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Review Article

Ischemia Time in Partial Nephrectomies for Kidney Cancer: Strategies to Preserve Parenchymal Function -

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ABSTRACT

There have been significant advances in the nephron sparing surgical techniques with the main aim of preserving as much parenchymal mass as possible in the management of renal cell carcinoma. While the issue of renal ischemia during these partial nephrectomy surgeries have been debated, latest improvements in minimizing the ischemia time, scoring for the ischemic injury on the basis of serum creatinine in patients with a still functioning contralateral kidney and basic and clinical research into the molecular mechanisms and markers for acute kidney injury have given rise to a clearer prediction of functional recovery in the patient. While the surgeon assessment of volume and parenchymal mass preservation is very critical in the prognosis and functional recovery, there are several molecular parameters that are still in the realm of pre-clinical research which, properly translated, would improve the quality of the parenchymal mass preserved and aid in the functional recovery of global parenchymal function. In this review, we focus on the molecular parameters such as induction of a hypo metabolic phenotype, the basis for the induction of hypothermia, the significance of remote ischemic preconditioning, the benefit of reducing inflammation with the use of ethyl pyruvate and finally the importance of reprogramming the inflamed kidney metabolism while emphasizing the basic premise that protecting the mitochondria from the ischemic and inflammatory damages may be the key to improve the quality of the parenchyma preserved. We discuss the benefits and pitfalls in each approach that will foster further translational research, ultimately benefiting the patient.

Keywords: Partial nephrectomy; Renal ischemia reperfusion injury; Warm and cold ischemia time; Global parenchymal preservation

INTRODUCTION

Renal cell carcinoma is one of the most common adult solid tumors occurring in almost 3% of the worldwide population, mostly in older adults between 50 and 70 years of age [1]. In recent years, patients with renal cell carcinoma are being detected at their early stages of the cancer due to the advent of sophisticated imaging techniques such as abdominal ultrasound, computed tomography and magnetic resonance imaging. As a result of the advanced and early detection, clinicians are increasingly relying upon more conservative approaches to the treatment of small renal cancer masses such as open or laparoscopic partial nephrectomies, with the ultimate aim of preserving the global parenchymal function [2,3]. Advances in nephron sparing surgical techniques, particularly in experienced medical centers, help maintain long term renal function and prevent the development of chronic renal disease in patients with small cancer masses [4,5]. Small renal cancer masses are currently described as having a size equal to or smaller than 4 cm (staged as T1a) while the tumors slightly bigger (between 4 and 7 cm) are staged at T1b [6]. The ultimate goal of nephron sparing surgery is to save and preserve as much healthy renal parenchyma as possible while at the same time achieving complete cancer resection and control with no positive surgical margins and without cancer recurrence [5,7]. These techniques include either open partial nephron sparing or laparoscopic partial nephron sparing surgeries and include polar resection, wedge resection, enucleation, excavation or enucleo-resection, with minimal damage to the nephrons that span the cortex and medulla while leaving the renal pelvis and the artery, vein and the ureter intact [5,7]. The surgical decision as to which route to take is mandated by several variables such as the clinical status of the patient, pathological findings, tumor stage, *etc.*, and is left entirely to the discretion of the surgeon involved. But, what is not variable is the prospect of the patient undergoing renal ischemia as a result of the renal pedicle occlusion during the surgery as it has to be performed in a bloodless field, followed by reperfusion, which causes a unique type of renal injury on its own [8]. In this connection, the warm ischemia or the cold ischemia duration and the resulting damages to the renal parenchyma has become a major subject of laboratory and clinical investigation in recent years [9-12]. Numerous studies have been published with respect to how to minimize the ischemia time and maximize the recovery of renal function during the past decade and readers are referred to several excellent reviews that are available in the literature [13-17]. In this review, we will focus on the parameters that are not in the control of the surgeon, namely the mechanisms

of renal ischemia and reperfusion injury, the rationale behind warm or cold ischemia, the critical nature of the time or duration of the blood/nutrient supply occlusion (which has given rise to the concept of “every minute counts”), issues with the mechanisms to preserve the functioning of the kidney after surgery [18]. We will take stock of some of the most recent animal and laboratory studies performed and lessons learned. We will make a case for timely and crucial studies in a clinical setting on the basis of some of the new information gained from these laboratory investigations in order to improve the recovery of parenchymal function post-surgery [19,20]. In this article, we focus on the second of the two most important factors that ultimately decide on the successful recovery of the parenchymal function: quantity and quality. While the “quantity” may be determined by “surgical precision”, the “quality” is determined solely by “molecular precision”, by one or more or all of the influencing factors that are described in this article. Since there is no “single” magic bullet that will positively affect the proper functioning and recovery of the parenchyma, we feel it is necessary to describe all these parameters and emphasize the most important ones that will help in future investigations and possibly foster clinical trials that would benefit the patient. Most of these strategies concentrate on minimizing the renal ischemia and reperfusion injury while some focus on modulating the innate immunity that may be activated once the insult to the kidney is started, such as renal artery occlusion.

Because most of the studies on the tolerance of the kidney to varying warm ischemia times were initially studied using animal systems, it is difficult to extrapolate the benefits of these studies to human conditions in terms of structural and functional loss [21]. For example, using mice and rat systems, it was found that any warm ischemia time more than 35 minutes caused irreversible damage to the parenchyma and compromised glomerular filtration parameters, increased serum creatinine and induced the expression of several ischemia specific markers, a finding which could relate to the human situation [22,23]. Interestingly, when patients were subjected to varying amounts of warm ischemia during their renal reconstruction surgeries (ranging from 30 to 60 minutes), it was found that humans were relatively resistant to the genesis of ischemic pathologies and underwent only limited structural compromise such as brush border loss and clubbing, fragmentation, desquamation and the secretion of ischemia markers such as creatinine and cystatin-C into the blood [24]. The ischemia induced structural changes were less dramatic compared to what was observed in animal systems for the same duration of the ischemic insult. However, when the ischemia time

