamic delivery of the corresponding transgene or ischemic preconditioning, as compared to the levels present in the control rats (Figure 10A and 10B). By densitometry of the

Western blot, IDH2 expression in the IDH2-delivered rats was 2.2 fold higher than in vehicle control rats. The IDH2 expression of preconditioned rats was 2.8 fold higher than in control rats (Figure 10C). The expression level of SULT1C2 in rats that received hydrodynamic delivery and ischemic pre-conditioned was respectively 5 fold and 3.5 fold higher than control rats (Figure 10D).

The Western analysis outlined above clearly demonstrates the upregulation of the mitochondrial enzymes facilitated both by hydrodynamic IDH2 and SULT1C2 gene delivery and by ischemic preconditioning.

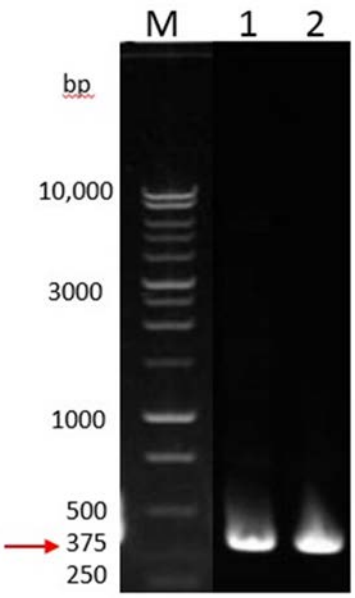


Figure 7. 1.2% Agarose gel electrophoresis of PCR amplified Kanamycin resistance gene from the plasmid vectors. Lane M, 1kb DNA Ladder Marker. Lane 1 and 2 showed PCR primers amplified the Kanamycin resistance gene sequence (375bp) of the plasmid.

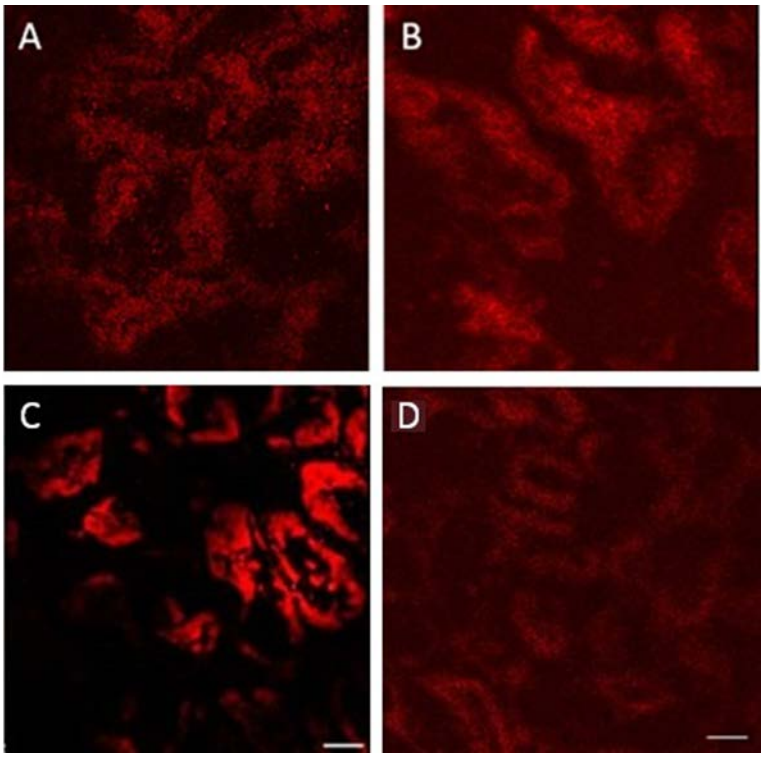


Figure 8. Optimization of direct in situ PCR in kidney tissue conditions using different digestion times. Before the in situ PCR reaction, hydrodynamic SULT1C2 delivered kidney tissue sections were digested by proteinase K for (A) 3min, (B) 2min, and (C) Null digestion.

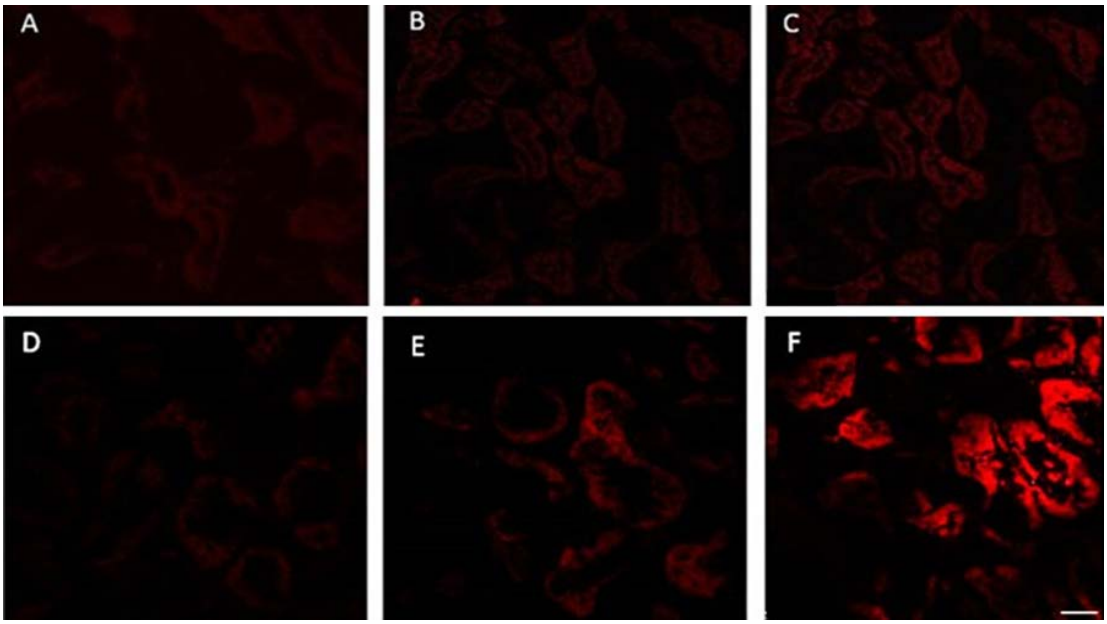


Figure 9. Direct in situ PCR in kidney tissue sections (1.5 min digestion). In saline injected kidney tissue sections, (A) Background fluorescence, no in situ PCR; (B) In situ PCR performed without Taq polymerase; (C) In situ PCR performed with all necessary primers and Taq polymerase. In hydrodynamic SULT1C2 delivered kidney tissue sections, (D) background fluorescence, no in situ PCR; (E) In situ PCR performed without Taq polymerase; (F) In situ PCR performed with all necessary primers and Taq polymerase. Bar = 10 µm

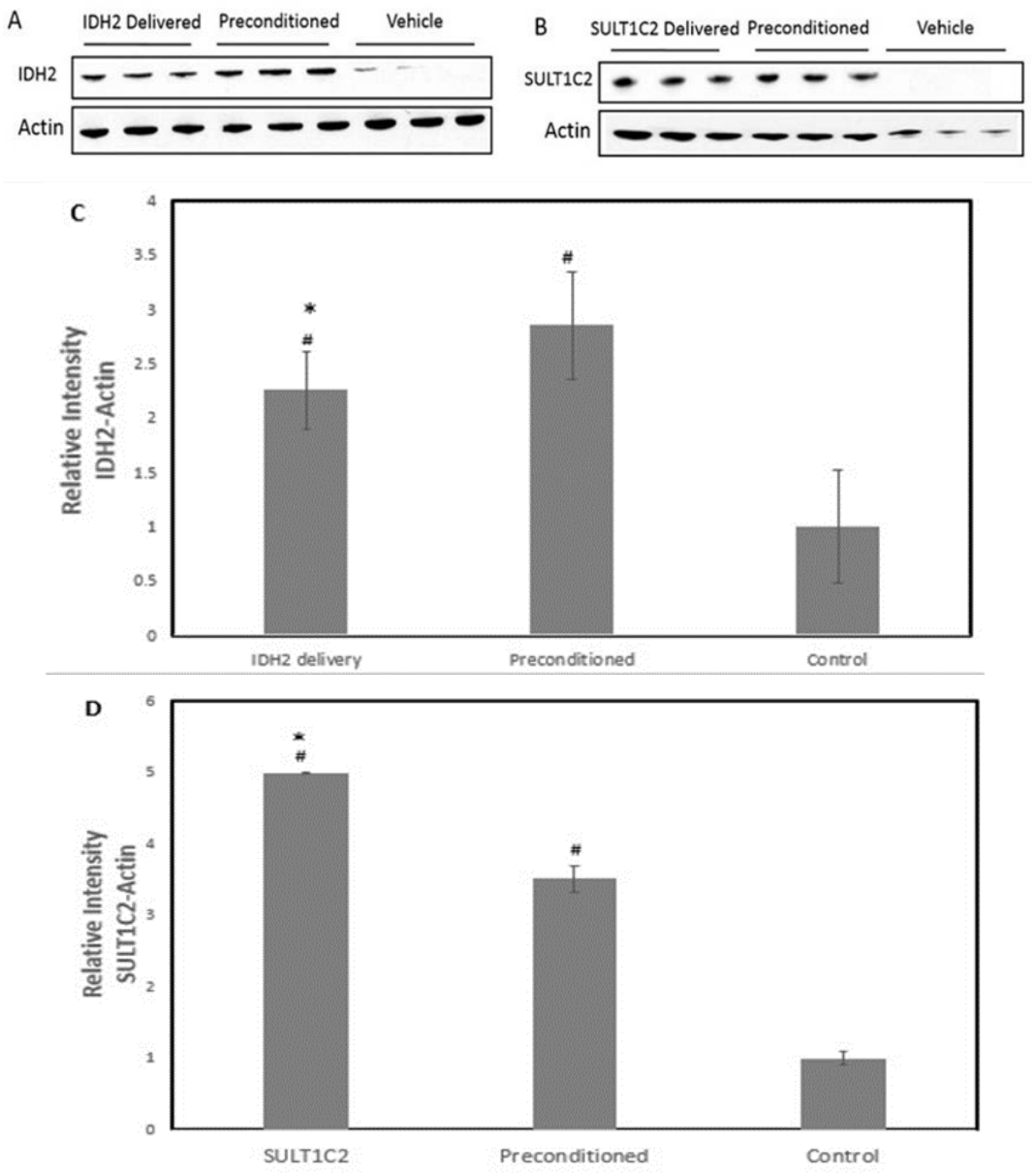


Figure 10. Western blot analysis of levels of IDH2 protein and SULT1C12 protein expression in rat kidneys. Western blot analysis was conducted on tissue sections obtained from rats after various treatment as indicated in the captions in (A) and (B): hydrodynamic injection of IDH2, SULT1C2 and saline, or rats that were subjected to ischemic preconditioning. Analyses of band intensity on films are presented as the relative ratio of IDH2 (C) and SULT1C2 (D) to actin. *P> 0.05 versus Preconditioned. #P<0.05 versus Vehicle.

