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The Effects of Renal Cold Storage and Transplantation on Immunoproteasome and the Complement System

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SENIOR THESIS APPROVAL

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The Ethical Component of Organ Donation

History of Giving the Gift of Life

The dream of curing illness and injury by transplanting organs, bone, and other tissues dates back to the Middle Ages. Unfortunately the scientific knowledge and surgical techniques that have made modern transplant medicine possible had to wait until the nineteenth and twentieth centuries to make their debut. Successful transplantation of bone, skin and corneas came early but was far from sophisticated. The first "miracle" transplantation of a kidney was performed between two monozygotic twins in 1954 by Dr. Joseph Murray and Dr. John Merrill of Peter Bent Brigham Hospital, and the question was quickly raised how to transplant organs between patients who are not identical (Jonsen, 2012). Although many obstacles remained at this point, the era of transplantation was on the horizon and people everywhere recognized it as an extraordinary leap in medicine. Important medical breakthroughs two decades or so later - such as tissue typing and immunosuppressant drugs - allowed for the successful transplant of larger organs between two people who were not related. Dr. Christian Barnard, in 1967, transplanted a still-beating heart into a patient who lived for eighteen days; less than a month later he attempted again and this time the patient lived for 594 days (Jonsen, 2012). By 1968 the first organ procurement organization was established along with the Uniform Donor Card as a legal document in all fifty states. What was once deemed impossible was now becoming a reality, but of course the roadblocks did not stop there. No longer is the problem how to perform organ transplantation surgeries or how to keep bodies from rejecting them, but the lack of supply due to a lack of registered donors. Ethical dilemmas were noticed lurking in the miracle, specifically with regard to physicians risking the health of a well person to save a sick one.

Physicians sign the Hippocratic Oath, promising to do no harm. One of the first predicaments of this breakthrough in medicine was that no matter how pure the motives, living organ donation attempts to make a sick person well by wagering the physical condition of a healthy person. People soon began to realize that this was not the only piercing question that needed to be addressed. How could consent be obtained without coercion (Jonsen, 2012)? Regarding deceased donors, what clinical evidence of death does there need to be? If the donor is not related to the one needing a transplant, should there be compensation? As transplants become more consummate, how should the recipients of these organs be fairly selected? How will there ever be enough organs or organ donors to meet the need? These were just the start of questions to be tackled and now they form the framework on the policy of organ transplantation.

There are many leading countries that have presumed consent laws for transplantation as opposed to the expressed consent laws that the United States currently has in place. Presumed consent is alternatively known as an 'opt-out' system and means that unless the deceased has expressed a wish in life not to be an organ donor, then consent will be assumed. Almost all that have adopted a presumed consent law as opposed to an expressed consent law have seen a very significant increase in organs for transplantation (Zink, 2005). The United States has not followed the way of the majority because they believe there is a loss of patient autonomy, and a potential violation of the 5th amendment. Although a presumed consent policy gets around the issue of coercion, it is understandable why the U.S. does not have that approach in place. For those countries that do, there is a legislative framework in which citizens must place their name on a national withdrawal registry, otherwise their organs will be removed for donation after they are pronounced deceased (Fabre, 2014).

In the 1960s, discussion arose on what clinical evidence is sufficient to determine whether or not a person is dead. It was proposed by Henry Beecher that a person could be diagnosed as dead when there was "irreversible cessation of the function of the entire brain" (Sade, 2011, p. 146). This status has since become known as brain death and has been codified in the law of every state by their adoption of the Uniform Determination of Death Act (UDDA). People now recognize that there is only one kind of death, but that it can be determined by the two different ways described in the law. A brain dead individual with a still-beating heart is just as dead, legally, as someone whose body has become stiff because their heart permanently stopped beating. The current system has been in effect for decades now, but there is still controversy over brain death checking the box for the dead donor rule, which requires a person to be declared dead before the removal of their organs for transplantation. Removing of organs would most definitely stop someone's heart from beating, whereas being brain dead would not. However, the UDDA assures patients, families, and health-care professionals that a patient who is brain dead is in fact dead, making the removal of organs for life-saving transplantation "legally and ethically acceptable" (Sade, 2011, p. 147). One thing that the law does not concede or accord is that the families of these deceased donors — or living donors — will receive any type of compensation for their organs.