was increased (> 60 minutes) the probability of inducing significant structural changes also increased [25]. These findings have important implications to the surgeon operating on the patient for small renal cancer masses using partial nephrectomy (PN) procedures. In particular, these studies reveal the intrinsic tolerance and/or resistance of the kidney to clamped ischemia during PN. While many reasons may be proposed, it is very likely that the human kidney possesses a better cellular response to ischemia and precise oxygen sensing and metabolic reprogramming mechanisms due to its capacity to upregulate reno-protective transcription factors such as Hypoxia Inducible Factor-1 α (HIF-1 α) among many other factors [26]. In spite of these impressive human studies, it is very imperative that the surgeon takes no solace in extending the warm or cold ischemia time to anything more than 30-35 minutes, since it is always better to bank on the side of caution and be safe than sorry in the consideration of time taken (ischemia time) in these renal reconstruction surgeries. But, in the case of solitary kidneys, a warm ischemia time of even 20 minutes was shown to result in poorer postoperative Glomerular Filtration Rate (GFR) and a decline of renal function after renal resection [27]. Therefore, this article assumes that there is considerable ischemia and reperfusion related damages and focuses on how to mitigate these damages and related pathologies using recently described animal and human studies as model systems with the main goal of preserving parenchymal function through molecular means.

THE THERAPEUTIC POTENTIAL OF INDUCING HYPOMETABOLISM: THE ROLE OF HYDROGEN SULFIDE (H₂S)

There are at the least 4 enzymes that are capable of generating hydrogen sulfide within the body, namely Cystathionine β Synthetase (CBS), cystathionine γ Lyase (CSE), 3-Mercaptopyruvate Sulfurtransferase (3MST) and Cysteine Amino Transferase (CAT). H₂S was initially regarded as an obnoxious and toxic gas produced as a byproduct of a few metabolic pathways, but recent research has shown that at very low concentrations, it is truly cytoprotective and its protective effects were first shown in brain and retinal systems [28]. Its beneficial effects on renal ischemia systems have been recognized only very recently [29-32]. The impressive effects of H₂S stems from the fact that it is a powerful, specific and reversible inhibitor of cytochrome c oxidase, also known as complex IV or the terminal enzyme complex of the electron transport chain in the mitochondria. Studies by Blackstone and coworkers showed that treatment of mice with 80 Parts Per Million (80 ppm) of H₂S created a deep suspended animation or hypometabolic state with a significant reduction in metabolic rate and core body temperature [33,34]. The translational benefit of this gas transmitter was immediately realized and it was proposed that it would be of great medical benefit in a variety of medical conditions including ischemia and reperfusion injury, transplantation, trauma such as gunshot wounds etc [35]. Later studies showed that apart from inducing a hibernation-like state, the beneficial effects of H₂S included to a reduction of inflammation and oxidative stress by facilitating the production of glutathione, the major intracellular anti-oxidant and by being a scavenger of reactive oxygen species in mitochondria [36]. H₂S also regulated accompanying endoplasmic reticulum stress by facilitating the translocation of the transcription factors nuclear factor erythroid 2-related factor-2 (Nrf2) and nuclear factor kappa light chain enhancer of activated B cells (NF κ B) into the nucleus to up regulate the transcription of anti-oxidant and anti-apoptotic genes respectively [36]. Most importantly, studies showed

that the hibernation-like state induced by H₂S protected mice from lethal hypoxia, a state that many organs such as the kidneys face when the renal pedicle is occluded during reconstruction surgeries [37,38]. H₂S mainly functions *in-vivo* by adding sulfane sulfur (sulfur with a zero oxidation state and a storage form of H₂S) to many proteins by a process called sulfhyration or sulfuration through the formation of polysulfides [39]. A fifth pathway for the production of hydrogen sulfide and the beneficial sulfane sulfur was discovered recently and is called the D-amino acid pathway [40]. Essentially, the human body also metabolizes D-cysteine (as opposed to the amino acid L-cysteine that is found in all proteins). Relevant to this discussion, the human kidney was shown to metabolize D-cysteine to produce H₂S and sulfane sulfur 80 times better than L-cysteine [39,40]. In experimental animal systems, such as mice, administration of D-cysteine increased the level of sulfane sulfur in the kidney 2.5 times the level of the control and decreased gradually to the control level around 12 hours after the administration of the D- amino acid [39,40]. This 12 hour window was reduced to a mere 3 hours when the same experiment was repeated with L-cysteine [39,40]. When the kidneys were analyzed after ischemia induction and reperfusion, there was significant protection of the kidney, as evidenced by several typical ischemia markers, when D-cysteine was administered compared to L-cysteine [39,40]. These studies highlight the important therapeutic and translational potential of D-cysteine whose administration increased the levels of H₂S and the formation of protein bound sulfane sulfur which protected the kidney from ischemia-reperfusion injury. During renal ischemia reperfusion injury, introduction of the hydrogen sulfide donor Sodium Hydrosulfide (NaSH) at clamping decreased serum creatinine, enhanced microvascular blood flow with a significant decrease in inflammation, necrosis and apoptosis in the tubule cells [37]. Bos and coworkers [37] created a hypometabolic state by gaseous H₂S but without hypothermia and showed that bilateral renal ischemic damages could be prevented by H₂S given before ischemia, compared to the controls. They also showed that pre-treatment with gaseous H₂S before ischemia caused less mitochondrial swelling and reduced oxidative stress and degeneration [37]. The role of the mitochondria in the action of H₂S was later confirmed by other investigators [38]. Later work by Bos and his group [41] showed that CSE was the major modulator of H₂S in the kidney. CSE knock out [+/-] animals showed low renal H₂S levels, increased morbidity and mortality, increased DNA damage response and renal functional loss upon bilateral renal ischemia [41]. It was also shown that some of these structural and functional damages to the kidney could be reversed by the administration of the H₂S donor NaSH. Further translational studies done in human transplant biopsies by Bos and coworkers [35] revealed that the level of CSE mRNA could be correlated with improved renal function after kidney transplantation. However, it is very important to note that H₂S may have both anti-inflammatory (the "good") as well as the pro-inflammatory ("the bad") effects in a context and concentration dependent manner which could explain some of the contrasting and conflicting evidence in the recent literature [42]. For example, the H₂S donor NaSH is known to release H₂S too quickly and its concentration fades over time, making interpretation of experiments difficult since this phenomenon does not correspond to a meaningful recapitulation of endogenous H₂S biosynthesis [43]. For this reason, the pharmacological developments of several "slow release" H₂S donors are gaining more significance [44]. Their slow and steady release of H₂S consistently over a period of time correlates with the physiological steady state concentrations of H₂S, allowing more meaningful investigations of the effects of H₂S in chronic and