3.2 Hydrodynamically Delivered Mitochondrial Protein IDH2 Protects Sprague Dawley Rat Kidneys against Mitochondrial Dysfunction Following Moderate Ischemia/Reperfusion Injury

Two vehicle groups of rats received hydrodynamic delivery of saline alone, while one group of rats received IDH2 plasmid delivery. 7 days later, one of the vehicle groups and the IDH2 delivered group were subjected to 40min unilateral ischemia (moderate injury in rats) and 1 h reperfusion. Mitochondrial fractions were isolated from the cortexes of the kidneys of the rats and applied to the mitochondrial activity assay.

After ischemic reperfusion injury, the mitochondrial oxygen consumption rate in State III was measured. The results of mitochondria respiration assay by O_2 consumption are shown (Figure 11). Using succinate as substrate, after 1h ischemic reperfusion injury, the mitochondria respiration remained significantly depressed compared to sham injury controls. Following IDH2 delivery and IRI, the mitochondria respiration was significantly higher than that of the IRI rats that received vehicle (saline) alone. Moreover, the mitochondrial respiratory rate was not markedly decreased as compared to sham controls (Figure 11A). We repeated the mitochondrial respiration assay using pyruvate as substrate. Overall, the same results were observed: overexpression of IDH2 protected the normal level of mitochondrial respiration when the kidney was subjected to IRI (Figure 11B).

These data demonstrate that upregulation of IDH2 facilitated by hydrodynamic IDH2 gene delivery results in a protection of mitochondrial respiratory function following IRI. Mitochondria are unique in that not only are they the site of energy production but also

a central locus in the regulation of cell death [141, 142]. Mitochondria respiration was markedly impaired after ischemic injury, while the overexpression of IDH2 facilitated by hydrodynamic delivery ameliorated the depression of respiration. This provides a plausible explanation for the reduced renal injury observed in previous studies using serum creatinine.

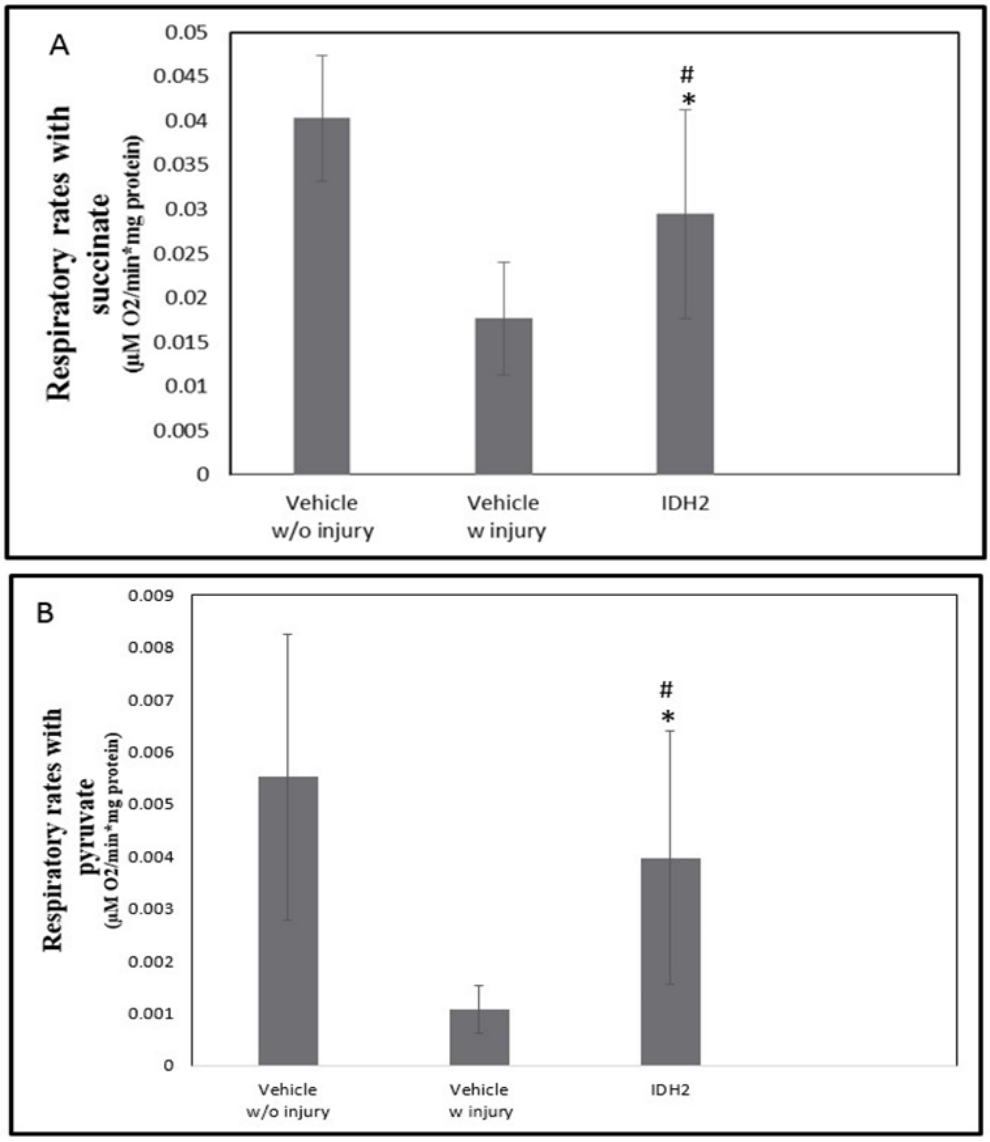


Figure 11. Overexpression of IDH2 increases mitochondrial respiratory rate. The mitochondrial fraction was extracted from the kidneys of the rats received hydrodynamic saline (control) injection IDH2 injection, or ischemic preconditioning. (A) Mitochondrial respiration with succinate with ADP (state III) is elevated (B) Mitochondrial respiration with succinate with ADP (state III) is elevated. The values are mean ± S.D. of nine male rats each.*P <0.05 versus vehicle w injury, #P > 0.05 versus vehicle.

3.3 Ischemic Preconditioning Alters the Mitochondrial Membrane Fluidity of Sprague Dawley Rat Kidneys Following Moderate IRI

Laurdan is a fluorescent probe that alters its fluorescent properties in response to membrane fluidity [143]. In this study, it was utilized to detect changes in mitochondrial membrane fluidity.

Mitochondrial damage is a major contributor to renal tubular cell death during acute kidney injury. During tubular cell injury, mitochondrial damage could involve impairment of respiration complexes, loss of mitochondrial membrane potential, and increasing permeability of the mitochondrial membrane. It is plausible that membrane fluidity changes could be closely linked to mitochondria dynamics. In theory, when the kidneys are subjected to ischemic reperfusion injury, the membrane of mitochondria could become more or less ordered (altered fraction of liquid-crystalline vs. gel-like state). We predicted that SULT1C2 could protect the kidney against ischemic injury by ordering the mitochondria membrane. To begin these studies, we first investigated the effect of ischemic preconditioning on mitochondria membrane fluidity.

The mitochondria fraction was isolated from the kidney cortexes of various groups of rats. From the data of Laurdan assay, the GP values of the sham rats with 1h IRI were markedly increased compared to the normal value (Figure 12). This indicates that the 1h ischemic reperfusion injury decreased mitochondrial membrane fluidity.

Though the GP value of the ischemic preconditioned rats was slightly higher than that of the normal rats, there was no significant difference of the fluidity between the two groups (Figure 12). The GP value of the preconditioned rats subjected to IRI was

remarkably higher than that of ischemic preconditioned rats. We can conclude that ischemic preconditioning affects makes the membrane more susceptible to alterations caused subsequent IRI leading to overall ordering of the mitochondria membrane.

Compared with the two groups subjected to IRI only, the GP value of the preconditioned rats was dramatically increased. Despite the evidence that ischemic preconditioning ordered the mitochondria membrane of the rats subjected to subsequent IRI, we cannot draw a hasty conclusion that reduced mitochondrial membrane fluidity play a role in protecting kidney from IRI. This is because the ischemic injury also led to a decrease in membrane fluidity. The 1h ischemic reperfusion injury could trigger the dysfunction of mitochondria by decreasing mitochondria membrane fluidity. Our expectation was that Ischemic preconditioning would counteract the effect of subsequent injury on membrane fluidity. This was clearly not supported by our laurdan assay. When the preconditioned kidney is subjected to IRI, the kidney is injured twice. The dramatically reduced mitochondrial membrane fluidity of the injured preconditioned kidney showed that the more injury events the kidney was subjected to, the more ordered the mitochondrial membrane was.

In conclusion, both ischemic preconditioning and ischemic injury reduced the fluidity of mitochondrial membrane against renal ischemic injury. In addition mitochondrial membrane fluidity decreased with increasing times of renal ischemic injury.

