Efficacy of Kidney Reperfusion Treatments Relative to Function and Survival Rates after Transplantation

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Abstract

Donor kidneys are damaged upon circulatory death before being preserved with machine perfusion until transplantation. During machine perfusion, blood is pumped back into the vasculature of the kidney, causing damage called ischemic reperfusion injury. The two forms of machine perfusion are hypothermic machine perfusion and normothermic machine perfusion. Different methods of treatment can be used during either hypothermic machine perfusion or normothermic machine perfusion to prevent ischemic reperfusion injury. Each of the treatment methods that were reviewed showed potential for preventing ischemic reperfusion injury, with hypothermic treatments having higher average levels of feasibility than normothermic treatments. The effect of the analyzed treatments on graft function after transplantation is likely positive, so a conclusion can be drawn that undergoing any one of the treatment methods will lead to positive results after transplantation.

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Current Methods of Kidney Preservation

Kidney transplantation is currently the best solution for patients with end-stage kidney disease. Care for transplant patients is more advanced than ever, and the overall survival rate for these patients has increased. However, with the current length of kidney transplant waitlists and the lack of available donor kidneys, patients are waiting for up to six years before they can have a kidney transplant. The wait is much longer for a patient to have a kidney transplantation with a deceased-donor (DD) organ. DD organs are either those that are donated after circulatory death (DCD), or those donated after brain death (DBD). In the situation of a DCD, the organ is already being damaged because of a lack of blood flow to its tissues. Typically, it is less ideal to use these organs because the graft is already damaged and may take some time to heal before it can undergo transplantation. Organs donated after brain death (DBD) are still being perfused in the donor's body, though the donor has died from brain death. Brain death results in the kidney sustaining metabolic and hormonal disturbances, while kidneys donated after cardiac death experience ischemia between the point of cardiac death and reperfusion. Surgeons can remove the organ before the circulation in the body stops, damaging the graft. Receiving a kidney from a living donor requires both the donor and patient to be immunologically compatible, meaning the recipient's immune system will not reject the foreign organ. It is especially hard to match with a diseased donor. Upon the death of a donor, it is paramount that the kidney is recovered and preserved.

The current procedure for preserving DCD kidneys is to put them in static cold storage (SCS). SCS is the storage of a kidney in a cold solution kept between 4-8 °C (Radajewska et al., 2022). Organs being stored in containers with ice are suspended in fluid inside a bag so the

kidney does not come in contact with the ice. A current mainstream cold storage method for preserving kidneys is the Lifeport Kidney Transporter (LKT). The LKT is a machine that houses a single kidney and keeps it at a cold temperature while allowing cold perfusate to circulate through the kidney vasculature. While a kidney is being preserved in a LKT, it can be transported to a hospital where it will await transplantation (*Lifeport Kidney Transporter*, n.d.). Though this method has been sufficient, there is an issue that presents itself during SCS. The cessation of blood flow through the organ causes small clots to form, which can damage the organ. This issue commonly leads to donated kidneys becoming unusable and decreased survival rate of kidneys after transplantation. Machine perfusion was introduced to combat this damage by continuing circulation in the kidney. The kidneys are connected to perfusion machines to rejuvenate blood flow through the organ. A perfusate solution kept at either a cold or warm temperature is pushed through the kidney, reducing the possibility of the formation of clots. Hypothermic machine perfusion (HMP) pushes a cold solution through the kidney, while normothermic machine perfusion (NMP) pushes a warm solution. It is preferred for machine perfusion to be done under near-physiological conditions. NMP keeps the temperature at approximately 37 °C (98.6 °F). A warmer temperature will allow for a better pre-transplant assessment because it simulates the organ being in the body (Pool et al., 2019).

This thesis aims to summarize and evaluate existing treatments for kidneys under temperature-sensitive experimental conditions and determine which treatment reduces reperfusion injury and improves graft function and overall survival after transplantation. Discovering an ideal method of treatment could lead to more DCD kidneys being available for transplantation. Wait times for kidney transplant waitlist patients would decrease exponentially, and the quality of graft function after transplantation could potentially be improved. The success

of these different types of treatment methods may have lasting effects and lead to a positive future for organ donation.

The act of removing an organ from a body and keeping it alive via perfusion so it can be assessed and evaluated for transplantation is referred to as *ex-vivo* perfusion. It is quickly being discovered that *ex-vivo* perfusion is one of the most effective ways to preserve organs as well as recondition and even improve their function before transplantation. *Ex-vivo* perfusion for kidneys can lead to improvement in organ quality because the organ can be put in simulated conditions resembling the conditions of a recipient's body. The organ can be treated for its reperfusion injuries and acclimate to a simulated version of its recipient's physiological conditions at the same time. A major benefit to putting kidneys on machine perfusion *ex-vivo* is that the kidney is not in a recipient's body where there is a possibility of rejection.