acute inflammation [44]. Recent studies with these advanced H₂S slow-release compounds have indicated that ischemia reperfusion injury could be minimized due to their specific mitochondria protective actions [44]. In fact, families of mitochondrially targeted hydrogen sulfide donors are being developed, specifically reducing mitochondrial oxidative stress *in-vitro* and reducing renal structural injury *in-vivo* [44]. Moreover, this type of specific H₂S treatment also increases its bioavailability to minimize renal endothelial cell dysfunction [45]. Thus, while the precise molecular mechanisms are still being worked out, H₂S releasing compounds reveal their therapeutic potential in minimizing renal ischemia reperfusion injury and dysfunction. Therefore, they can be considered therapeutically not only for decreasing the metabolic demand of the kidney (creating a state of renal hypometabolism), but also for significantly reducing the oxidative stress, particularly in the renal cell mitochondria by increasing their chances of survival of the parenchyma under hypoxic conditions.

THE THERAPEUTIC POTENTIAL OF INDUCING HYPOMETABOLISM: THE ROLE OF 5'-AMP

The second concept that is relevant to the topic of inducing a hypometabolic state arises from the work of Lee and his group as well as work from our own laboratory [46,47]. In response to severe external stressors such as lack of food and water supply, extreme heat, cold or lack of oxygen supply, several species including certain mammals undergo metabolic adjustment to sustain life throughout their life cycle. This type of hypometabolism, similar to what is described for the therapeutic potential of hydrogen sulfide (see above) is central to the survival of hibernators such as bears, arctic ground squirrels and even in some mammals closer to man, such as tropical Malagasy lemurs [48]. Their entrance into a hypometabolic or hibernation state is the body's attempt to minimize energy expenditure under metabolic stress caused by insufficient nutrient supply [49-52]. However, the uniqueness of these hibernators is that these hypo metabolic changes are rapidly reverted to normal metabolism when these animals are aroused again when the spring season arrives without any signs of cellular or organ injury. Relevant to this discussion, all their organ systems including the brain, heart and kidneys start to function in a normal fashion, in spite of the fact that they underwent long and near ischemic episodes. Studies by Lee's group deciphered this mechanism and showed that a small molecule that is available in every cell in the body and namely 5'-Adenosine Monophosphate (5'-AMP) and allowed the non-hibernators such as mice to rapidly, safely and most importantly reversibly enter a deep hypo metabolic and hibernation-like state [46]. These studies pave the way for the adoption of hypothermia and hypometabolism as a routine clinical tool in several conditions in the future such as renal reconstruction surgeries and renal transplantation etc. Recent biochemical studies from the same group proved that AMP caused hypometabolism by reducing the oxygen carrying capacity of hemoglobin in erythrocytes which forces all the tissues to reprogram their metabolism and reduce all their energy intensive (*i.e.*, ATP requiring) programs such as protein synthesis, functioning of transporters, *etc.* [53]. Recent work from our laboratory expanded on this concept and showed that non-hibernators such as mice could be protected from renal ischemia reperfusion injury by the administration of 5'-AMP before ischemia by reprogramming the kidney metabolism to a hypometabolic state [47]. This is mainly done by a significant reduction of oxidative stress markers and ischemia specific parameters brought out by a specific

activation of the master metabolic stress sensor, namely Adenosine Monophosphate Kinase (AMPK) [47]. Our work on the benefits of pre-conditioning with 5'AMP also showed that it enhanced the expression of the renoprotective transcription factor HIF-1 α and enhanced the expression of the anti-oxidant response genes such as Nrf2, which is already known to be protective in experimental acute kidney injury. Our studies also indicate that the benefits of inducing hypometabolism by 5'-AMP are several fold which could be applicable in a future clinical setting:

1. It positively benefits the functioning as well as recovery of the parenchymal function as evidenced by the reduced expression of several ischemia markers.
2. It reveals an "extended window of opportunity" (around 120 minutes, in our system) in which the blood flow to the kidney could be naturally reduced because of a significant reduction in heart rate. This may help the surgeon to work in a relatively bloodless field during PN surgery.
3. As a result of this natural hypometabolism, the kidney may withstand the effects of additional clamping of the renal pedicle with no significant effects of structural damage.
4. This 2 hour window of opportunity potentially extends the warm ischemia time that could be tolerated by the kidney without any additional damage, offering the surgeon precious extra minutes.
5. The marked reduction in core body temperature of the animal under surgery naturally minimizes the extra metabolic and energy demands, reducing the resultant stresses in the kidney, including severe oxidative stress due to the generation of Reactive Oxygen Species (ROS)
6. Most importantly, the recovery of the blood flow, oxygen consumption and reperfusion in a "gradual fashion" may markedly reduce the effects of fast blood re-flow upon clamp release that causes severe reperfusion injury.

Thus, this method allows for reperfusion to occur in an incremental manner which may allow for a significant reduction in the oxidative stresses that overwhelm the anti-oxidant responses as a result of abrupt blood flow back into the kidney. We also feel that the introduction of these novel concepts in a clinical setting in the future may allow the surgeon to perform renal reconstruction surgeries for small cancer masses with artery clamping and yet operate under an environment of "near-zero ischemia" (as opposed to "severe ischemia" using the current procedures) resulting in improved clinical outcomes. These strategies may help increase the quality of the parenchymal mass preserved. The renoprotective cocktail described in our studies was designed to induce both hypometabolism and hypothermia and we propose that both clamping and flushing the kidney with the renoprotective cocktail will create not only a relatively blood-less field but also a near-zero ischemia field, which surgeons can exploit to their advantage in enhancing renal function recovery as the best way to avoid ischemic injury is to avoid ischemia itself [22,23,47]. There are two more avenues to experimentally induce hypometabolism, namely, the use of ghrelin and 3-Iodo-Thyronamine [T₁AM] [54,55]. But, these experimental concepts are still in their infancy to be seriously considered for significantly reducing ischemia reperfusion injuries during PN.

THE CONCEPT OF INDUCING HYPOTHERMIA

As noted in the discussion above, the two beneficial factors that

make the administration of either hydrogen sulfide or 5'-AMP very attractive are:

1. Both are capable of inducing a hypometabolic state.
2. Both are capable of reducing the Core Body Temperature (CBT) which will reduce the metabolic rate in a natural way.