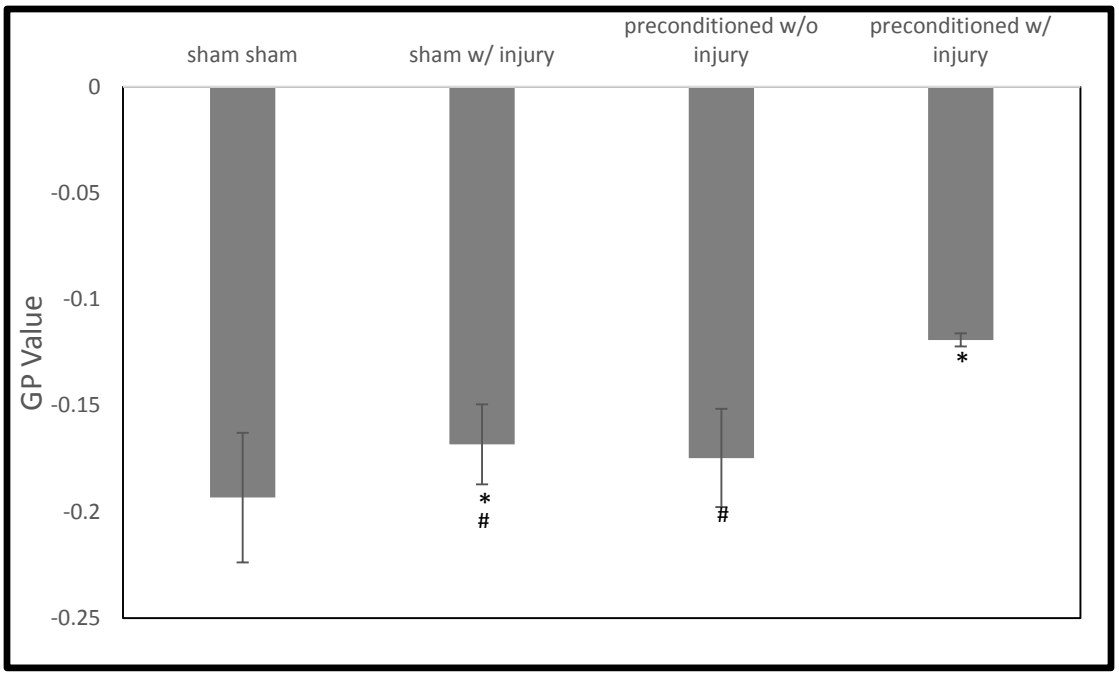


Figure 12. Mitochondrial membrane fluidity changes resulting from varied treatment to kidneys. Mitochondrial membrane fluidity was quantified from the GP value of Laurdan in mitochondrial extracts. The values are mean ± S. D. of four male rats each. *P< 0.05 versus sham, #P< 0.05 versus preconditioning w injury.

3.4 Effects of Ischemic Preconditioning on the Mitochondria Isolation

From our laurdan data, we cannot draw a conclusion that IP can protect kidney against IRI via regulating mitochondria membrane fluidity. Ischemic preconditioning should take effect on the kidney by other pathways. There is evidence that mitochondrial structures is altered in kidney cells following ischemic injury. This has the potential to affect the yield of mitochondria in our preparations. We measured the yield of mitochondria in the fractions used for the laurdan assay, and attempted to investigate whether ischemic preconditioning can increase the yield of mitochondria in kidney, for example by causing fragmentation or aggregation of mitochondria.

As mentioned earlier, the mitochondrial fraction was extracted from kidney cortices. The fraction was resuspended in 1mL lysis buffer. Bradford protein assay was applied to measure the concentration of the mitochondrial fraction. The total yield of mitochondria for each group were calculated (Figure 13).

The yield of mitochondria in the fraction extracted from the preconditioned kidneys with subsequent IRI was significantly higher than that either from the sham kidneys or that from the preconditioned kidneys without subsequent IRI. The yield of mitochondria extracted from the ischemic injured kidneys was larger than that of sham kidneys and the preconditioned kidneys without subsequent IRI, but less than that of the preconditioned kidneys with subsequent IRI. There was no significant difference between the yield from ischemic injured kidneys and preconditioned kidneys with subsequent injury (Figure 13).

Our data demonstrates that when kidneys were preconditioned and subjected to subsequent IRI, there was an increase in isolated mitochondria yield from these kidneys. But the kidneys subjected to IRI also had relatively higher yield of mitochondria. It is not clear that the changes in mitochondrial yield reflect systematic differences between treatments. The amount of mitochondria we measured could be influenced by many other factors. The size of each kidney was different and the total weight of the cortices shaved from the kidney was also different. These confounding variables make it impossible to draw conclusions about the differences in isolated mitochondria fractions.

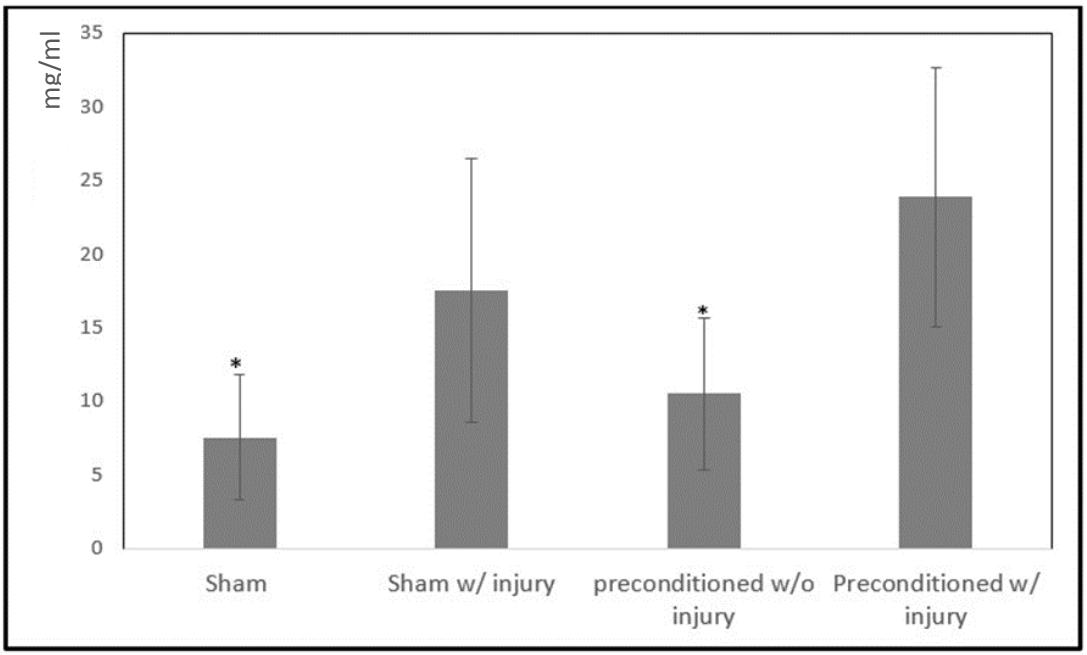


Figure 13. Yield of extracted mitochondrial fraction varied by different treatment. The values are mean ± S. D. of four male rats each. *P< 0.05 versus Preconditioned w/ injury.

CHAPTER 4. DISCUSSION

4.1 Summary

Firstly, with the help of Dr. Corridon, we tested the efficiency of hydrodynamic plasmid delivery technique by delivering fluorescent plasmid vectors to the kidney. The EGFP-occludin and H2BtdTomato plasmids were expressed in the proper location and morphology. This shows the usefulness of this technique for expression fluorescent probes and biomarkers in the kidney.

Secondly, I presented results obtained from the in situ PCR and Western blot analysis that were conducted to prove that hydrodynamic fluid method facilitated non-viral transgene delivery in the rat kidney. In these studies, we delivered plasmid vectors encoding two genes, IDH2 and SULT1C2 to Sprague Dawley rat kidneys, using hydrodynamic retrograde venous injections.

These genes were previously identified to be upregulated in Sprague Dawley rats subjected to ischemic preconditioning. Immune blot analysis confirmed the upregulation of protein expression of these mitochondria-associated proteins in rats that received hydrodynamic injections of the IDH2 and SULT1C2 plasmids. Western blot

analysis also provided the first independent confirmation of the proteomic results, showing that ischemic preconditioning does significantly increase the levels of IDH2 and SULT1C2.

Thirdly, we identified that hydrodynamic injection of IDH2 plasmid protected rat kidneys against moderate IRI. This protective effect was seen both with serum assays of kidney function (serum creatinine) and using in vitro assays of mitochondrial respiration. As an essential enzyme in TCA cycle, IDH2 is supposed to affect mitochondria activity through mitochondrial respiration. Sprague Dawley rats that received hydrodynamic venous injections of IDH2 plasmid were exposed to moderate IRI. Strikingly these rats appeared to be resistant to this injury, as their mitochondrial respiration was normal.

Fourthly, we focused on the mitochondrial membrane fluidity study. We also expected that preconditioning could counteract the effect of IRI on membrane fluidity. Our results actually ran counter to the latter hypothesis. GP values showed that ischemic preconditioning actually potentiated the changes in membrane fluidity resulting from IRI. Thus, it seems unlikely that preconditioning or levels of IDH2 and SULT1C2 protect mitochondria against IRI by regulating mitochondrial membrane fluidity.

Finally, we measured the total amount of the mitochondrial fraction extracted for the fluidity study to investigate the effect of ischemic preconditioning on the quantity of renal mitochondria. Since many variables can affect mitochondria levels in the kidney, future studies are needed to determine if ischemic preconditioning can increase the amount of mitochondria present in the kidney.

4.2 The Effect of Hydrodynamic Fluid Delivery on Transgene Uptake in Normal Rats

Hydrodynamic retrograde fine-needle renal vein injection has been shown to be efficient and to generate minimal injury and disruption to regular renal function in previous studies in our lab. We first verified the quality of our plasmid transgene vectors in cell culture before conducting in vivo studies. EGFP-occludin fluorescence signals ran between adjacent nuclei as punctate fluorescent bands along regions that correspond to tight junctions. Fluorescent H2B-tdTomato signals colocalized with Hoechst counterstained nuclei.

After completing these in vitro studies, we began our transgene delivery studies in live animals. Transgene expression was examined in live animal kidneys with intravital two-photon fluorescence microscopy. Using hydrodynamic retrograde venous injections to deliver plasmid vectors, we detected fluorescent protein expression in the kidneys.

We hypothesized that forces produced by this hydrodynamic delivery method may facilitate endocytic uptake and expression of exogenous transgenes encoding IDH2 and SULT1C2 throughout the kidneys, although at present we do not have evidence that supports any particular mechanism by which this procedure facilitates expression.