A general literary review of known preservative treatment methods for kidneys during *ex-vivo* machine perfusion was derived from empirical studies. The various treatment methods show influence on graft function as well as the kidney's rate of survival after transplantation.

Ischemic Reperfusion Injury

With the use of HMP and NMP in the preservation of kidneys, a new problem presents itself in the form of IRI. IRI is defined as cellular dysfunction and death following the restoration of blood flow to ischemic tissues. This damage is paradoxical because the only way to save ischemic tissues is to reinstate blood flow despite the damage that is caused. Ischemia is a challenging problem because of the effects of its three main phases: hypoxia, cell death, endothelial dysfunction (Knijff et al., 2022).

The first phase is hypoxia, or the lack of oxygen. Hypoxia is the result of a lack of blood flow to the organ. One would think that during reperfusion, the tissues that are receiving blood again would react positively. However, IRI occurs upon the reoxygenation of these tissues. This phenomenon occurs because of the processes taking place in the kidney cells. When the cells are receiving oxygen, hypoxia inducible factor (HIF) α proteins are produced. These proteins (HIF-1α and HIF-2α) are marked with oxygen by enzymes called prolyl hydroxylases (PHD). The oxygen acts as a cofactor to identify the proteins so they can be degraded by a proteasome. When the cells are in a state of hypoxia, there is no oxygen for the PHDs to mark the HIF-α proteins, and they are not degraded. HIF-1α and HIF-2α can then translocate to the nucleus with HIF-1β, where they bind to HIF response elements on the DNA. This causes many genes that regulate angiogenesis, metabolism, and cell growth to be disrupted (Knijff et al., 2022).

The second phase of IRI is cell death. Cell death takes place when blood is being reperfused through the vessels because a large number of reactive oxygen species (ROS) are released. Dying cells release damage-associated molecular patterns (DAMPs), which activate the body's immune response. This could cause a negative effect on graft function as the organ is undergoing the process of being accepted by the recipient. Apoptosis and necrosis are the main forms of cell death that occur upon reperfusion. Once a cell's death is initiated, surrounding cells begin to systematically dismantle. While there is still cell death that occurs, HMP has shown to reduce the amount of cell death from apoptosis and necrosis. When fewer cells are dying, fewer DAMPs are released, and there is lower activation of immune response. Though HMP may not drastically lessen cell death, it is still much preferred to static cold storage because graft survival rates are increased due to there being less activation of the immune system. Other forms of cell death, such as necroptosis, pyroptosis, and autophagy, have not been studied extensively yet,

though it is known that these different forms of cell death may occur in different immunological pathways. Necroptosis is similar to apoptosis because it is a type of programmed cell death that involves the formation of a necrosome, a complex consisting of pseudokinases. Apoptosis is referred to as programmed cell death because the death of the cell is mediated by multiple enzymes leading to a non-lytic cell death (Chaouhan et al., 2022). Pyroptosis is a type of programmed cell death relating to tumor progression. Pyroptosis is also described as involving microorganism invasion (Wang et al., 2023). Autophagy is the process of a cell that delivers intracellular components to the lysosome for degradation. Autophagy is typically a healthy process, but when a cell is old or damaged and could possibly lead to the development of cancer, autophagy facilitates the removal of the bad cell (D'Arcy, 2019)

The third phase is endothelial dysfunction. The endothelium of the kidney can be a target of inflammation. Leukocytes adhere to the endothelium and insert neutrophils into the cells. Fibrosis in the connective tissue of the graft is a common result of endothelial dysfunction. Therefore, one can assume there is endothelial dysfunction taking place in a graft where fibrosis is observed (Knijff et al., 2022). P-selectin was found to play an important role in the invasion of the tissue by leukocytes, and intercellular adhesion molecule 1 (ICAM-1) is a key factor in the adhesion of leukocytes to the endothelium. Several studies have been conducted in which organs were preserved using HMP or static cold storage, and when compared, the organs that underwent HMP had a lower expression of P-selectin and ICAM-1 (Henry et al., 2012; Zeng et al., 2019; Zhao et al., 2017).