One of the most easily answered questions that was posed at the start of organ transplantation involved compensation — mainly financial — for donors, primarily those who were living. Leading transplanters and scholars in the law at that time decided that organs must always be donated and explicitly granted by the donor (Jonsen, 2012). A spirit of altruism and volunteerism are necessary to use a natural resource for the common good. This was ultimately

an ethical decision that would prevent commercialization of organs, exploitation of the healthy poor, and promote equality in organ distribution. However, there is now a continually widening gap between the number of patients needing an organ donation and the number of those available. For those who have not been waiting near as long or who are not in as dire of a medical need, the gap is significantly wider.

Organs are limited resources in a majority of countries, and the justice-based system in place right now prioritizes patients based on their medical status and urgency of their need (Cameron, 2008). Under the National Organ Transplant Act, organ transplantation in the United States is overseen by the U.S. Department of Health and Human Services. Organs are allocated — blind to name, race, sex, and wealth — according to strict rules that take into account physical matching, tissue and blood type matching, medical criteria, waiting time, and severity of illness. The allocation system's rules have been developed over many years by physicians and transplantation experts and continue to update as there are new changes in the field of medicine. One of the largest issues facing the department right now is that the success of organ transplants in treating end-stage organ failure has generated an unprecedented demand for transplantable organs that unfortunately remain in short supply. More than 116,000 patients were on the United States transplant waiting list, but only 28,000 transplants were performed (Razdan et al., 2015). Those statistics reveal that less than twenty-five percent of people needing an organ donation received one, and sadly there has not been a significant increase in that number over the last eight years. The gap is so wide that there has been discussion of not allowing prisoners to be placed on the organ transplant waiting list. Increasing the availability of transplantable organs and willing donors is therefore critical in preventing deaths from organ failure.

There is a continually widening gap between the number of patients needing an organ donation and the number of those available. This growing shortage will continue to persist — and there will never be enough organs to meet the need — unless something is changed. The ability of physicians to treat patients facing organ failure is largely dependent on public willingness to supply them (Laden, 2016). Although there is a strong support for organ donation across the United States, there is still a lack of organs for treatment of organ failure. There are a lot of concerns that need to be addressed to increase the supply of organs, and all of the questions addressed above are just the beginning. Many people believe that physicians have an obligation in facilitating education and discussion with their patients regarding organ donation (Ladin, 2016). Although, health authorities should be morally compelled to intervene and make sure that the public is aware of how they can have an impact on organ donation, the weight should not only be on their shoulders. The reality is that there are simply not enough organ donors to meet the demand, but there are several explanations for this ongoing shortage.

Present Policies are Falling Short

Despite the slight increase in the rate of organ donations from deceased donors, the demand still exceeds the supply of transplantable organs. And public policies have done little to close this gap. Changing policies to account for the lack of post-medical care costs and lost wages given to living organ donors by organ recipients is a start. A change in the current philosophy of social education policies regarding organ donation and transplants is clearly necessary, as recognition and support by health and education organizations would undoubtedly make people aware of this issue. A “no-give, no-take” policy could be enforced that requires people to be an organ donor before they can receive one from someone else. In addition,

formulating a public policy that promotes organ donation through state incentives. Present policies seem to be falling short when it comes to increasing the number of available organ donors, but the proposed alterations and additions above could make a difference.

In the United States and other countries, live organ donation tends to be costly and burdensome for those people who are willing to give their organs. While the majority of medical costs are covered, many donors still face lost wages, travel expenses, and medical expenses that may come from complications after organ removal. Despite the widespread agreement that living donors should not be left with any financial burden from their donation, not a lot has been accomplished to alleviate those costs. The failure of policies to eliminate these out-of-pocket costs for living donors may be a contributing factor to the lack of willing patrons. A goal of financial neutrality for the living donor should be sought and upheld; coverage/reimbursement of all medical, travel, and lodging costs in addition to lost wages and any other expenses related to the organ donation (Hays et al., 2016). Risks of job loss and insurability after donation can only be addressed and taken care of with policy and legislative improvements. Although the notion of financial neutrality for living donors is not controversial, nothing has changed. Achieving total compensation for those willing to give their organs should be considered a policy priority if there is to be a decrease between the gap of needed and available organs. Until such policies are developed, transplant hospitals should ensure that contracts with those paying for the organ relocation include adequate coverage for the living donor.