It is standard practice to “cool” the kidney by a slush of ice in PN procedures. In transplantation surgeries too, it is common to transport the kidney on ice, which in fact offers clinical benefit to some extent. But, we feel that cooling the kidney, as practiced clinically, is not based on sound scientific reasoning and in fact the benefits of cooling can be improved with the following modifications, the rationale behind which is described below. Traditionally, cooling the kidney through ice is thought to generate therapeutic hypothermia, giving rise to the concept of targeted temperature management. But, in non-hibernating species such as man, the mechanisms of this targeted hypothermia are complex and are still poorly understood. The aim of cooling the tissue is “metabolic rate depression” with an eye towards reducing the cascade of secondary inflammation and injury mechanisms which start immediately after the initial insult. By cooling the tissue, there is an expectation that there would be less oxygen consumption and a decreased metabolism when the oxygen and nutrient supply is impaired. Moreover, it is also expected that kidney cooling may reduce the production of free radicals injurious to the kidney. While all the above factors may function giving the surgeon some clinical benefit during open PN surgery, there are still a number of theoretical and practical matters that should be considered. Several clinical studies have been performed comparing cold ischemia to warm ischemia in patients undergoing PN [56-58]. These studies indicate that while cold ischemia provides a better recovery particularly when the ischemia times were prolonged compared to warm ischemia, the overall benefits appear to be relatively modest [56-58]. We feel that this is mainly because the potential benefit of hypothermia is counteracted by its negative effects, offsetting its overall benefit.

First, because one puts the kidney on ice, it does not mean that the mitochondrial bioenergetics which is responsible for producing ATP as well as heat (through electron transport and uncoupling mechanisms) will also slow or shut down. In fact, the opposite happens upon cooling, particularly in non-hibernators such as man [59,60]. The ultimate aim of ice-cooling during PN is to produce a slower metabolic state which may have certain potential clinical benefits. But, this type of ice-induced low metabolic rate has no connection with the naturally induced hypometabolism and has little relevance/benefit when one considers what really happens to the kidney, particularly to its mitochondria upon cooling [61-63]. Second, humans, unlike hibernators, are not designed metabolically to facilitate cooling beyond a very mild hypothermia (less than 32-34°C, which can be accomplished by certain anesthesia regimens). Any cooling below this temperature may cause severe shivering and possibly cardiac arrest in man. But, the work of Lee and his group, as described earlier, has shown that it is possible to reduce the core body temperature of a non-hibernating mammal (mice) to 26°C, yet without inducing shivering and/or cardiac arrest [46]. This is a major conceptual advancement in itself to make a statement that non-hibernators are perfectly capable of generating and withstanding extreme hypometabolism.

But, the studies done through 5'-AMP or hydrogen sulfide have one parameter in common. They all slow down the progress

of electrons through the electron transport chain during oxidative phosphorylation in the mitochondria, whereas hypometabolism through 2-deoxy glucose decreases ATP production through the inhibition of glycolysis. Thus, lowering of ATP levels by any means would down regulate energy intensive processes and biochemical reactions necessary for thermal regulation defense mechanisms. But, hypothermia induced by ice cooling is a result of undefended heat loss from the kidney to the outside. As a result, upon cooling, the kidney mitochondria, which have not inherently slowed down, are kept under severe stress as they continually try to compensate for the cooling effect by producing more heat through the action of uncoupling proteins (UCPs). But, in the absence of nutrient supply and more importantly oxygen supply (oxygen being the final acceptor of electrons in the electron transport chain), the mitochondria are kept under severe and exacerbated stress, not only by the loss of nutrients and oxygen supply but also by cooling, increasing the necessity to produce more heat to compensate [61-63]. Each hibernating mammalian species (such as bears, ground squirrels or Malagasy lemurs) has an optimum core body temperature and a range of temperature that they can tolerate. Once this threshold is crossed and temperature lowered further, their hypometabolic pathways are activated for their survival. But, in man, such temperature compensation and its tight regulation mechanisms to maintain a core body temperature with all the metabolic activities (both biosynthetic and catabolic) comes at an enormous price, *i.e.*, at a significant cost of energy which requires a continuous supply of nutrients, oxygen and other supplies [49-52]. As a result of the significant lack of thermoregulatory compensation mechanisms in man, any external cooling will only aggravate and exacerbate the mitochondrial distress [61-63]. Viewed from this angle, it is possible to further appreciate the significance of the work of Lee and his group which showed for the first time the potential of non-hibernators to undergo hypometabolism. In the light of their studies, one can infer that a proper cooling (*i.e.*, a drop in core body temperature) and hypometabolism can be achieved by agents such as H₂S or 5'-AMP while at the same time protecting the mitochondria (from undergoing severe oxidative stress) by “cooling” the system from the “inside” while ice only cools the system from the “outside” while leaving the mitochondria all the room to undergo oxidative stress. Thus, preserving the kidney by forcing it to undergo hypometabolism either by hydrogen sulfide or 5'-Adenosine monophosphate could be better at protecting the kidney compared to cooling on ice. As a practical alternative, one can consider the administration of one or both of these hypometabolism inducing agents while still mildly cooling the kidney on ice. Whether this combination has synergistic benefits needs to be investigated in laboratory studies before it could be applied clinically.

THERAPEUTIC POTENTIAL OF A RENO-PROTECTIVE COCKTAIL

Since the optimal function of the postoperative kidney is determined by the integrity of the renal parenchyma preserved and by limiting the duration of the Ischemia Reperfusion Injury (IRI) our laboratory has developed a reno-protective cocktail which was shown to mitigate the negative effects of IRI and improve renal recovery and function. The work of Villanueva and his group indicated that during ischemia and reperfusion, the kidney expresses a set of genes and proteins closely resembling embryonic kidney development markers [64,65]. These developmental markers are very likely an effort by the kidney to protect itself during IR injury. These proteins include Basic Fibroblast Growth Factor (BFGF), Bone Morphogenic Protein-7

(BMP-7) and Vascular Endothelial Growth Factor (VEGF). Among these proteins, BFGF participates in the early kidney development as a morphogen and is expressed in the recovery phase of IR. After IR injury, the kidney is also known to upregulate Stromal Cell Derived Factor-1 (SDF-1) which mobilizes bone marrow derived cells into the circulation to repopulate and repair the damaged renal tissue [64-67]. For these reasons, we included BFGF, BMP-7, SDF-1 and VEGF in the renoprotective cocktail [22,23]. We also supplemented the cocktail with hypoxia inducible gene products such as Erythropoietin (EPO) which has been demonstrated to be protective in a murine acute renal failure model [22,23,68-70]. EPO is apparently protective possibly because of the positive feedback loop by which EPO itself upregulated the expression of the Hypoxia Inducible Factor-1 α (HIF-1 α) under hypoxic conditions [71].