The glycocalyx is also a part of the endothelium that is damaged by IRI (Chappell et al., 2009). The glycocalyx covers the endothelium and is essential for leukocyte and platelet adhesion. Most of the damage to the glycocalyx takes place during reperfusion (Abassi et al.,

2020). A study was performed to examine the degradation of the glycocalyx of a human liver during transplantation, in which biomarkers for degradation of the glycocalyx were discovered to be released when the liver was damaged during reperfusion. The levels of one of the biomarkers called heparin-sulfate in effluent veins were found to be much lower than those in venous blood. This could be a marker showing that the damaged graft is being repaired. Another study supported glycocalyx regeneration during HMP by showing how the thickness of the glycocalyx increased over time (Snoeijs et al., 2010). According to these results, there could be benefits to longer periods of HMP to give the glycocalyx more time to regenerate. Although more research is necessary to confirm its feasibility, these examples of the effect of HMP on the glycocalyx of human livers could have similar results on renal grafts because the same type of damage was done from IRI to the glycocalyces of both organs. Membrane permeability is increased when there is a sudden interruption of blood flow and tissue oxygenation. Resumed oxygen supply during reperfusion facilitates the generation of reactive oxygen species, which contributes specifically to glycocalyx damage (Abassi et al., 2020).

The fourth phase of IRI is its effect on immune response. A key element of kidney transplantation is finding a kidney that will be compatible with the recipient's immune system. There is also the issue that when the kidney and recipient are a match, the recipient must take immunosuppressive medications daily to ensure the body does not reject the graft. Immunological response most often appears in the form of inflammation. Toll-like receptors (TLRs) are proteins that are responsible for directing immune response in the gut. A damage-associated molecular pattern (DAMP) called HMBG1 located in the nucleus of the cell is released during cell death and is sensed by TLR4. HMBG1 binds to TLR4, causing inflammation (Leventhal & Schröppel, 2012).

Examination of IRI has revealed there to be an upregulation of TLR2 and TLR4 in the kidney. TLR4 activation has been found to lead directly to higher concentrations of pro-inflammatory cytokines and adhesion molecules and the attraction of a host of immune cells (Arslan et al., 2010; Nieuwenhuijs-Moeke et al., 2020)**.** TLR4 is strongly correlated to renal graft dysfunction, according to studies in which its effect on rat kidneys was examined (Krezdorn et al., 2018)**.** HMP has been shown to cause TLR4 levels to drop, leading to less damage from inflammation caused by IRI (Xue et al., 2018). HMP in porcine kidneys has also been shown to reduce the expression of other pro-inflammatory cytokines like IL-6 and IL-8 (Hosgood et al., 2017).

HMP has been studied and determined to be the gold standard for donor kidney preservation. Knijff et al. (2022) conducted a review on HMP and how it prevents much of the damage caused by IRI. The purpose of the study was to analyze the effect that HMP can have on the pathophysiology of IRI while comparing HMP to static cold storage. IRI is inevitably going to happen in donor organs that are reperfused, but HMP has been shown to cause less damage when compared with other forms of organ preservation.

The ongoing organ shortage calls for an increase in the use of marginal kidneys. Marginal kidneys are those that are suboptimal but still can be used for transplantation. There are not enough healthy kidneys ready to be donated. The high number of marginal kidneys is a direct result of IRI, which leads to delayed graft function. There is currently no FDA-approved therapy for recipients experiencing delayed graft function (Bahl et al., 2019). However, research is being done to find potential treatment methods to ameliorate delayed graft function. For example, one study has found that estradiol can be used before transplantation as an *ex-vivo* treatment for dysfunction in the kidney caused by IRI. The results of the study showed that estradiol works as

a protective mechanism against the pro-inflammatory and IRI-related effects in rat kidneys when it is implemented during preservation time (Zhou et al., 2022).

IRI is an extremely complex process that has a negative effect on donor kidneys before and after transplantation. Researchers have noticed that proximal tubular cells (PTCs), cells that are heavily involved in renal homeostasis, detoxification, and drug elimination, are common victims of the damage caused by IRI. PTCs in the kidney are more sensitive to IRI than other renal structures. They are sensitive because they are responsible for the expression of many transporters that make up important electrolytes, glucose, amino acids, anions, uremic toxins, and xenobiotics (Faucher et al., 2020). Certain Xenobiotics are used after transplantation, so their preservation is essential to prevent delayed graft function.

Research has discovered many different regenerative therapies to treat kidneys while they are undergoing NMP or HMP. Treatments such as cellular, gene, and biological therapies have been studied during HMP (Gregorini et al., 2017; Moser et al., 2016; Sedigh et al., 2014), while the use of nanoparticles and antibodies, along with some of these treatments, have been studied during NMP (Rogers et al., 2016; Tietjen et al., 2017). Experimentations using rodent, porcine, and human kidneys have shown that each of the various methods of treatment has had positive results in healing IRI and kidney survival after transplantation. However, little research has been conducted to determine which treatment method provides the best results after transplantation.

Cellular Therapies

The first method being assessed is cellular therapies. Cellular therapies can be utilized in both HMP and NMP. The most prominent method for cellular treatment is the use of mesenchymal stromal cells (MSC). MSCs are cells that are able to produce tissue such as bone marrow in a fibroblast-like manner (Casiraghi & Remuzzi, 2019). MSCs have previously shown

to multiply to clinically applicable levels in-vitro. This means the use of MSCs in an ex-vivo environment has the potential to become a common high-quality form of treatment for donor organs that have gone through IRI.