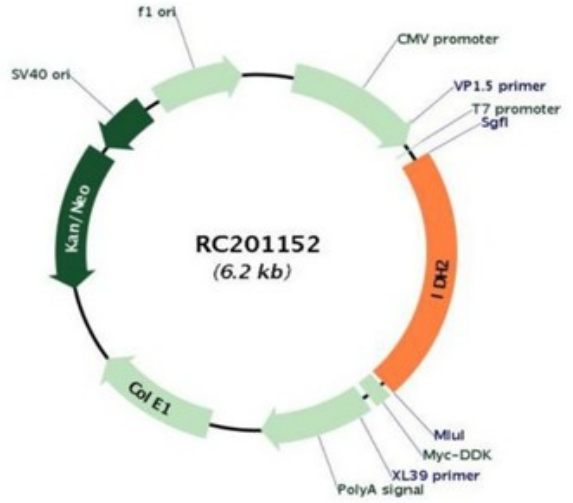
Once we established that this delivery method could reliably facilitate efficient fluorescent protein expression in the rat kidney, we considered whether this method could be used as a way to help physicians in the management of AKI. In doing so we investigated the possibility of delivering genes encoding SULT1C1 or IDH2. The hydrodynamic delivery of plasmid vectors that encoded these proteins provided resistance against AKI in rats (in rats with two kidneys and uninephrectomized rats) that

were subjected to moderate IRI, based on serum creatinine measurements by Dr. Corridon. This demonstrates the ability of this method to achieve sufficient expression of transgenes to affect the pathophysiology of injury.

Transferred exogenous genes was detected in the rats subjected to hydrodynamic transgene injection via in situ PCR. The primers were designed against the gene of Kanamycin resistance carried by the plasmid vector of IDH2 and SULT1C2 (Figure 13). The kanamycin resistance gene is used as a selective marker for exogenous plasmids not naturally occurring in mammalian cells and organisms such as bacteria or yeast. This design solved the problem of nonspecific amplification or of amplification of the endogenous IDH2 or SULT1C2. The upper primer was labeled with fluorescent dye Cy 5, which was used as probe for detection and localization of target transgene in tissue sections. When the in situ PCR reaction was performed without Taq polymerase, weak fluorescence signals were imaged in the tissue sections. In contrast, when direct in situ PCR was performed on the sections with all necessary primers and Taq polymerase, significantly stronger fluorescent signals were observed in proximal tubule segments of the rats that received hydrodynamic SULT1C2 plasmid delivery compared to that of control rats with saline injection. Since the primers were designed for the kanamycin resistance gene which was also contained in IDH2 plasmid, we can predict that stronger fluorescent signals should be observed in IDH2 delivered tissue sections. This result indicates that hydrodynamic injection is sufficient to deliver transgenes to the kidney proximal tubules and facilitate the endocytosis of exogenous plasmids.

This result is also in agreement with the Western blot data, which shows the expression levels of IDH2 or SULT1C2 of the plasmid delivered rats are significantly higher than that of the control rats. In addition, IDH2 and SULT1C2 is overexpressed in the rats that subjected to ischemic preconditioning. The upregulated expression of IDH2 or SULT1C2 confirmed to us that hydrodynamic fluid delivery facilitates the expression of transgenes and was also used to confirm the results of the proteomic studies, showing increase expression of these proteins following ischemic preconditioning.

A



B

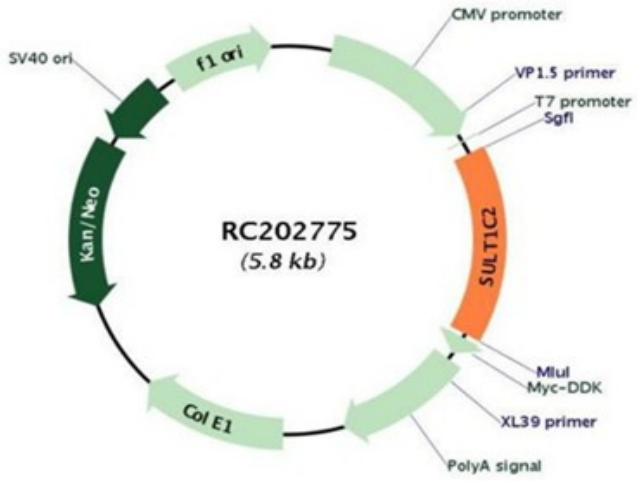


Figure 14. pCMV6-Entry Plasmid vector maps of IDH2 (A) and SULT1C2 (B).

4.3 Hydrodynamic-based Transgene Expression Enhances Mitochondrial Activity in Rats with Moderate IRI

The hydrodynamic delivery of plasmid vectors that encoded IDH2 and SULT1C2 provided resistance against AKI in rats that were subjected from moderate IRI, based on serum creatinine measurements. These genes have been suggested to enhance the activation of specific pathways in mitochondria that provide protection from IRI.

This phenomenon was first suggested from ischemic preconditioning studies conducted in rats. Ischemic preconditioning is an experimental technique that has been applied to produce resistance to induce resistance reductions in blood and oxygen supplies. From a mechanistic perspective, ischemic preconditioning has been studied for the past 50 years, during which time many ideas have been put forward to outline its organ protective role [144].

For instance, in the context of myocardial ischemia, there has been much debate about whether the mechanism involves vascular recruitment as a means of improving myocardial oxygenation by bypassing the site of occlusion [145]. Other studies have implicated mitochondria as key mediators of ischemic preconditioning. Specifically, it is proposed that the opening the mitochondrial ATP-sensitive potassium channel or mitoK_{ATP} plays an important role in producing the protective effect of ischemic precondition. Ischemic preconditioning has been shown to reduce post-ischemic tissue hyper-oxygenation as it preserves NADH dehydrogenase and cytochrome c oxidase, thus maintaining more normal consumption of oxygen.

These events work to prevent oxidative stress, and increase tissue viability on reperfusion [146].Mitochondrial enzymes IDH2 and SULT1C2 have been shown to be upregulated in Sprague Dawley rats kidneys subjected to ischemic preconditioning, and aids in the animal's ability to resist IRI.

Once we confirmed that the delivery method could reliably facilitate efficient IDH2 and SULT1C2 expression in the rat kidney, we considered whether IDH2 or SULT1C2 transgene treatments were sufficient to blunt the effect of IRI in Sprague Dawley rats. We delivered relatively large doses of plasmid vectors that expressed these genes to normal Sprague Dawley rat kidneys. The Sprague Dawley is not genetically resistant to IRI as compared to, for example, the Brown-Norway rat [147]. Ischemic preconditioning is generally done 3-7 days prior to IRI. We waited seven days after the gene delivery process and then induced AKI by subjecting these rats to 40 minute unilateral renal pedicle cross-clamps.

In the previous study of our lab, hydrodynamic retrograde venous injections of either plasmid blunts the serum creatinine increases and keeps mitochondrial membrane potential in rats with moderate IRI. Upregulation of either mitochondria modifier enzyme appeared to have protected renal function from the IRI. So we expected to explore how these enzymes protect the kidney from AKI.

IDH2 could clearly change mitochondrial function based on its role in the TCA cycle. In cellular respiration, metabolites, such as glucose, fatty acids and ketone bodies, are oxidized via the TCA cycle to produce reduced NADH and FADH2. Electrons are transferred by NADH and FADH2 to ETC complexes embedded in mitochondrial inner

membrane. The supply of substrates and oxygen are essential for mitochondria respiration. Export of NADH and FADH2 derived from the tricarboxylic acid (TCA) cycle plays an important metabolic role in mitochondrial respiration. The oxidation of isocitrate by IDH2 is one step in the TCA cycle. This reaction generates not only NADH for the electron transport chain, but also α-ketoglutarate delivered to the following reactions of TCA cycle.

From our mitochondrial respiration data, we determined that IDH2 transgene treatment was sufficient to blunt the effect of IRI on mitochondrial respiration in these Sprague Dawley rats. Mitochondrial respiration rates in rats that received hydrodynamic retrograde venous injections of IDH2 plasmid remained within normal levels despite being subjected to moderate IRI, when compared to rats that received saline injections. Overexpression of the mitochondrial enzyme IDH2 appeared to have protected renal function from IRI by enhancing mitochondrial respiration. Our prediction is that increased IDH2 can trigger certain pathways involved in mitochondria respiration, such as TCA cycle, electron transport system, and oxidative phosphorylation, to enhance the respiration rate against AKI, although considerable work is needed to understand the exact mechanisms involved.

The potential role that SULT1C2 plays is not obvious from its known function. SULT1C2, belongs to sulfotransferase family, is a cytosolic enzyme catalyzing the transfer a sulfo-moiety from PAPS to phenol-containing compounds. Since SULT1C2 was upregulated in mitochondria resulting from the renal ischemic preconditioning, we speculated that the sulfation of the mitochondrial membrane catalyzed by SULT1C2

could regulate the fluidity of mitochondria membrane. The fluidity changes of mitochondria can be detected by shifts in laurdan emission maximums spectrum from 440nm to 490nm. The shift of the emission maximum of Laurdan can be quantified by the Generalized Polarization (GP) function defined as: $GP = \frac{\mathrm{I440-I490}}{\mathrm{I440+I490}}$. The more positive the GP value is, the less fluid the mitochondrial membrane is.

Ischemic reperfusion injury can trigger an increase in mitochondrial membrane permeability. The mitochondrial permeability transition is a key contributor in cell apoptosis or necrosis. Therefore we speculated that ischemic reperfusion injury could increase the fluidity of mitochondrial membrane and ischemic preconditioning could maintain mitochondrial membrane order, which inhibits the mitochondrial permeability induced by AKI.

The GP value of all the groups of rats were negative, which meant that the mitochondria membrane was in the fluid state. Ischemic reperfusion injury led to the increasing of the GP value, compared to the normal level. It implied that the ischemic injury decreased the mitochondria membrane fluidity. Surprisingly, the mitochondrial membrane of the preconditioned rats with subsequent ischemic reperfusion injury was significantly more ordered than the ischemic reperfusion injured rats.

If ischemic preconditioning plays a role in protecting kidney by regulating mitochondria membrane fluidity, we expected it to increase the membrane fluidity, acting in the opposite direction to ischemic injury. However, from our laurdan data, like ischemic injury, ischemic preconditioning ordered the mitochondrial membrane, which

indicates that ischemic preconditioning may not protect mitochondrial function by regulating mitochondrial membrane fluidity. Our existing Laurdan data therefore show that multiple successive ischemic events decreased the fluidity of mitochondrial membrane.