The widening gap between needed and available organs for transplantation is not going to decrease if the public is not aware of this issue. Ignorance is one of the leading causes of society's lack of response to the demand for organ donation, especially in regard to deceased

donation (Ladin, 2016). The media is a large contributor to this ignorance with false reports on organs going to the rich first and then to the poor, the lack of properly trained medical officials, fears concerning a misdiagnosis of brain death, and even stories about criminal organ commerce. With proper education and information on all of the logistics regarding organ donation, people would understand that posts by media outlets — such as those listed above — are not to be taken seriously. Polices that require public education, mainly through proper media coverage, non-governmental organizations and lectures by experts, should be implemented as a strategy to change social attitudes toward organ donation. A social education policy that promotes the incorporation of topics on donation and transplants in curricular programs, periodically carried out in schools, colleges and universities, would certify that people understand the seriousness of the need for registered organ donors. Furthermore, implementing information to modify the current reluctant negative behavior toward organ donation by society constitutes a potential possibility to improve this urgent medical-social crisis. Policies that focus on the education and awareness of the public regarding organ donation would potentially increase the number of registered donors.

The death of thousands of Americans on the organ transplant waiting list could be prevented if more people were registered donors; therefore, changes to the present policies or addition of new policies regarding donation is necessary. To solve this organ shortage, many nations have considered implementing a “no-give, no-take” allocation policy that would prevent people who are not registered donors from receiving an organ transplant. Under the current system, non-donors have just as much access to the organ pool as donors because the United Network for Organ Sharing regards organs as being a natural resource. However, it is not morally

just for a non-donor to receive this resource before a registered donor. Giving priority for organ transplants to those who have already agreed to donate creates an incentive to sign an organ donor card and imposes a penalty for those who do not sign, thereby increasing the number of transplantable organs. There are several advantages to this "no-give, no-take" policy — one being that is satisfies peoples moral intuitions. Many find the idea of paying for organs as distasteful but are comfortable with the morality of reciprocity; it does not deem human beings as a commodity. Adopting policies like "no-give, no-take" increase the public incentive to donate, which in turn increases the total number of organs available for transplantation.

The miracle of organ transplantation saves the lives of thousands every year, however, the chronic organ shortage that leads to substantially more deaths overshadows this success. This dilemma is a public health problem due to its grave consequences on transplant patients and society as a whole. Promotion of a public policy through various state incentives that express gratitude for the solidarity act of the donor could potentially curve this issue. It would provide the necessary stimulus to overcome individual's negligence and apathy, specifically the majority who are inclined to donate but have not taken action yet to be a donor. Some of these incentives might include — but are not limited to — tax credits, discounts on health insurance premiums, and contributions to funeral costs for deceased donors (Levy, 2018). There is a public interest in improving organ donation rates to decrease the cost burden on health care systems and social security systems, and also to fight against organ trafficking. Consequently, the state assumes multiple roles in transplantation medicine, one of them being to encourage donation and increase the number of available organs. Public policies through various state incentives would accomplish that task; however, the common contention that incentives are not ethical presents a

road block. The question now becomes not whether the state should encourage individuals' willingness to donate but how.

Getting more Organs — Are Incentives Ethical?

It cannot be denied that the shortage of organs available for transplant is a serious problem worldwide. Incentives for organ donation are currently prohibited in most countries, but the truth is that they may increase donation and save lives. Discussions of increasing the number of available organs with the help of incentives has been focused on two reservations: whether or not there are ethical principles that justify the current prohibition and whether incentives would do more good than harm. Legally, the sale or purchase of human organs is not allowed. Currently, the only form of compensation that is permissible is the reimbursement of expenses acquired by the donors and related to their donation. The purpose of these payments is to alleviate financial loss living donors might experience as a result of their much-appreciated donation, as opposed to the financial incentive used to encourage a person to donate. Essentially the difference is when this compensation is given and how it is used; to a willing-donor after their donation to cover medical costs or to a financially unstable person before their donation to pay monthly bills. A lot of this discussion is focused towards living donors but can also be applied to deceased donors who may seek coverage of their funeral costs. The ethical landscape regarding incentives for organ donation is convoluted with some people proposing that any form of inducement is not permissible, while there are others who think that any un-harmful way of encouraging organ donation is necessary.