Cellular therapy started to grow into a viable form of treatment during the last decade. The reason stem cells were a particularly exciting new medium for treatment was because of cell-to-cell communication between MSCs. Extracellular Vesicles (EV) are released by MSCs to transport biologically active molecules and genetic information to target cells to influence their function. MSCs have been shown to positively influence renal regeneration by limiting apoptosis and promoting kidney repair by enhancing proliferation of tubular cells after a toxic injury (Grange et al., 2019).

A study was conducted in which cellular therapy was administered to rat kidneys which were donated after circulatory death (DCD). The kidneys underwent HMP for four hours with Belzer solution (BS), which improves their viability, though leaving the clinical graft course in a critical state. EV-producing MSCs were added during HMP to facilitate tissue repair. The study evaluated whether the MSC-derived EV protects the rat kidneys during HMP from ischemic injury. The kidneys treated with MSC/EV showed significantly less ischemic damage. It was concluded that the addition of MCS/EV to BS during HMP works to prevent ischemic injury by preserving the enzymes which are essential for cell viability and protect the kidney from reperfusion injury (Gregorini et al., 2017).

One effect that IR has on the cells that make up the kidney and other organs is that the mitochondria are shifted into a fission state. This causes the mitochondrial fragmentation and induces the cell to undergo apoptosis (Anzell et al., 2018). Gu et al. discovered that when MSCs are administered right after reperfusion, the EVs that are produced from the MSCs are able to

suppress mitochondrial fission and subsequently prevent cell apoptosis in rat kidney models (Gu et al., 2016). The use of MSCs during HMP has been shown to be even more beneficial than MSCs alone (Henry & Guarrera, 2012). The protective effect of MSCs was further observed in a combination with HMP on DCD rat kidneys. After 4 hours of perfusion, $3x10^6$ MSCs were detected in the vessels, tubules, and tissues of the kidneys. A histologic evaluation revealed that MSCs are able to significantly heal severe lesions such as tubular necrosis and tubular lumen obstructions when compared to kidneys that are only perfused (Gregorini et al., 2017). The examiners suggested that MSCs ameliorated the cellular metabolism and IRI of the rat kidneys during HMP. Increased pyruvate levels and reduced levels of lactate dehydrogenase, malonaldehyde, lactate, and glucose were all factors that supported this suggestion. The most fundamental finding was that the MSC-EVs were found to heal renal IRI during HMP more effectively and more rapidly, which could be attributed to the quick action of mediators contained in EVs (Gregorini et al., 2017).

Cellular therapy during NMP was analyzed. In the study, five pairs of human kidneys donated after cardiac death (DCD) were studied. One kidney was perfused for 24 hours using warm NMP as the control, while its opposite kidney was perfused with MSCs. The two kidneys were analyzed for DNA synthesis, cytokine/chemokine synthesis, cytoskeletal regeneration, and mitosis. NMP with MSCs led to reduced inflammatory cytokines synthesized by the kidneys. MSC treatment also showed a significant increase in the number of renal cells undergoing mitosis (26%) compared to the control group. The study concludes that it is the first study to observe actual renal regeneration while ischemically damaged human kidneys undergo ex vivo NMP for 24 hours. Factors of regeneration such as increased synthesis of ATP, a reduced

inflammatory response, increased synthesis of growth factors, normalization of the cytoskeleton, and mitosis were observed (Brasile et al., 2019).

A secondary study was also conducted with NMP and porcine kidneys in 2019 (Pool et al., 2019). The purpose of the study was to determine, much like Brasile's study, if administering MSCs to kidneys during NMP is feasible. This study also seeks to find out if MSCs are retained after NMP and what structures they stay in. The study was conducted by obtaining four viable porcine kidneys and machine perfusing them for 7 hours. After 1 hour, MSCs derived from human adipose tissue were added to each. The first received 0 MSCs; the second received 10^5, the third 10^6, and the fourth 10^7. Pre-labeled bone marrow-derived MSCs with a fluorescent stain were also perfused into the kidneys to assess the location of surviving MSCs once NMP was finished.

After NMP, immunohistochemistry revealed that surviving MSCs had been detected in the lumen of glomerular capillaries. This discovery, however, was only seen in the 10^7 MSC group. The fluorescent pre-labeled MSCs were only seen in a small number of glomeruli, while other glomeruli housed multiple MSCs at once (Pool et al., 2019). Flow cytometry showed that only 10% of the MSCs infused during NMP remained after perfusion had ceased. During NMP, all the kidneys were functional. They were consuming oxygen and glucose and producing urine properly. The conclusion of the study is that the number of infused MSCs decreases over time, and after NMP, only about 10% of MSCs survived in a minority of glomeruli (Pool et al., 2019).