Additionally, our cocktail also included a set of amino acids known to protect the mitochondria from excessive oxidative stress (reactive oxygen species, ROS) generated due to sudden oxygen loss during IRI [22,23,72]. Weinberg and associates showed that mitochondrial dysfunction could be prevented to a significant extent by the use of specific citric acid cycle intermediates such as a mixture of α -ketoglutarate, L-Aspartate and malate that help anaerobically maintain electron flux and proton extrusion at the level of Complex I in the electron transport chain [72]. These types of ATP production at the substrate level at the least help retain the structural and functional integrity of the mitochondria by reducing ROS production [72]. Later studies however, have placed the mitochondrial defect upstream of complex I [73]. Our cocktail also included other biochemicals known to protect the mitochondrial membrane and enhance glutathione synthesis namely α -lipoic acid, N-Acetyl cysteine and N-Acetyl carnitine [74-76]. Finally, we also incorporated glutamine as a renoprotective agent since it has been safely used in critical care medicine with significant benefits such as tissue protection, immune modulation, preservation of glutathione and anti-oxidant capacity and most importantly, in reprogramming and preserving the metabolic pathways that are deranged during IRI [77,78]. Glutamine donor pretreatment of rat kidney transplants upregulated Heat Shock Protein-70 (HSP-70) and strongly attenuated the early structural damage suggesting a cytoprotective role for HSP-70 [79]. Very recent studies have shown that glutamine induces HSP-70 expression *via* N-Acetyl glucosamine modification and subsequent increased expression of the transcription factor Heat Shock Factor (HSF) and also by increasing the phosphorylation of HSF [80,81]. This was verified by our own earlier studies using this renoprotective cocktail [22,23]. Very likely, the benefit of glutamine lies in the expression of protective factors of the heat shock response, to protect the proteins that are unfolded and dysfunctional after (IRI) [82]. These results support our earlier hypothesis that empowering and protecting the mitochondria during IR injury and generating a robust cytoprotective response may be the key to reverse ischemic damage during PN. While we are aware that these animal models only approximate human physiology and human kidney's tolerance and response to ischemia, we feel that the lessons learned in these animal studies will pave the way for future clinical trials [83]. It is interesting to note that similar "cocktail approach" has been used by other investigators. In one such study, a mixture of allopurinol, Vitamin E and C and glutathione precursors such as N-Acetyl cysteine used, showed promise in a lower torso ischemia system [84]. But again, its potential role in the benefit of patients undergoing PN mediated IRI has not been investigated using our renoprotective cocktail.

Other than the above potential treatments that might benefit the kidney during IRI, there are a few more and as yet unresolved issues in the pharmacological manipulation of the kidney, in order to improve its function after PN. For example, use of mannitol has been widespread and it appears to protect the kidney from ischemic injury [85,86]. But recent clinical studies tend to question the rationale behind the use of this compound as a free radical scavenger, as an agent that induces osmotic nephrosis and its overall clinical benefit [85,86]. In the same way, use of dopamine or dopamine receptor agonists such as fenoldopam have also failed to show impressive renoprotective effects [87].

ROLE OF SUPEROXIDE DISMUTASES IN RENAL ISCHEMIA REPERFUSION INJURY

Earlier work of MacMillan-Crow and her coworkers have suggested that the loss of the anti-oxidant mechanisms including the inactivation of the mitochondrial Manganese Superoxide Dismutase (MnSOD) might play an important role in renal injury after IR [88]. Saba and colleagues have shown that there was a therapeutic benefit in using the catalytic anti-oxidant and SOD mimetic Manganese porphyrin (MnTnHex-2-PyP5+, Compound I) in animal models of oxidative stress injury [89]. They showed that this porphyrin derivative administered to rats 24 hours before surgery protected the animals against Adenosine Triphosphate (ATP) depletion, nitration of tyrosine residues in proteins, MnSOD inactivation and renal dysfunction. Work of Batinic-Haberle and her group has shown that these Mn porphyrin derivatives are mitochondriotropic due to its higher lipophilicity where it prevents the inactivation of SOD which occurs under oxidative stress [90,91]. In collaboration with the Batinic-Haberle and her group, [22,23] we have used Compound I in our own studies on the development of the renoprotective cocktail. Our studies showed that inclusion of Compound I in the cocktail upregulated several endogenous anti-oxidant defenses, such as glutathione peroxidases, peroxiredoxins, thioredoxin reductase and mitochondrial superoxide dismutase. These data highly suggest that the Mn porphyrin compound I not only could act as a SOD mimetic but also acts as an Nrf2 inducer which in turn upregulated these anti-oxidant enzymes. It is very relevant to note here that a few next-generation derivatives of compound I, such as MnTnBuOE-2-PyP5+ have entered Phase I and II clinical trials to treat brain cancers such as glioma and similar novel derivatives are currently employed in other clinical trials to treat Amyelotrophic Lateral Sclerosis (ALS) and in protect pancreatic β cells during islet cell transplants [92,93]. Therefore, we feel that such novel derivatives of compound I have tremendous translational potential in protecting the kidney during IRI after PN surgery. However, such novel applications are yet to be tested in clinical trials involving renal reconstruction surgeries.

THE FEASIBILITY OF ZERO ISCHEMIA DURING PN

As the best way to avoid renal IRI is to take ischemia out of consideration, several investigators have attempted to develop what is called "zero ischemia" PN using special manipulations such as segmental clamping [94,95]. As this technique requires sophisticated vascular micro dissection maneuvers to isolate the branch vessels leading into the tumor, intraoperative three dimensional CT reconstructions and color Doppler ultrasonography, these zero ischemia techniques have proven to require experienced hands and can be performed only at a few established medical centers

[97]. In spite of all its promise, there was no overall advantage in performing this novel procedure when compared to open PN when the recovery of global renal function was estimated after normalizing for parenchymal loss [97].