There are problems with our laurdan assay that may make this method unsuitable to gauge the effect that ischemic preconditioning has on mitochondrial membrane fluidity. The fluidity of mitochondria membrane assayed by Laurdan presented the fluidity of the total mitochondrial membranes, since laurdan likely partitions into both the outer membrane and inner membrane. But the fluidity of the inner membrane of mitochondria is most likely the more important parameter to assay.

The inner mitochondrial membrane (IMM) is the active site for the electron transport chain and ATP production. Its fluidity is largely determined by the composition of phospholipids is crucial for mitochondrial function. For example, the activity and stability of the oxidative phosphorylation proteins, a significant portion of the IMM proteins, are affected by their interaction with phospholipids in the IMM. It has been found that complexes III and IV of the oxidative phosphorylation system were destabilized in a disordered IMM.

Among the IMM proteins, a significant portion of proteins comprises the oxidative phosphorylation system, which contains five multi-protein complexes, illustrating the importance of fluidity of inner mitochondria membrane for mitochondrial respiration. In addition, an ordered inner mitochondria membrane is also involved in the import and assembly of proteins. The majority of mitochondrial proteins are encoded in the nucleus

and require protein translocases in the mitochondrial membranes in order to be imported [148]. The changes of mitochondrial membrane fluidity of both outer membrane and inner membrane complicated our analysis and did not allow us to isolate effects on the inner membrane alone.

As ischemic preconditioning protects kidney from AKI, we investigated other potentially protective pathway – whether ischemic preconditioning can increase the amount of mitochondria or their organization in a way that affected the yield of mitochondria in our assays. We measured the total yields of the mitochondrial fractions extracted for the laurdan assay. The data showed that preconditioned kidneys with subsequent IRI had dramatically increased mitochondria yield, while the kidneys subjected to IRI also had increased yields of mitochondria. The changes in yields of the mitochondrial fraction cannot be solely accounted for by ischemic preconditioning treatment. Different kidney size and various weights of the cortices shaved from different kidneys were the variables that contributed to the results. In addition, if ischemic preconditioning treatment could increase the amount of mitochondria in kidney, there should be similar amount of mitochondria in the preconditioned kidneys with subsequent IRI and the preconditioned kidneys without subsequent IRI. From our data, the kidneys treated with ischemic preconditioning alone unexpectedly had lower yields of mitochondria.

At present, little is known about mitochondrial adaptations in experimental or genetic models of resistance. This is an important gap in our knowledge since a variety of organisms and tissues have demonstrated adaptations to ischemia/hypoxia or

anaerobic conditions by altering mitochondria protein expression. There are knowledge gaps in how mitochondria adaptations influence hemodynamics. However, proximal tubules are highly dependent on mitochondrial oxidative phosphorylation. Hypoxia or I/R injury leads to impaired mitochondrial energetic capacity and/or activation of cell death pathways. Post-hypoxic mitochondria are a source of reactive oxygen species, which represent a contributing factor toward cellular injury, and may cause vasoconstriction. For example, superoxide enhances the effects of other vasoactive factors and may have direct actions in reducing renal blood flow [149]. As a result, we hypothesized that adaptations in mitochondrial composition confer protection against ischemia by altering baseline mitochondria function. These adaptations preserve mitochondria integrity in response to injury, leading to cytoprotection and preservation of renal hemodynamics.

In conclusion, we investigated that hydrodynamic fluid delivery of IDH2 plasmid protects kidneys from AKI via enhancing mitochondria respiration. Further studies need to be carried out to explore the role SULT1C2 and ischemic preconditioning play in protecting kidney from AKI. The potential therapeutic benefit observed in these results also provides an exciting platform to facilitate the future management of IRI. Though there is long way to go to being able to apply the hydrodynamic-based gene delivery for gene therapy, this technique facilitates the study of the protective mechanism pathways of ischemic preconditioning.

CHAPTER 5. CONCLUSIONS

We first showed that hydrodynamic fluid delivery could facilitate the delivery of plasmid vectors into live rodent kidneys. Fluorescent plasmids EGFP-occludin and H2B-tdTomato delivered by hydrodynamic fluid renal injection widely expressed in proper localization. The widespread expression of fluorescent plasmid vectors confirmed the efficiency of the hydrodynamic fluid delivery technique. Then IDH2 and SULT1C2 plasmids were delivered to the kidneys of live Sprague Dawley rats. Via in situ PCR, we observed amplified expression of the plasmids in the tissue sections of kidneys administered through hydrodynamic transgene delivery. From the immune blot, the overexpression of IDH2 and SULT1C2 facilitated by the hydrodynamic gene delivery was also observed.

Second, we found that hydrodynamic gene delivery could be used to protect rat kidneys against moderate IRI by facilitating the upregulation of IDH2 in Sprague Dawley rats. Mitochondrial respiration was maintained in the rats that received hydrodynamic delivery of IDH2 plasmid and subsequent IRI. The overexpression of IDH2 protects the ischemic injured rats through the mitochondria respiration pathway.

Third, in order to explore what role SULTL1C2 plays in mitochondria, we utilized Laurdan assay to detect if overexpression of SULT1C2 can alter the mitochondria membrane fluidity. To begin with, we completed the study on whether ischemic preconditioning can alter mitochondria membrane fluidity to resist subsequent ischemic injury. The result is not convincing, because when subjected to renal ischemic injury, the fluidity of mitochondrial membrane both ischemic preconditioning treated rats and sham rats was decreased. It cannot be concluded that ischemic preconditioning can protect kidneys against AKI by regulating mitochondria membrane fluidity. In the future study of membrane fluidity, other experimental methods need to be employed to investigate the changes of mitochondria membrane fluidity.

In conclusion, our studies demonstrated that hydrodynamic fluid delivery of IDH2 plasmid can lessen cell damage caused AKI due to improved mitochondria respiration. Further studies are needed to explore p methods to measure mitochondria membrane fluidity and investigate how ischemic preconditioning and overexpression of SULT1C2 can alter the fluidity of membrane to protect the kidney from AKI.

CHAPTER 6. FURTHER STUDIES

How the ischemic preconditioning and hydrodynamic-based SULT1C2 plasmid delivery prevents kidneys from AKI will be the major tasks for the following study. We will continue to utilize the mitochondrial oxygen consumption study of the Sprague Dawley rats administered through ischemic preconditioning or hydrodynamic fluid delivery of SULT1C2 plasmid to examine whether overexpressed SULT1C2 or ischemic preconditioning can enhance mitochondrial respiration to protect kidney against AKI.

For the mitochondrial membrane fluidity study, polarized-fluorescence spectroscopy is a rapid and traditional method to detect overall changes in membrane fluidity. Besides Laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene), many other fluorescent dyes can be used to detect the membrane fluidity. DPH (1, 6-diphenyl-hexa-1, 3, 5-triene), which is one of the classical probes used for fluorescence polarization measurements [150].

The spin-labeling technique with electron spin resonance can be introduced to determine membrane fluidity in plasma membranes, too [151]. But the fluidity measurement of inner mitochondria membrane is more complicated. One method is to use a polarity-sensitive fluoroprobe to selectively bind to inner mitochondrial

membrane. Cardiolipin, known as the signature phospholipid of the inner mitochondrial membrane, can be used as a potential target of inner mitochondrial membrane [152].

Though our data indicate that hydrodynamic-based IDH2 plasmid delivery increases mitochondria respiration, the role IDH2 plays in the respiration pathway still needs in-depth analysis. For example, by testing whether the catalytic activity of the enzyme is actually required.

Our study demonstrates our hypothesis that hydrodynamic retrograde venous injections can be used to effectively deliver mitochondrial specific genes that can protect mammalian kidneys from acute kidney injury generated from ischemia/reperfusion injury. In the future, this technique can by employed to facilitate the delivery of other genes that have been reported to be upregulated in kidneys of rats that are resistant to moderate ischemia-reperfusion injury. This gene delivery method can enable scientist to investigate the pathways of the genes take effect in kidneys. Hydrodynamic gene delivery has the potential to aid researchers in their pre-clinical and translational gene therapy studies in other fields.

REFERENCES

REFERENCES

1. Bellomo R, Kellum JA, Ronco C. Acute Kidney Injury. The Lancet. 2012; 380: 756–66.
2. Kellum JA, Mehta RL, Angus DC, Palevsky P, Ronco C. The First International Consensus Conference on Continuous Renal Replacement Therapy. Kidney International. 2002; 62:1855-1863.
3. Mehta RL, Kellum JA, Shah SV. Acute Kidney Injury Network (AKIN): Report of an Initiative to Improve Outcomes in Acute Kidney Injury. Critical care. 2007; 11, R 31.
4. Molitoris BA, Levin A, Warnock D. Improving Outcomes of Acute Kidney Injury: Report of an Initiative. Nature Clinical Practice Nephrology. 2007; 3 (8): 439-442.
5. Torio CM, Andrews RM. National Inpatient Hospital Costs: The Most Expensive Conditions by Payer. Agency for Healthcare Research and Quality. 2013; HCUP Statistical Brief #160.
6. Lameire N, Van Biesen W, Vanholder R. The Changing Epidemiology of Acute Renal Failure. Nature Clinic Practice Nephrology. 2006; 2(7): 364–377.
7. Waikar SS, Liu KD, Chertow GM. Diagnosis, Epidemiology and Outcomes of Acute Kidney Injury. Clinical Journal of the American Society of Nephrology. 2008; 3(3): 844–861.
8. Hsu CY, McCulloch CE, Fan D, Ordonez JD, Chertow GM, Go AS. Community-Based Incidence of Acute Renal Failure. Kidney International. 2007; 72(2): 208–212.
9. Ronco C, Bellomo R. Acute Renal Failure and Multiple Organ Dysfunction in the ICU: from Renal Replacement Therapy (RRT) to Multiple Organ Support Therapy (MOST). The International Journal of Artificial Organs. 2002; 25(8):733-747.
10. Hsu CY, Chertow GM, McCulloch CE, Fan D, Ordonez JD, Go AS. Nonrecovery of Kidney Function and Death after Acute on Chronic Renal Failure. Clinic Journal of American Society of Nephrology. 2009; 4(5): 891–898.
11. Ishani A. Acute Kidney Injury Increases Risk of ESRD Among Elderly. Journal of the American Society of Nephrology. 2009; 20(1): 223–228.
12. Coca SG, Yusuf B, Shlipak MG, Garg AX, Parikh CR. Long-Term Risk of Mortality and Other Adverse Outcomes After Acute Kidney Injury: A Systematic Review And Meta-Analysis. American Journal of Kidney Diseases. 2009; 53(6): 961–973.
13. Wooley JA, Btaiche IF, Good KL. Metabolic and Nutritional Aspects of Acute Renal Failure in Critically Ill Patients Requiring Continuous Renal Replacement Therapy. Nutrition in Clinical Practice. 2005; 20(2): 176–191.

14. Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P. Acute Renal Failure – Definition, Outcome Measures, Animal Models, Fluid Therapy And Information Technology Needs. Critical Care. 2004; 8: 204–212.
15. Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury Work Group. KDIGO Clinical Practice Guideline for Acute Kidney Injury. Kidney International. 2012; 2: 19.
16. Weisberg LS. Management of Severe Hyperkalemia. Critical Care of Medicine. 2008; 36 (12): 3246–3251.
17. Tierney LM, McPhee SJ, Papadakis MA. Current Medical Diagnosis and Treatment (44th ed.). McGraw- Hill Medical Publishing Division. 2005; p. 871.
18. Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL. Harrison's Principles of Internal Medicine (16th ed.). McGraw-Hill Medical Publishing Division. 2005; 1644–1653.
19. Wailar SS, Bonventre JV. Biomarkers for the Diagnosis of Acute Kidney Injury. Nephron Clinical Practice. 2008; 109(4): 192–197.
20. Schrier RW, Wang W. Acute Renal Failure and Sepsis. The New England Journal Medicine. 2004; 351: 159–169.
21. Bywaters EG, Beall D. Crush Injuries with Impairment of Renal Function. British Medicine Journal. 1941; 1(4185): 427–432.
22. Abassi ZA, Hoffman A, Better OS. Acute Renal Failure Complicating Muscle Crush Injury. Seminars in Nephrology. 1998; 18(5): 558–565.
23. Parfrey PS, Griffiths SM, Barrett BJ. Contrast Material-Induced Renal Failure in Patients with Diabetes Mellitus, Renal Insufficiency, or Both: A Prospective Controlled Study. The New England Journal of Medicine. 1989; 320: 143–149.
24. Rich MW, Crecelius CA. Incidence, Risk Factors, and Clinical Course of Acute Renal Insufficiency after Cardiac Catheterization in Patients 70 Years of Age or Older: A Prospective Study. Archives of Internal Medicine. 1990; 150: 1237–1242.
25. Schwab SJ, Hlatky MA, Pieper KS. Contrast Nephrotoxicity: A Randomized Controlled Trial of a Nonionic and an Ionic Radiographic Contrast Agent. The New England Journal of Medicine. 1989; 320: 149–153.
26. Bentley ML, Corwin HL, Dasta J. Drug-Induced Acute Kidney Injury in the Critically Ill Adult: Recognition and Prevention Strategies. Critical Care Medicine. 2010; 38(6): 169–174.
27. Sutton TA, Molitoris BA. Mechanisms of Cellular Injury in Ischemic Acute Renal Failure. Seminars Nephrology. 1998; 18 (5): 490–497.
28. Goldsmith D, Jayawardene S, Ackland P. ABC of Kidney Disease. Blackwelln Publishing, Ltd. 2007; p. 34.
29. Liano F, Pascual J. Epidemiology of Acute Renal Failure: A Prospective, Multicenter, Community-Based Study. Kidney International. 1996; 50(3): 811–818.
30. Mehta RL. Spectrum of Acute Renal Failure in the Intensive Care Unit: The PICARD Experience. Kidney International. 2004; 66(4): 1613–1621.
31. Le Dorze M, Legrand M, Payen D, Ince C. The Role of the Microcirculation in Acute Kidney Injury. Current Opinion in Critical Care. 2009; 15(6): 503–508.

32. Zuk A, Bonventre JV, Brown D. Polarity, Integrin, and Extracellular Matrix Dynamics in the Postischemic Rat Kidney. American Journal of Physiology. 1998; 275: 711–731.
33. Thadhani R, Pascual M, Bonventre JV. Acute Renal Failure. The New England Journal of Medicine. 1996; 334(22): 1448–1460.
34. Zuk A, Bonventre JV, Matlin KS. Expression of Fibronectin Splice Variants in the Postischemic Rat Kidney. American Journal of Physiology. 2001; 280(6): 1037–1053.
35. Kwon O, Hong SM, Ramesh G. Diminished NO Generation by Injured Endothelium and Loss of Macula Densa nNOS May Contribute to Sustained Acute Kidney Injury after Ischemia-Reperfusion. American Journal of Physiology. 2009; 296(1): 25–33.
36. Sprague AH, Khalil RA. Inflammatory Cytokines in Vascular Dysfunction and Vascular Disease. Biochemical Pharmacology. 2009; 78(6): 539–552.
37. Rabelink TJ, de Boer HC, van Zonneveld AJ. Endothelial Activation and Circulating Markers of Endothelial Activation in Kidney Disease. Nature Reviews Nephrology. 2010; 6(7): 404–414.
38. Basile DP, Fredrich K, Chelladurai B, Leonard EC, Parrish AR. Renal Ischemia Reperfusion Inhibits VEGF Expression and Induces ADAMTS-1, a Novel VEGF Inhibitor. American Journal of Physiology. 2008; 294(4): 928–936.
39. Basile DP. The Endothelial Cell in Ischemic Acute Kidney Injury: Implications for Acute and Chronic Function. Kidney International. 2007; 72(2): 151-156.
40. Bonventre JV, Yang L. Cellular Pathophysiology of Ischemic Acute Kidney Injury. The Journal of Clinical Investigation. 2011; 121(11): 4210–4221.
41. Johnson GB, Brunn GJ, Platt JL. Activation of Mammalian Toll-Like Receptors by Endogenous Agonists. Critical Reviews in Immunology. 2003; 23(2): 15–44.
42. Kaisho T, Akira S. Toll-Like Receptor Function and Signaling. Journal of Allergy Clinical Immunology. 2006; 117(5): 979–987.
43. Kelly KJ, Williams WW, Colvin RB. Antibody to Intercellular Adhesion Molecule-1 Protects the Kidney Against Ischemic Injury. Proceedings of National Academic Sciences. 1994; 91: 812–816.
44. Wahl P. Renal Tubular Epithelial Expression of the Costimulatory Molecule B7RP-1 (Inducible Costimulator Ligand). Journal of the American Society of Nephrology. 2002; 13(6): 1517–1526.
45. Bonventre JV, Zuk A. Ischemic Acute Renal Failure: an Inflammatory Disease? Kidney International. 2004; 66(2): 480–485.
46. Jang HR, Rabb H. The Innate Immune Response in Ischemic Acute Kidney Injury. Clinical Immunology. 2009; 130(1): 41–50.
47. Awad AS. Compartmentalization 0f Neutrophils in the Kidney and Lung Following Acute Ischemic Kidney Injury. Kidney International. 2009; 75(7):689–698.

48. Claure-Del GR, Bouchard J. Acid-Base and Electrolyte Abnormalities during Renal Support for Acute Kidney Injury: Recognition and Management. Blood Purification. 2012; 34(2): 186–193.
49. Murry CE, Jennings RB, Reimer KA. Preconditioning with Ischemia: a Delay of Lethal Cell Injury in Ischemic Myocardium. Circulation. 1986; 74: 1124–1136.
50. Jennings RB, Murry CE, Reimer KA. Energy Metabolism in Preconditioned and Control Myocardium: Effect of Total Ischemia. Journal of Molecular Cell Cardiology. 1991; 23: 1449–1458.
51. Schott N, Rohman S, Braun ER. Ischemic Preconditioning Reduces Infarct Size in Swine Myocardium. Circulation Research. 1990; 66: 1133–1142.
52. Yin DP, Sankary HN, Chong AS, Ma LL, Shen J, Foster P, Williams JW. Protective Effect of Ischemic Preconditioning on Liver Preservation-Reperfusion Injury in Rats. Transplantation. 1998; 66(2): 152–157.
53. Fan LH, He L, Cao ZQ, Xiang B, Liu L. Effect of Ischemia Preconditioning on Renal Ischemia/Reperfusion Injury in Rats. International Brazilian Journal of Urology. 2012; 38(6): 842–854.
54. Soncul H, Öz E, Kalaycioglu S. Role of Ischemic Preconditioning on Ischemia-Reperfusion Injury of the Lung. Chest Jounal. 1999; 115(6): 1672–1677.
55. Bonventre JV. Kidney ischemic preconditioning. Current Opinion in Nephrology & Hypertension. 2002; 11(1): 43–48.
56. Safian RD, Textor SC. Renal-Artery Stenosis. The New England Journal of Medicine. 2001; 344: 431–442.
57. Schrier RW, Wang W. Acute Renal Failure and Sepsis. The New England Journal of Medicine. 2004; 351: 159–169.
58. Cochrane J, Williams BT, Banerjee A, Harken AH, Burke TJ, Cairns CB. Ischemic Preconditioning Attenuates Functional, Metabolic, and Morphologic Injury From Ischemic Acute Renal Failure in The Rat. Renal Failure. 1999; 21: 135–145.
59. Wever KE, Warlé MC, Wagener FA, van der Hoorn JW, Masereeuw R, van der Vliet JA. Remote Ischaemic Preconditioning by Brief Hind Limb Ischaemia Protects Against Renal Ischaemia-Reperfusion Injury: The Role of Adenosine. Nephrology Dialysis Transplantation. 2011; 26: 3108–3117.
60. Liu GS, Thornton J, Van Winkle DM. Protection Against Infarction Afforded by Preconditioning Is Mediated by A1 Adenosine Receptors in Rabbit Hearts. Circulation. 1991; 84: 350–356.
61. Grover GJ, Sleph PG, Dzwonczyk S. Role of Myocardial ATP-Sensitive Potassium Channels in Mediating Preconditioning in the Dog Heart and Their Possible Interaction with Adenosine A1-Receptors. Circulation 1992; 86: 1310–1316.
62. Ardehali H, O'Rourke B. Mitochondrial K (ATP) Channels in Cell Survival and Death. Journal of Molecular and Cellular Cardiology. 2005; 39: 7–16.
63. Xu M, Wang Y, Ayub A, Ashraf M. Mitochondrial K(ATP) Channel Activation Reduces Anoxic Injury by Restoring Mitochondrial Membrane Potential. American Journal of Physiology— Heart and Circulatory Physiology. 2001; 281(3): 1295–1303