Pool at al. conducted another study using porcine kidneys in 2020. The goal of this study was to determine which cytokines are secreted by MSCs during NMP of a porcine kidney. The kidneys underwent warm ischemia at a standard time of 20 minutes. The ischemia is necessary, so the kidneys are injured at the beginning of the experiment. Ischemia is followed by HMP for

2-3 hours. The kidneys were then machine perfused at 37°C for 7 hours. After the first hour of NMP, MSCs derived from human adipose tissue or from human bone marrow were added to some of the kidneys, while the other control kidneys did not have MSCs added to them. NMP in a fourth group was performed for 7 hours with adipose-derived MSCs, except there was no kidney in the circuit.

The kidneys perfused with MSCs ended up with lower levels of lactate dehydrogenase and neutrophil gelatinase-associated lipocalin (NGAL) when compared to the control group. The Kidneys perfused with MSCs also showed higher levels of human hepatocyte growth factor, interleukin (IL) found in the form of IL-6, and IL-8 than the control group. The conclusion of the study is that an injured kidney, when perfused with MSCs during NMP, could lead to a more proliferous release of immunomodulatory cytokines as well as a decrease in markers of injury (Pool et al., 2020). An increase in immunomodulatory cytokines will result in regulation of immune function, which will not allow the kidney tissues to be damaged as badly. A less damaged kidney will be more viable for transplantation.

The effect of MSCs during NMP on porcine kidneys after autotransplantation was studied. Autotransplantation is the act of removing a patient's kidney, treating the kidney, and transplanting the kidney back into the same patient. Kidneys that are autotransplanted have no need for immunosuppression because the patient's body will accept its own kidney. In the study, after the operation the examiners observed the number of MSCs that remained in the transplanted kidneys. On postoperative day 14 a drastic decline in the number of MSCs was observed. The examiners failed to see evidence of MSC-induced recovery in the kidneys. They saw a lack of improvement in renal function early fibrosis markers and histology in the posttransplant phase (Lohmann et al., 2021).

Another avenue of cellular therapy that is being researched for NMP is the use of Multipotent Adult Progenitor Cells (MAPCs). A study by Dr. Emily Thompson was conducted in 2020 in which five pairs of kidneys, each from the same donor, were simultaneously perfused for 7 hours. Kidneys were randomly assigned to receive MAPC treatment, while the other kidneys remained as a control. Samples of perfusate, urine, and tissue were taken from each of the kidneys and compared.

Kidneys that received MAPC treatment showed better urine output (P= .009), decreased expression of neutrophil gelatinase-associated lipocalin (P= .012), improved microvascular perfusion when examined using contrast-enhanced ultrasound (cortexP= .019, medullaP= .001), downregulation of IL-1 beta (P= .050), and upregulation of IL-10 (P< .047), and indolamine-2, 3-dioxygenase (P= .050). Immunofluorescence showed prelabeled MAPC cells in the perivascular space of kidneys during NMP. The examiners proudly reported that they are the first to ever successfully deliver cellular therapy to a human kidney during NMP. Results of the study showed that kidneys treated with MAPC cells were found to have fewer biomarkers for injury as well as all-around clinical improvement (Thompson et al., 2021)

Cellular therapy has been an extremely promising method of treating *ex-vivo* kidneys during HMP and NMP. Treatment using mesenchymal stromal cells has been researched and consistently yields positive results. Treatment using multipotent Adult Progenitor Cells, though having been researched less, is also showing excellent results. Thompson was able to successfully treat human kidneys with MAPC cells during NMP for the first time in 2020. If successful studies continue to emerge using cellular therapy, it may become a preferred method of treatment in the future.

MSCs are very important because they could be the next step toward finding a way to better preserve ex-vivo kidneys and improve graft function and survival rates after transplantation. MSCs are known to stimulate the repair of damaged tissue. It is suggested by current analyses that organ transplantation is vastly improved by treatment using MSCs. The point at which Pool's (2019) study fell short was that the MSCs were mostly gone by the end of NMP. However, the fact that a certain amount was infused (10^7) suggests there may be a feasible way for more MSCs to survive NMP in future studies.

Gene Therapies

Another exciting new method of machine perfusion treatment for *ex-vivo* kidneys is gene therapy. Gene therapy can be defined as the translation of healthy genes into a cell to correct genetic disorders by replacing genes that are missing or defective. In the kidney, there are enzymes called matrix metalloproteinases (MMPs). MMP-2 and MMP-9 have been found to be closely correlated with ischemic heart injury. A study was performed to determine if MMP-2 and MMP-9 also play a significant role in ischemic injury to the kidneys, and if MMP inhibitors are introduced during hypothermic machine perfusion, will the inhibition of MMP-2 and MMP-9 result in fewer markers of ischemic injury (Moser et al., 2016). Perfusates were analyzed for the presence of cytochrome c oxidase, lactate dehydrogenase, NGAL, and measurements were taken of MMP-2 and MM-9. A similar study was also performed on the effects of MMP inhibitors MMP-2 siRNA and doxycycline in the perfusate of rat kidneys which acted as models of donation after circulatory determination of death (DCDD).