On the basis of the laboratory studies and clinical studies done so far, it is tempting to propose that there is an urgent need to develop an “ischemia prevention solution” that can be used when the surgeon performs PN for small renal cancer masses, that is similar in design to “renal preservation solution” used in transplantation studies [98-100]. If the arteries are clamped and the ischemia prevention solution is perfused, the surgeon can not only perform the surgery in a significantly bloodless field, but also aim to protect the renal parenchymal function in a non-ischemic fashion during PN for small renal cancer masses. However, these concepts are still in their infancy and need to be clinically investigated.

POTENTIAL ROLE OF REMOTE ISCHEMIC PRE-CONDITIONING [RIPC] DURING PN SURGERY FOR SMALL RENAL CANCER MASSES

Remote ischemic preconditioning is defined as brief periods of intermittent ischemia followed by reperfusion of (usually forearm) one tissue (skeletal muscle) that can potentially confer subsequent protection against ischemia reperfusion injury in remote organs such as heart and kidneys. The potential of this concept was investigated in animal models with significant and encouraging results [101]. This inexpensive way of protecting tissues against ischemic damage inflicted by a number of procedures such as cardiac surgery and intravenous administration of contrast media in human patients holds much promise [102]. Several human studies with healthy volunteers and clinical trials with patients undergoing PN surgeries have been done, after promising initial clinical trials focusing on the mitigations of the effects of cardiac surgery [103,104]. Particularly in renal medicine, tissue damage extends beyond the initial ischemic insult well into reperfusion due to overwhelming of antioxidant mechanisms by the onslaught of the reactive oxygen species produced. RIPC is thought to be clinically therapeutic since it is an application of a transient and non-lethal episodes of ischemia preferably to the skeletal muscle that reduces the effect of a subsequent and much larger ischemic episode at a distant organ and limiting the reperfusion injury that follows in that organ [105-109]. Incidentally, several studies with patients undergoing laparoscopic partial nephrectomy for small renal cancer masses revealed a very different picture with potentially conflicting results about this renoprotective strategy [110,111]. We feel that these differing results are mainly due to differences in study design, duration and frequency of the ischemic pre-conditioning method applied among many other variables [112-114]. Therefore, caution must be applied before ruling out this procedure entirely as a potential for parenchymal preservation after PN.

Nevertheless, it is important to consider here the molecular mechanisms by which RIPC might work exerting its potential beneficial effects. As with the initial descriptions on the importance of protecting the mitochondria in this article, RIPC might protect the tissue by reducing the oxidative and the apoptosis inducing stresses in the remote organ(s) [115,116]. Extensive laboratory studies indicate that RIPC may offer benefit through a multitude of protective mechanisms [117,118].

1. The protective signal may be conveyed to the remote site

following intermittent limb ischemia by several humoral, neuronal and systemic mechanisms [117,118]. These diverse communicating pathways have been shown to converge on Glycogen Synthase Kinase 3 β GSK 3 β [119]. Inhibition of GSK 3 β subsequent to RIPC reinforces the Nrf2 mediated anti-oxidant defenses, reduces the transcription factor NF κ B dependent pro-inflammatory response and most importantly enhanced the pro-survival tendencies by desensitizing the mitochondrial permeability transition pore, limiting apoptosis [119]. These studies again highlight the critical role played by the mitochondria in generating and propagating the ischemia reperfusion injury, a molecular theme that was repeatedly stressed in our earlier studies.

2. In a rat system, Hussein, et al [120] showed that RIPC activated the anti-oxidant Nrf2, Heme Oxygenase-1 (HO-1) and NAD (P) H : Quinone Oxidoreductase 1 (NQO1) and anti-apoptotic genes (bcl-2 and bcl-X_i) and inhibited the expression of pro-inflammatory cytokine genes TNF- α , IL-1 β and Intercellular Adhesion Molecule1 (ICAM1) in the kidney, underscoring the renoprotective effects of RIPC.

3. As a corollary, these anti-inflammatory responses are proposed to be the ultimate effect of inhibition of the “inflammasomes” of the kidney that receive their pro-inflammatory signals through the activation of toll-like receptors 2 and 4 on renal cell epithelia by binding with Damage Associated Molecular Patterns (DAMPs) [121,122]. Work of Zarbock and his group [106,108] established that these DAMP signaling are the main reason for the pro-inflammatory environment in the post-ischemic and re-perfused kidney. Zarbock and his group have further shown [Zarbock A, personal communication] that a low level of production of the DAMP signals from the muscle during RIPC “sub-lethally” activates the inflammasome pathway in the remote organ [*i.e.*, the kidney] so that the organ is “primed” but not “fully activated”. Such “pre-conditioning” of the kidney with respect to the inflammasome activation produces a protection of the organ to a subsequent and lethal ischemic insult, which falls well within the boundaries of the classical definition of the term “pre-conditioning” the only exception being that such pre-conditioning is done *via* a remote organ.

4. Another mechanism for the protective benefits of RIPC has been proposed, which deals with the kidney generated renoprotective factor Erythropoietin (EPO). It is highly possible that during RIPC and ensuing brief hypoxia, a small quantity of Hypoxia Inducible Factor-1 α (HIF-1 α) is generated which in turn upregulates its target gene EPO in the remote organ [*i.e.*, the kidney]. The generated EPO pre-conditions the kidney and protects it from further damage by a subsequent bigger ischemic insult [69-71].

5. As a corollary to the above reasoning, it has also been shown that either remote pre-conditioning or EPO pre-treatment before surgery primes the kidneys to clear out the ischemia-reperfusion damaged cells in a pig renal ischemia model [123]. This study apparently provides enough justification for the potential reno-protective effects of RIPC and EPO treatment and offers a possible connection between the two mechanisms. Incidentally, EPO is an established drug for the clinical treatment of anemia. Gardner and his group also showed that the renoprotective value of either RIPC or EPO treatment may also relate to their influence on the renal cortical cell handling and clearance of the apoptotic cellular debris that is generated during ischemia and reperfusion as described above [123]. It is worth noting that we had introduced the EPO mediated pre-conditioning in the

development of our own renoprotective cocktail as shown by our previous studies [22,23].