64. Jaburek M, Costa ADT, Burton JR, Costa CL, Garlid KD. Mitochondrial PKC and Mitokatp Copurify and Coreconstitute to Form A Functioning Signaling Module in Proteoliposomes. Circulation Reseach. 2006; 99: 878–883.
65. Murriel CL, Mochly-Rosen D. Opposing Roles of Delta and Epsilonpkc in Cardiac Ischemia and Reperfusion: Targeting the Apoptotic Machinery. Archives of Biochemistry and Biophysics. 2003; 420: 246–254.
66. Friederich M, Hansell P, Palm F. Diabetes, Oxidative Stress, Nitric Oxide and Mitochondria Function. Current Diabetes Reviews. 2009; 5(2): 120–144.
67. Campbell NA, Williamson B, Heyden RJ. Biology: Exploring Life. Pearson Prentice Hall Publishing. 2006; 430–452.
68. Saraste M. Oxidative Phosphorylation at the Fin De Siecle. Science. 1999; 283: 1488–1493.
69. Mitchell P, Moyle J. Chemiosmotic Hypothesis of Oxidative Phosphorylation. Nature. 1967; 213(5072): 137–139.
70. Mitchell P. Coupling of Phosphorylation to Electron and Hydrogen Transfer by a Chemi-osmotic Type of Mechanism. Nature. 1961; 191: 144–147.
71. Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, Scha¨gger H. Cardiolipin Stabilizes Respiratory Chain Supercomplexes. Journal of Biological Chemistry. 2003; 278: 52873–52880.
72. Benard G, Bellance N, James D, Parrone P, Fernandez H, Letellier T, Rossignol R. Mitochondrial Bioenergetics and Structural Network Organization. Journal of Cell Science. 2007; 120: 838–848.
73. Curtis MT, Gilfor D, Farber JL. Lipid Peroxidation Increases the Molecular Order of Microsomal Membranes. Archives of Biochemistry and Biophysics. 1989; 235: 644-649.
74. Rouslin W. Mitochondrial Complexes I, II, III, IV, and V in Myocardial Ischemia and Autolysis. American Journal of Physiology. 1983; 244: 743–748.
75. Bolli R. The Late Phase of Preconditioning. Circulation Research. 2000; 87: 972–983.
76. Crompton M, Costi A. Kinetic Evidence for a Heart Mitochondrial Pore Activated By Ca2+, Inorganic Phosphate and Oxidative Stress. A Potential Mechanism for Mitochondrial Dysfunction during Cellular Ca2+ Overload. European Journal of Biochemistry. 1988; 178(2): 489–501.
77. Crompton M, Costi A. A Heart Mitochondrial Ca2+-Dependent Pore of Possible Relevance to Re-Perfusion-Induced Injury. Evidence that ADP Facilitates Pore Interconversion between the Closed and Open States. Biochemical Journal. 1990; 266(1): 33–39.
78. Crompton M. The Mitochondrial Permeability Transition Pore and Its Role in Cell Death. Biochemical Journal. 1999; 341: 233–249.
79. Kroemer G, Dallaporta B, Resche-Rigon M. The Mitochondria Death/Life Regulator in Apoptosis and Necrosis. Annual Review of Physiology. 1998; 60: 619–642.
80. Martinou JC, Green DR. Breaking the Mitochondrial Barrier. Nature Reviews. 2001; 2: 63–67.

81. Ravagnan L, Roumier T, Kromer G. Mitochondria, the Killer Organelles and Their Weapons. Journal of Cellular Physiology. 2002; 192: 131–137.
82. Childers MK, Joubert R, Poulard K, Frange Robert W. Gene Therapy Prolongs Survival and Restores Function in Murine and Canine Models of Myotubular Myopathy. Science Translational Medicine. 2014; 6(220): 220–231.
83. Verma IM, Weitzman MD. Gene Therapy: Twenty-First Century Medicine. Annual Review of Biochemistry. 2005; 74: 711–738.
84. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, Greenblatt JJ, Rosenberg SA, Klein H, Berger M, Mullen CA, Ramsey WJ, Muul L, Morgan RA, Anderson WF. T Lymphocyte-Directed Gene Therapy for ADA- SCID: Initial Trial Results after 4 Years. Science. 1995; 270(5235): 475–480.
85. Felgner PL. Nonviral Strategies for Gene Therapy. Scientific American. 1997; 276(6): 102–106.
86. Verma IM, Somia N. Gene Therapy - Promises, Problems and Prospects. Nature. 1997; 389: 239–242.
87. Thomas CE, Ehrhardt A, Kay MA. Progress and Problems with the Use of Viral Vectors for Gene Therapy. Nature Reviews Genetics. 2003; 4: 346–358.
88. Lam AP, Dean DA. Progress and Prospects: Nuclear Import of Nonviral Vectors. Gene Therapy. 2010; 17: 439–447.
89. Atkinson H, Chalmers R. Delivering the Goods: Viral and Non-Viral Gene Therapy Systems and the Inherent Limits on Cargo DNA and Internal Sequences. Genetica. 2010; 138 (5): 485–498.
90. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct Gene Transfer Into Muscle. Science. 1990; 247: 1465–1468.
91. Niidome T, Huang L. Gene Therapy Progress and Prospects: Non-Viral Vectors. Gene Therapy. 2002; 9: 1647–1652.
92. Al-Dosari MS, Gao X. Nonviral Gene Delivery: Principle, Limitations, and Recent Progress. American Association of Pharmaceutical Scientisits Journal. 2009; 11(4): 671–681.
93. Xing Y, Pua EC, Lu X, Zhong P. Low-Amplitude Ultrasound Enhances Hydrodynamic-Based Gene Delivery to Rat Kidney. Biochemical and Biophysical Research Communications. 2009; 386: 217-222.
94. Verkman AS, Yang B. Aquaporin Gene Delivery to Kidney. Kidney International. 2002; 61: 120–124.
95. Lien YH, Lai LW. Renal Gene Transfer: Nonviral Approaches. Molecular Biotechnology. 2003; 24: 283–294.
96. Kelley VR, Sukhatme VP. Gene Transfer in the Kidney. American Journal of Physiology-Renal Physiology. 1999; 276: 1–9.
97. Tanner GA, Sandoval RM, Molitoris BA, Bamburg JR, Ashworth SL. Micropuncture Gene Delivery and Intravital Two-Photon Visualization of Protein Expression in Rat Kidney. American Journal of Physiology-Renal Physiology. 2005; 289: 638-643.

98. Moullier P, Friedlander G, Calise D, Ronco P, Perricaudet M, Ferry N. Adenoviral-Mediated Gene Transfer to Renal Tubular Cells in Vivo. Kidney International. 1994; 45: 1220–1225.
99. Zhu G, Nicolson AG, Cowley BD, Rosen S, Sukhatme VP. In Vivo Adenovirus-Mediated Gene Transfer Into Normal and Cystic Rat Kidneys. Gene Therapy. 199; 63: 298–304,
100. Budker V, Zhang G, Danko I, Williams P, Wolff J. The Efficient Expression of Intravascularly Delivered DNA in Rat Muscle. Gene Therapy. 1998; 5: 272–276.
101. Liu F, Song Y, Liu D. Hydrodynamics-Based Transfection in Animals by Systemic Administration of Plasmid DNA. Gene Therapy. 1999; 6: 1258–1266.
102. Zhang G, Budker V, Wolff JA. High Levels of Foreign Gene Expression in Hepatocytes after Tail Vein Injections of Naked Plasmid DNA. Human Gene Therapy Journal. 1999; 10: 1735–1737.
103. Miao CH, Thompson AR, Loeb K, Ye X. Long-Term and Therapeutic-Level Hepatic Gene Expression of Human Factor IX after Naked Plasmid Transfer in Vivo. Molecular Therapy. 2001; 3: 947–957.
104. Alino SF, Crespo A, Dasi F. Long-Term Therapeutic Levels of Human Alpha-1 Antitrypsin in Plasma after Hydrodynamic Injection of Nonviral DNA. Gene Therapy. 2003; 10: 1672–1679.
105. Dai C, Yang J, Liu Y. Single Injection of Naked Plasmid Encoding Hepatocyte Growth Factor Prevents Cell Death and Ameliorates Acute Renal Failure in Mice. Journal of the American Society of Nephrology. 2002; 13: 411–422.
106. Montini E, Held PK, Noll M, Morcinek N, Al Dhalimy M, Finegold M. In Vivo Correction of Murine Tyrosinemia Type I by DNA-Mediated Transposition. Molecular Therapy. 2002; 6: 759–769.
107. Kishida T, Asada H, Itokawa Y, Cui FD, Shin-Ya M, Gojo S. Interleukin (IL)-21 and IL-15 Genetic Transfer Synergistically Augments Therapeutic Antitumor Immunity and Promotes Regression of Metastatic Lymphoma. Molecular Therapy. 2003; 8: 552–558.
108. Ye X, Loeb KR, Stafford DW, Thompson AR, Miao CH. Complete and Sustained Phenotypic Correction of Hemophilia B in Mice Following Hepatic Gene Transfer of A High-Expressing Human Factor IX Plasmid. Journal of Thrombosis Haemostasis. 2003; 1: 103–111.
109. Chen L, Woo SL. Complete and Persistent Phenotypic Correction of Phenylketonuria in Mice by Site-Specific Genome Integration of Murine Phenylalanine Hydroxylase cDNA. Proceedings of National Academy of Sciences of the United States of America. 2005; 102: 15581–15586.
110. Held PK, Olivares EC, Aguilar CP, Finegold M, Calos MP, Grompe M. In Vivo Correction of Murine Hereditary Tyrosinemia Type I by Phic31 Integrase-Mediated Gene Delivery. Molecular Therapy. 2005; 11: 399–408.
111. Zhang G, Budker V, Williams P, Subbotin V, Wolff JA. Efficient Expression of Naked DNA Delivered Intra arterially to Limb Muscles of Nonhuman Primates. Human Gene Therapy. 2001; 12: 427–438.