Each of the perfusates from both human and rat kidneys revealed the presence of markers of injury, with human kidneys having higher levels in kidneys which were DCDD, and those with delayed graft function. The rat kidneys were perfused with MMP inhibitors at 4 °C for 22

hours, and the result was a marked reduction of MMP-2 and MMP-9 levels and coinciding injury markers. The study concluded that MMPs are involved in ischemic kidney injury, and the supplementation of a preservation solution with MMP inhibitors is potentially a positive method of protecting ex-vivo kidneys.

A study was conducted in 2016 to determine the feasibility of protecting grafts from extended cold IRI as applied to kidney transplantation by downgrading IRI-associated genes using small interference RNA (siRNA) (Zheng et al.). They previously found that the treatment of animals with siRNA results in the prevention of warm IRI in nontransplant models and cold IRI in heart transplantation. In this study, donor kidneys were perfused with a siRNA solution and preserved in a siRNA solution. These siRNA-treated donor organs were then implanted into immunologically compatible mice. The siRNA solution's effect on extended cold IRI was assessed by examining renal function, histopathological change, cell apoptosis, and inflammation.

The results of the study revealed that the siRNA solution reduced levels of blood urea nitrogen and serum creatinine when compared to control groups. The solution also decreased cell apoptosis inflammatory response and histopathological changes. All these factors lead to prolonged graft survival. The examiners concluded that this was the first demonstration that perfusing donor organs with a siRNA solution can induce gene suppression in the kidney and prevent kidneys from undergoing extended cold IRI in kidney transplantation (Zheng et al., 2016). This breakthrough application of siRNA solutions shows great promise toward extending the time of preservation of donor kidneys and other organs.

The purpose behind another gene therapy study was to determine if there is a way to genetically engineer a kidney to permanently silence HMC antigens in a rat model (Yuzefovych

et al., 2020). Previously, it was demonstrated that HMC-silenced cells are protected against allogenic immune responses. In a kidney, this would protect against IRI by not allowing the immune system to damage the kidney tissues. They constructed a sub-NMP system to deliver lentiviral vectors which encode shRNAs into the kidney. The shRNAs target β2-microglobulin and the class II transactivator in the kidney. The vector also contained the sequence for secreted nanoluciferase.

Results from this study showed that there was a detection of bioluminescence in the plasma and urine of recipients of a genetically engineered kidney during the 6th week of post-transplantation monitoring. This indicated that transgene expression is stable. Kidneys that were expressing shRNAs showed a significant decrease of 70% in β2-microglobulin and class II transactivator levels. After NMP, the kidneys that underwent genetic modification did not show as much cell death as the control kidneys. The study concluded that this method of gene therapy shows great potential that donor kidneys can be genetically engineered to silence HMC transcripts. This will support overall graft survival after transplantation as well as reduce levels of immunosuppression (Yuzefovych et al., 2020).

Bioengineered Therapies

Along with the inhibition of immune responses in the kidney during and after transplantation is the treatment of the epithelial cells (EC) that are the first point of contact the kidney makes with the recipient's body. A study has shown how nanoparticles (NPs) are used to provide extended therapy to *ex-vivo* kidneys undergoing NMP. NPs have previously been shown to act as a means of long-term drug release (Tietjen et al., 2017). The forefront of damage caused by ischemia is located in the EC of the kidney. By providing a way for treatment to be delivered in specific sections of the ECs, damage from ischemia can potentially be lessened. In a treatment

setting, the use of NPs would be greatly beneficial because a kidney's ECs can be treated as soon as possible without the need for systemic drug delivery.

In this study, an anti-CD31 antibody is shown to further improve the targeting of NPs to the graft ECs of human kidneys during NMP. Two-color quantitative microscopy revealed that the accumulation of NPs to targeted ECs can be increased by 5- to 10-fold in concentrated areas of renal vasculature. NPs were also observed to accumulate in other parts of the renal vasculature that are poorly perfused. Out of the eight kidneys being treated, six showed at least a moderate level of perfusion, and the last two displayed excellent perfusion (Tietjen et al., 2017). The results from this study showcase how targeted nanomedicines have the potential to become an essential method for preserving *ex-vivo* kidneys and other transplant organs.