6. As an extension of the hypoxia mediated beneficial effects of RIPC, it was also shown that hypoxia at the remote organ induced the expression and release of extracellular vesicles (exosomes) that protected the kidney remotely, very likely through its cargo namely, specific protective microRNAs [124]. However, these studies are still under investigation.

7. Very recent work of Kaelin and his group showed another important angle to the protective benefits of RIPC through extensive metabolomics, proteomic and parabiosis studies that the inhibition of the Prolyl Hydroxylase 2 (PHD2) in the remote organ (*i.e.*, skeletal muscle) by hypoxia, or by a pharmacological inhibitor or by specific genetic ablation techniques produced specific protection against myocardial infarction following ischemia [125]. They characterized the circulating protective factor to be a metabolite of the amino acid tryptophan namely Kynurenic Acid (KYNA). Their studies also showed KYNA to be a mediator of ischemic preconditioning, which was further proved by abrogating the effects of RIPC through the pharmacological inhibition of the tryptophan metabolic pathway [125]. PHD2 inactivation in the skeletal muscle during RIPC apparently increases the circulating KYNA levels and causes a parallel increase in the obligatory PHD co-substrate α -Keto-Glutarate (α -KG), which in its transit through the liver, produced enhanced levels of KYNA, which exerted its reno-protective actions [125]. In confirmatory studies on the fundamental role of α -KG, these authors also showed that systemic α -KG administration protected hearts from ischemia reperfusion injury.

8. As further evidence supporting the protective role of RIPC, Davidson, et al [126] showed that RIPC involves signaling through the Stromal Derived Factor-1 α (SDF-1 α)/CXCR4 signaling axis. It is very important to note here that even before all these molecular studies were published, our earlier work had included EPO, α -KG as well as SDF-1 α in our renoprotective cocktail [22,23]. Therefore, it is tempting to speculate that, on the basis of all these recently published studies on RIPC, the renoprotective cocktail designated as “GPM” in our previous studies was in fact a “remote preconditioning” cocktail designed to mimic what happens in the muscle [104,106,116]. Therefore, the GPM cocktail could in reality be a cocktail designed to induce RIPC beside its many other renoprotective and mitochondriocentric functions [22,23].

It must be noted here that in spite of all these highly plausible and exciting mechanistic studies, it is still a challenge to translate RIPC studies on animal models into clinically meaningful studies in humans, particularly in patients undergoing PN for renal cell cancer masses. We feel that it is only a matter of time before the right conditions for application of RIPC to patients are worked out in reproducible detail and introduced into the clinic.

THE BENEFITS OF ETHYL PYRUVATE IN ISCHEMIA REPERFUSION INJURY

Even though it may not be detectable by accepted measures, it is safe to assume that ischemic injury starts at the moment the renal vessels are occluded. This injury only intensifies over time and becomes noticeable beyond 30 minutes of ischemia by following the ischemia specific markers such as Kidney Injury Molecule-1 (KIM-1), Neutrophil Gelatinase Associated Lipocalin-2 (NGAL), Galectin-3 (Gal-3), creatinine and cystatin-c etc either in the tissue

or in urine [127,128]. But, the innate immunity mechanisms do not have this time lapse luxury of waiting beyond 30 minutes. As a first line of defense, the innate immune cells are activated the moment the ischemic injury markers are produced, even at undetectable concentrations. This is mainly because most of the ischemia injury markers are “Damage Associated Molecular Patterns” (DAMPs) [121,122]. Therefore, as mentioned at the beginning of this article, we have to not only mitigate the “ischemia reperfusion injury” that occurs, but also have to minimize the “innate immune cell attack” that is initiated by DAMPs. These ischemia specific markers that are capable of activating innate immune cells include extracellular produced Heat Shock Proteins (HSPs), small molecules like Uric Acid (UA), Adenosine Triphosphate (ATP), S-100 protein, and dissolution of the extracellular matrix producing small Molecular Weight Hyaluronic Acid (LMW-HA), High Mobility Group Box Protein 1 (HMGB1) to name a few [121,122]. Among these ischemia specific markers, HMGB1 has received much attention with respect to its role in activating the immune cell attack [129,130]. HMGB1 was shown to be a multifunctional protein [131]. Intracellularly, it acts as an architectural chromatin binding factor responsible for the stability of chromosomes. However, it can be passively released by any cell during cellular damage or virus infection or it can be actively secreted by innate immune cells in response to exogenous stimuli such as bacterial pathogens or by endogenous stimuli such as DAMPs, Tumor Necrosis Factor α (TNF α) or by Interferon- γ (IFN- γ). Thus, HMGB1, once released into the extracellular environment, acts as another DAMP and interacts with Toll-like receptors-2 and 4 (TLR2 and TLR4) and with the Receptor for Advanced Glycation End Products (RAGE) [132]. The signal transmitted through this activation culminates in the intracellular activation of the NF κ B signaling pathway and subsequently the proteolytic cascade platform called the “inflammasome” [133]. These inflammasomes, in turn accelerate the extracellular release of more HMGB1 apart from the release of active inflammatory mediators such as Interleukin-1 β (IL-1 β) which activates the inflammasome pathway in the manner of a vicious cycle. Therefore, many investigators have focused on inhibitors of HMGB1 release as a potential therapeutic mechanism to mitigate ischemia and innate immune cell mediated damage that occurs during partial nephrectomy procedures that require prolonged periods of warm or cold ischemia. One such inhibitor, characterized to a great extent is the small molecule Ethyl Pyruvate (EP) which is a simple ester of pyruvic acid and ethyl alcohol [134-136]. This is shown to be superior to the effects of using pyruvic acid or sodium pyruvate alone because of its enhanced stability, hydrophobicity and cell penetrating property. Recent results from several laboratories have shown that EP treatment reduced the severity of ischemia mediated damages in many experimental systems [134-136]. The most notable mechanism is the capacity of EP to inhibit the release of HMGB1 from the nucleus into the cytoplasm which has to be accomplished before its release into the extracellular environment where it can act as a DAMP, initiating inflammation and ischemia mediated damaging signals downstream [137]. Other potentially beneficial mechanisms of EP include:

1. Its capacity to act as a scavenger for the Reactive Oxygen Species (ROS) [137].
2. Its induction of the anti-oxidant transcription factor Nrf2 and the anti-oxidant protein Heme Oxygenase-1 (HO-1) [138].
3. Competing with the inflammatory transcription factor NF- κ B for binding to its co-activator protein p300, thus preventing the entry of the active NF- κ B subunit complex p65/p50 into the nucleus [139-141].