112. Eastman SJ, Baskin KM, Hodges BL, Chu Q, Gates A, Dreusicke R. Development of Catheter-Based Procedures for Transducing the Isolated Rabbit Liver With Plasmid DNA. Human Gene Therapy. 2002; 13: 2065–2077.
113. Maruyama H, Higuchi N, Nishikawa Y, Hirahara H, Iino N, Kameda S. Kidney-Targeted Naked DNA Transfer by Retrograde Renal Vein Injection in Rats. Human Gene Therapy. 2002; 13: 455–468.
114. Hagstrom JE, Hegge J, Zhang G, Noble M, Budker V, Lewis DL. A Facile Nonviral Method for Delivering Genes and Sirnas to Skeletal Muscle of Mammalian Limbs. Molecular Therapy. 2004; 10: 386–398.
115. Tsoulfas G, Takahashi Y, Liu D, Yagnik G, Wu T, Murase N. Hydrodynamic Plasmid DNA Gene Therapy Model in Liver Transplantation. Journal of Surgical Research. 2006; 135: 242–249.
116. Liang X, Graf BW, Boppart SA. In Vivo Multiphoton Microscopy for Investigating Biomechanical Properties of Human Skin. Cellular and Molecular Bioengineering. 2011; 4: 231–238.
117. Molitoris BA. siRNA Targeted to p53 Attenuates Ischemic and Cisplatininduced Acute Kidney Injury. Journal of American Society of Nephrology. 2009; 20: 1754-1764.
118. Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ. Hydroporation as the Mechanism of Hydrodynamic Delivery. Gene Therapy. 2004; 11: 675–682.
119. Andrianaivo F, Lecocq M, Wattiaux-De CS, Wattiaux R, Jadot M. Hydrodynamics-Based Transfection of the Liver: Entrance Into Hepatocytes of DNA That Causes Expression Takes Place Very Early After Injection. Journal of Gene Medicine. 2004; 6: 877–883.
120. Kobayashi N, Nishikawa M, Hirata K, Takakura Y. Hydrodynamics-Based Procedure Involves Transient Hyperpermeability in The Hepatic Cellular Membrane: Implication of A Nonspecific Process in Efficient Intracellular Gene Delivery. Journal of Gene Medicine. 2004; 6: 584–592.
121. Hall AM, Rhodes GJ, Sandoval RM, Corridon PR, Molitoris BA. In Vivo Multiphoton Imaging of Mitochondrial Structure and Function during Acute Kidney Injury. Kidney International. 2013; 83: 72-83.
122. Corridon PR, Rhodes GJ, Leonard EC, Basile DP, Gattone VH, Bacallao RL, Atkinson SJ. A Method to Facilitate and Monitor Expression of Exogenous Genes in the Rat Kidney Using Plasmid and Viral Vectors. American Journal of Physiology-Renal Physiology. 2013; 304: 1217–1229.
123. Xu X, Zhao J, Xu Z, Peng B, Huang Q, Arnold E, Ding J. Structures of Human Cytosolic NADP-Dependent Isocitrate Dehydrogenase Reveal a Novel Self-Regulatory Mechanism of Activity. The Journal of Biological Chemistry. 2004; 279: 33946–33957.
124. Denton RM. Regulation of mitochondrial dehydrogenases by calcium ions. Biochimica et Biophysica Acta. 2009; 1787(11): 1309–1316.

125. Reitman ZJ, Yan H. Isocitrate Dehydrogenase 1 and 2 Mutations in Cancer: Alterations at a Crossroads of Cellular Metabolism. Journal of the National Cancer Institute.2010; 102(13): 932–941.
126. Joseph JW, Jensen MV, Ilkayeva O, Palmieri F, Alarcon C. The Mitochondrial Citrate/Isocitrate Carrier Plays A Regulatory Role in Glucose-Stimulated Insulin Secretion. Journal of Biological Chemistry. 2006; 281: 35624–35632.
127. Minard KI, McAlister-Henn L. Dependence of Peroxisomal Beta-Oxidation on Cytosolic Sources of NADPH. Journal of Biological Chemistry. 1999; 274: 3402–3406.
128. Lee SM, Park SY, Shin SW, Kil IS, Yang ES. Silencing of Cytosolic NADP(+)-Dependent Isocitrate Dehydrogenase by Small Interfering RNA Enhances the Sensitivity of Hela Cells Toward Staurosporine. Free Radical Research. 2009; 43: 165–173.
129. Lee SH, Jo SH, Lee SM, Koh HJ, Song H. Role of NADP+-Dependent Isocitrate Dehydrogenase (NADP+-ICDH) on Cellular Defence Against Oxidative Injury by Gamma-Rays. International Journal of Radiation Biology. 2004; 80: 635–642.
130. Kil IS, Kim SY, Lee SJ, Park JW. Small Interfering RNA-Mediated Silencing of Mitochondrial NADP+-Dependent Isocitrate Dehydrogenase Enhances the Sensitivity of Hela Cells Toward Tumour Necrosis Factor-Alpha and Anticancer Drugs. Free Radical Biology and Medicine. 2007; 43: 1197–1207.
131. Jennings GT, Sechi S, Stevenson PM, Tuckey RC, Parmelee D, McAlister-Henn L. Cytosolic NADP(+)-Dependent Isocitrate Dehydrogenase. Isolation of Rat Cdna and Study of Tissue-Specific and Developmental Expression of Mrna. Journal of Biological Chemistry. 1994; 269(37): 23128–23134. 1
132. Yang ES, Park JW. Regulation of Ethanol-Induced Toxicity by Mitochondrial NADP(+)-Dependent Isocitrate Dehydrogenase. Biochimie. 2009; 91: 1020–1028.
133. Her C, Kaur GP, Athwal RS, Weinshilboum RM. Human Sulfotransferase SULT1C1: Cdna Cloning, Tissue-Specific Expression, and Chromosomal Localization. Genomics. 1997; 41: 467–470.
134. Ichimura K, Pearson DM, Kocialkowski S. IDH1 Mutations Are Present in the Majority of Common Adult Gliomas But Are Rare in Primary Glioblastomas. Neuro-Oncology. 2009; 11(4): 341–347.
135. Bonventre JV. Kidney Ischemic Preconditioning. Current Opinion in Nephrology & Hypertension. 2002; 11(1): 43–48.
136. Waikar SS, Betensky RA, Bonventre JV. Creatinine as the Gold Standard for Kidney Injury Biomarker Studies? Nephrology Dialysis Transplantation. 2009; 24: 3263–3265.
137. Chance B, Williams GR. Respiratory Enzymes in Oxidative Phosphorylation I. Kinetics of Oxygen Utilization. The Journal of Biological Chemistry. 1955; 217: 383–394.
138. Weber G, Farris FJ. Synthesis and Spectral Properties of a Hydrophobic Fluorescent Probe: 2-Dimethylamino-6-Propionylnaphthalene. Biochemistry. 1979; 18: 3075–3078.

139. Parasassi T, Gratton E. Membrane Lipid Domains and Dynamics as Detected by Laurdan Fluorescence. Journal of Fluorescence. 1995; 8: 365–373.
140. Kaunitz JD, Cummins VPS, Mishler D, Nagami GT. Inhibition of Gentamicin Uptake Into Cultured Mouse Proximal Tubule Epithelial-Cells by L-Lysine. Journal of Clinic Pharmacology. 1993; 33: 63–69.
141. Foo RS, Mani K, Kitsis RN. Death Begets Failure in the Heart. Journal of Clinical Investigation. 2005; 115: 565–571.
142. Kroemer G, Galluzzi L, Brenner C. Mitochondrial Membrane Permeabilization in Cell Death. Physiological Reviews. 2007; 87: 99–163.
143. Sanchez SA, Tricerri MA, Gunther G, Gratton E. Laurdan Generalized Polarization: from Cuvette to Microscope. Modern Research and Educational Topics in Microscopy. 2007; 48: 1689–1700.
144. Basile DP, Donohoe D, Cao X, Van Why SK. Resistance to Ischemic Acute Renal Failure in the Brown Norway Rat: A New Model to Study Cytoprotection. Kidney International. 2004; 65: 2201–2211.
145. Murry CE, Jennings RB, Reimer KA. Preconditioning With Ischemia: A Delay of Lethal Cell Injury in Ischemic Myocardium. Circulation. 1986; 74: 1124–1136.
146. Nilakantan V. Favorable Balance of Anti-Oxidant/Pro-Oxidant Systems and Lated Oxidative Stress in Brown Norway Rats in Renal Ischemia-Reperfusion Injury. Molecular and Cellular Biochemistry. 2007; 304: 1–11.
147. Basile DP, Dwinell MR, Wang SJ, Shames BD, Donohoe DL, Chen S, Sreedharan R, VanWhy SK. Chromosome Substitution Modulates Resistance to Ischemia Reperfusion Injury in Brown Norway Rats. Kidney International. 2013; 83: 242–250.
148. Schmidt O, Pfanner N, Meisinger C. Mitochondrial Protein Import: from Proteomics to Functional Mechanisms. Nature Reviews Molecular Cell Biology. 2010; 11(9): 655– 667.
149. Just A, Olson AJ, Whitten CL, Arendshorst WJ. Superoxide Mediates Acute Renal Vasoconstriction Produced by Angiotensin II and Catecholamines by A Mechanism Independent of Nitric Oxide. American Journal of Physiology- Heart Circulation Physiology. 2007; 292: 83–92.
150. Harris BA, Simon FR. What Should Hepatologists Know About Membrane Fluidity? Hepatology. 1987; 7: 17–180.
151. Betty JG, Robert JM. A New Measurement of Surface Charge in Model and Biological Lipid Membranes. Journal of American Chemical Society. 1976; 98(10): 3044–3045.
152. Alexander VB, Liu S, Soong Y, Mills W, Singh P, Warren JD, Seshan SV, Pardee JD, Szeto HH. The Mitochondrial-Targeted Compound SS-31 Re-Energizes Ischemic Mitochondria by Interacting With Cardiolipin. Journal of American Society of Nephrology. 2013; 24: 1250–1261.