Due to the rising need for transplant organs, there is a need for the improved treatment and rehabilitation of kidneys from high-risk donors. These kidneys would otherwise be discarded due to an increased likelihood of complications after transplantation. A previous study showed how microvascular obstructions were found in 8 transplant-declined human kidneys undergoing NMP (DiRito et al., 2020). The obstructions impeded microvascular blood flow. In a similar manner, another study was done using 39 human kidneys. It was observed that long cold storage of the kidneys caused an accumulation of fibrinogen within the tubular epithelium. The return to normal warm temperatures by either NMP or transplantation caused the fibrinogen to release and allow red blood cells to aggregate and plug up microvascular vessels. A combined delivery of plasminogen and tissue plasminogen activator was added during NMP to lyse the plugs. A significant reduction of markers of injury as well as improved renal function and improved delivery of vascular-targeted nanoparticles were observed as a result of this treatment. The study concluded that the next step is to develop a new method of cold storage for high-risk donor

organs that will cause less injury and a form of treatment that shows immediate translational potential (DiRito et al., 2020).

An additional study utilized a similar but alternate approach regarding nanoparticles (Brasile et al., 2010). A major obstacle to overcome in transplantation is the immunological response of the recipient. The examiners sought to determine if pretreatment of canine renal allografts would be feasible for preventing early allorecognition in the recipient's immune system. The vasculature of the allografts was treated with a bioengineered nano-barrier membrane during 3 hours of NMP. Untreated renal allografts were given to control dogs to compare to the treated group. The results of the study revealed that the bioengineered nano-barrier was able to cover approximately 90% of the vasculature during the 3 hours of NMP. Examination of the reaction of mixed lymphocyte-vascular endothelium revealed that the covering of the luminal surfaces prevented allorecognition. Renal function is not inhibited by the nano-barrier based on autotransplant outcomes, and the length of time before rejection of the graft is significantly extended in canine kidneys that underwent the bioengineered treatment. Dogs in the control group lasted until day six on average before graft rejection, while dogs in the treatment group lasted until day 30 before showing any signs of graft rejection. The results of this study indicate that postponement and eventual elimination of allorecognition is feasible without the need to manipulate the immune system of the recipient. The examiners claim this study could provide the foundation for new therapies that support the cooperative acceptance of transplant organs (Brasile et al., 2010).

Biological Therapies

A study was undertaken in 2014 concerning the endothelial glycocalyx of kidneys (Sedigh et al., 2014). The glycocalyx regulates endothelial function and helps to maintain

homeostasis in vascular tissues. Ischemia and reperfusion of the kidney cause the glycocalyx to shed and be absorbed into the bloodstream. A Corline heparin conjugate (CHC) is suggested as the solution to the shedding of the glycocalyx. A CHC is made of 70 heparin molecules that can bond well with certain tissues that have an affinity to heparin. The examiners have a hypothesis that CHC could be used to prevent and possibly reverse the destruction of the glycocalyx of the *in vivo* kidneys of brain-dead pigs. After inducing brain death, the kidneys were recovered and perfused with HMP. A volume of 50 mg of CHC was added to the perfusate of treated kidneys, and 50 mg of unfractionated heparin was added to the perfusate of control kidneys. Immunofluorescence and confocal microscopy were used to detect the CHC on the inside of the blood vessel walls (Sedigh et al., 2014). This binding of CHC to the vessel walls of the porcine kidneys is a positive step toward being able to restore the glycocalyx of kidneys undergoing reperfusion and ischemia. It is an approach that has the potential to become a vital way to protect renal vascular endothelium from IRI.

The same examiners performed a follow-up study after the initial study demonstrated how it is possible to coat the inner lining of the renal arteries with CHC Solution. The new study performed in 2019 with the goal of proving whether this treatment assessed in the previous study has any effect on reducing IRI (Sedigh et al., 2019). Porcine kidneys were once again obtained by inducing brain death in male landrace pigs via expansion of an epidural balloon catheter. Six pairs were obtained from the pigs and preserved for 20 hours using HMP. During the HMP, 50 mg of CHC was added to one of the systems as the treatment group. After the 20 hours of HMP, the kidneys were evaluated for kidney function via a customized NMP system.

After 3 hours of NMP, the results of the treatment revealed that the treated kidneys weighed less ($P=0.017$), had faster levels of creatine decline ($P=0.024$), higher urinary volume

(P=0.031), and a lower level of neutrophil gelatinase-associated lipocalin (NGAL) than the control group. The conclusions of the study are that treating porcine kidneys with CHC during HMP reduces IRI and improves organ function shortly after reperfusion. The examiners made a point to say that this novel method of treatment has the potential to improve the quality of transplanted kidneys in the clinical setting (Sedigh et al., 2019).