4. By inhibiting the immune modulator enzyme Indolamine Dioxygenase (IDO) [142].

HOW METABOLIC REPROGRAMMING CAN PROTECT RENAL PARENCHYMA FROM ISCHEMIA-REPERFUSION INJURY AND ACTIVATION OF INNATE IMMUNITY MECHANISMS?

Unmasking the real renal dysfunction/Acute Kidney Injury (AKI) mechanisms in the operated kidney while the healthy contralateral kidney is still functional has been a challenge after partial nephrectomy/renal reconstruction procedures. Even though patients develop varying degrees of AKI during and after PN due to ischemia, most of these patients exhibit a significant recovery of renal function. The development of AKI after surgeries such as PN is qualitatively and quantitatively different from the AKI that develops after Chronic Kidney Disease (CKD) [143]. Recovery from AKI after PN is a function of the parenchyma preserved among many other parameters such as ischemia time, warm/cold ischemia and the route of approach to the surgery itself. Several recent studies have shown that the extent or percentage of recovery depends upon the metabolic status of the operated kidney and how well it readjusts or achieves metabolic homeostasis by reprogramming its metabolism after the initial trauma of the inevitable surgical procedure of PN itself. Therefore, it is worth taking stock of the recent advances in the metabolic pathways that are deranged when surgery happens and the metabolic efforts made by the kidney to revert back to normalcy. The extent of this reversion to normal state will determine the functional recovery of the parenchymal mass that is spared. Irrespective of the surgical route taken, inflammation, ischemia, subsequent reperfusion and the activation of the innate immunity mechanisms appear to be common denominators that ultimately determine the extent of AKI and functional recovery after PN. Therefore, the metabolic pathways that regulate the above processes are very important determining factors for the functional recovery of the patient.

It is important to note that these metabolic pathways are deranged (leading to varying degrees of AKI) the moment the surgery is started, starting from trauma, inflammation and activation of innate immunity effectors. Our earlier studies with the development of a renoprotective cocktail and studies from other laboratories emphasized the importance of protecting the mitochondria by preventing its production of Reactive Oxygen Species (ROS) in unacceptable amounts [22,23,144]. Moreover, other studies have indicated that the tumor suppressor protein p53 induced the target protein TIGAR that acted in a pro-apoptotic or pro-survival mechanism by reprogramming the cellular metabolism in kidney proximal tubules depending upon the ischemic burden [145]. And, at the same time, the role of mitochondria in regulating the cell metabolism should be considered. As the level of ischemic injury depends upon the ischemia time, it stands to reason that the metabolic reprogramming that occurs in the parenchyma after PN is critically dependent upon and directly proportional to the extent of ischemic injury. Since the tumor suppressor protein p53 is the gate keeper of metabolic homeostasis inside the cell, recovery of the global parenchymal function after PN may be a function of the p53 status in the kidney and how this tumor suppressor protein directs its effector signaling pathways downstream [146]. Studies have indicated that the final effect of p53 in renal ischemia mitigation is context dependent

[147,148]. Therefore, the issue of metabolic reprogramming should be viewed with caution as it can function as a double edged sword in regulating the effects of ischemic injury [149]. Apart from p53, proteins such as mammalian Target of Rapamycin Complex-1 (mTORC1) and HIF-1 α also help in maintaining renal tubular metabolic homeostasis and are essential in response to ischemic stress particularly in regulating the tubular energy and glycolysis metabolism [150,151]. Most of the metabolic reprogramming that occurs after PN [during ischemia and reperfusion] can be classified as those pathways such as

1. Impaired purine metabolism
2. Impaired arginine metabolism
3. Transition from glucose to lipid metabolism
4. Reprogramming of taurine and hypotaurine metabolism [152-154].

These metabolic reprogramming pathways are associated with the lesions that occur as a result of early injury, alternative energy sources, inflammation, activation of innate immunity pathways such as inflammasomes and late phase kidney recovery, ultimately determining the level of reversal to homeostasis and parenchymal function. These shifting metabolic pathways (particularly towards glycolysis) explain why some regenerating proximal tubules undergo atrophy as they are influenced by factors such as HIF-1 α , mTORC1 and pro-fibrotic transforming growth factor- β (TGF- β) and the mitochondrial alterations [155]. Since these tubules support a high level of transport functions which require a constant ATP supply, any change in the mitochondrial metabolism from oxidative phosphorylation to glycolysis, particularly as a result of HIF-1 α induction during hypoxia (due to surgery, which depends upon the ischemia time) will force the tubule cells to dedifferentiate [155]. Parallel input from the pro-fibrotic TGF- β will force these cells to atrophy if the metabolic reprogramming due to dedifferentiation is not reversed in time. Redifferentiation mechanisms promoting survival of these tubules again depends upon a specific metabolic reprogramming whose signatures have been worked out recently [155,156]. While metabolic programming through HIF-1 α mediated shift to glycolysis and fatty acid utilization are a characteristic feature of dedifferentiated proximal tubules, the level of insult from pro-fibrotic TGF- β forces these tubules to fail to redifferentiate, reverse their mitochondrial pathologies and fail to regenerate, causing fibrotic complications after surgery as a result of maladaptive repair [155-157]. Thus, these complications will determine the quality of the parenchymal function after PN as described earlier.

CONCLUDING REMARKS

In this review, we have attempted to give a comprehensive listing of the molecular parameters that may influence the “quality” of the parenchymal mass preserved during the nephron sparing surgeries, while the “quantity” is entirely in the control of the surgeon. The final functional outcome of patients undergoing partial nephrectomy is very likely determined by the preserved parenchymal volume as well as the extent of warm or cold ischemia time, tumor complexity and the precision of excision. However, this matter is still debated. It is our view that apart from the surgical parameters that are being extensively studied, there is still more to be done in the name of improvement, particularly in the strategies to minimize ischemia

time, protecting the parenchymal mass from inflammatory and oxidative injury resulting from occlusion of blood flow. It is our hope that the parameters described in this review will stimulate further discussions and translational research in this field.

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