Another excellent example of a potentially useful biological therapy was examined in a study from 2016 (Hamaoui et al., 2016). The purpose of the study was to examine whether treatment using thrombalexin, a cell-binding thrombin inhibitor, was a feasible way to reduce coagulation in the blood vessels of ex vivo kidneys. This intervention could prevent microvascular thrombotic sequelae after transplantation. Both porcine and human kidneys were used as ex vivo HMP models. Thirty-eight porcine kidneys were used. Fifteen of the kidneys underwent normal HMP As the control group, while 19 of the kidneys underwent HMP with a thrombalexin-treated solution.

Upon the cessation of treatment, the kidneys were assessed, and the thrombalexin-treated kidneys showed improved levels of perfusion when compared with control kidneys (26.4%), superior flow, and higher perfusion flow indexes by 28.9%. Thrombalexin-treated kidneys also showed higher levels of microvascular capillary perfusion. Thrombalexin-treated kidneys also had lower levels of markers for tissue ischemia when examined with rapid-sampling microdialysis and overall reduced fibrin generation. The examiners concluded that their data suggested that pretreatment with thrombalexin as a cytotopic anticoagulant is a feasible way to protect against IRI in *ex-vivo* hemoreperfusion models. This study concluded with a statement that this method of delivering locally active anticoagulants to the vasculature of grafts prior to

HMP has the potential to be a superior technique for decreasing microvascular clotting in the future (Hamaoui et al., 2016).

Treatment Using Antibodies

A different potential form of treatment being assessed is the use of antibodies. Researchers observed how IRI in *ex-vivo* kidneys was being contributed to by defects in renal tubular epithelial cells while they underwent repair. A study was conducted in which thrombospondin-1 and its receptor CD47 were discovered to be involved in the IRI of kidneys. Certain mice who were CD47 negative (CD47-/-) after IRI were found to have an enhanced proliferation of self-renewal genes being produced in the kidneys. CD47-/- renal tubular epithelial cells were found to express self-renewal genes at an increased rate. Thrombospondin-1 was also found to inhibit the expression of self-renewal genes in mice.

An experiment was conducted in which mice with CD47 (CD47+/+) were compared to wild-type mice that were CD47-/-. Six groups of mice kidneys underwent warm ischemia. Three groups of CD47+/+ kidneys were put through 10, 15, and 20 minutes respectively, while three groups of CD47-/- kidneys were each put through warm ischemia for the same times. Each of the groups was assessed at the end of its time for renal injury before being put on 24-hour reperfusion for 7 days. The kidneys were assessed again for renal injury after 1, 3, and 7 days of reperfusion.

After 10 minutes, there was no notable effect in any of the kidneys. After 15 minutes, serum creatinine levels were moderately increased. The kidneys that were ischemic for 15 minutes went on to be perfused for 24 hours, and by the time reperfusion was done, the serum creatinine levels had returned to baseline. The kidneys that were subject to 20 minutes of warm ischemia experienced a dramatic drop in serum creatinine levels. After 24 hours of reperfusion,

the serum creatinine levels of these two groups also returned to baseline. After reperfusion, the proliferating cells were analyzed with Ki67 staining of sections of the renal tissue, and the degree of injury to the tissues was scored with hematoxylin and eosin-stained sections. The results of the assessments of the injury showed that 20 minutes of warm ischemia is sufficient for kidneys to experience robust renal injury.

A treatment using a CD47-blocking antibody was also performed on the tubular epithelial cells in the kidneys of wild-type mice to prevent CD47 from inhibiting the production of self-renewal transcription factors that promote cell proliferation. In a syngeneic model, treatment with a CD47-blocking antibody increased self-renewal gene transcription, decreased damage to renal tissues, and increased renal function when compared to control kidneys. The conclusion of their study was that inhibition of self-renewal genes by thrombospondin-1 via CD47 after IRI can be ameliorated by perfusing the kidney with a CD47-blocking antibody (Rogers et al., 2016).

Conclusion

There is a greater need for more donor kidneys now than ever before. People in desperate need of a new kidney are told either they must wait too long to get one or that there are not enough. The current preservation methods of static cold storage, HMP, and NMP are not sufficient for the mass preservation and protection of kidneys from IRI. Promising new treatment methods during HMP and NMP, such as cellular therapies, gene therapies, bioengineered therapies, biological therapies, and therapies using antibodies, must be further researched to discover new and more efficient ways to protect donor kidneys against the effects of IRI.

Numerous studies have shown how novel treatments have exciting potential for preservation and protection against IRI, but there is a gap in the research about how the treatments will affect the health of kidneys post-transplantation. Most of the studies on machine

perfusion treatments have been done using kidneys from pigs and rats. It is imperative that empirical research is performed on more human kidneys to observe machine perfusion treatments and their effect on graft function before and after transplantation. The improvement of donor kidney treatment during reperfusion is essential for the medical field to be able to provide a higher number of viable kidneys to patients who need transplantation.

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