Incentives are defined as being a stimulant that encourages or motivates a person to do something. In terms of organ donation, these incentives can either be financial by having

material gain or value or non-financial by having no material gain or value. The field of organ donation focuses heavily on making sure that their standards and policies are ethically upstanding, as to promote the welfare of society. Regarding incentives—both financial and non-financial—there are a lot of people who hold to the opinion that neither is ethically objectionable in terms of organ donation; whatever lawfully increases the number of available organs is equitable. Ultimately, people promoting these for donors conclude that their motives are ethical because they are solely based on the concern for the well-being of patients and saving the lives of those who desperately need a transplantation. In turn, incentives would also decrease or eliminate unregulated and illegal organ trade markets. Trafficking in human beings for the purpose of organ removal and organ trade is universally condemned, yet it is presumed that almost 10,000 transplants occur annually in this way (Caulfield, 2016). A carefully regulated and principled system of incentives for donation could not only diminish organ commerce, but it could also potentially provide extensive benefits to both recipients and donors. Therefore, it is worthy of systematic investigation. Furthermore, surveys have shown that the public not only supports incentives but would be more likely to donate if they were offered incentives. (Incentives for Organ Donation: Proposed Standards for an Internationally Acceptable System, 2011). Arguments in favor of incentives or organ donation are founded in hope that this system would increase the supply of organs and thereby validate the ethical concern of saving lives that may otherwise be lost due to lack of this resource. Despite these seemingly accurate points, compelling arguments against financial and non-financial incentives for organ donation have been persuasively made as well.

Living and deceased organ donation poses a fundamental ethical challenge: should incentives be used to increase transplantation numbers or by using them are people being viewed as a commodity. Selling organs, mostly kidneys, is banned by law in practically every Western country (Sells, 2004). This is not the case for Iran where payment for kidneys has been legalized and capitalized by their government. Although they are one of the very few countries with no waiting list for kidney transplants, the United States and others cannot ethically wrap their heads around this type of incentive. Many opponents point out that there would not only be a loss of emotional gain and personal linkage, but also a decreased respect for life and sanctity of the human body. The argument is that incentives and paid donation do not embrace the Hippocratic Oath or protect the interests of the donor. This shift in balance of responsibility to the paying recipient may increase the number of organs available for transplant, but many view this as disturbing and unethical. If payment for donation was legalized, those with a greater financial need would be more likely to donate, and there does not need to be a potential rich versus poor contingency. To understand this fully let us look at the demographic of the donors that would result from this type of situation. Donors are lacking financial stability, most of them below the poverty line. Most are uneducated and in low-paid manual jobs — not seeking to donate out of altruism or a feeling of moral obligation but to pay their bills. In fact, most living donors who do sell their kidneys fall back into debt due to a decline in general health from losing that organ. Most people, although eager to increase the number of transplants, would argue that the buying and selling of human organs can never be made ethical because it will always somehow penalize the weakest in society or exploit the poor. Financial gain should never take precedence over patient care, and that is why the current system promotes altruistic donation.

Altruism and Moral Obligations

Altruism can be defined as the belief in or practice of selfless concern for the well-being of other people. Living and deceased organ donation are formulated around this idea of giving without seeking reciprocation. Altruism has been an integral part of transplantation from the beginning: the gift of life and science to humanity; grieving family members who offer to donate the organ of a deceased loved one; and recipients who consent to participate in life-saving scientific research. In fact, many organ procurement organizations and medical associations such as American Society of Transplantation (AST) and United Network for Organ Sharing (UNOS) clearly assert that organ donation and transplantation should maintain voluntariness of choice and an altruistic foundation (Fortin et al., 2010). It is believed that a donation is only considered altruistically motivated if the organs are available for allocation to recipients with whom the donor does not have a personal connection, or if they are receiving no direct-benefit. However, it is almost impossible to determine what a donor's motives are. Therefore, many believe that altruism should be desired in donation, but in order to increase transplantation numbers, it should not be a requirement. Altruism has been the guiding principle of ethical organ donation and has been used as a justification for rejecting or allowing certain types of donation (Moorlock et al., 2014).

Organ donation and transplantation is deemed by many to be the gift of life and science to humanity; it is not just a complex medical process but a personal human deed. This form of altruism touches people closely and intimately, and through which the recipient gains a lease on life, an enhanced quality of living, and a newfound respect for humanity. Many people believe that if there was no longer a moral obligation or conscious desire to give out of selfless concern,

then there would be a lack of this personal connection; it would no longer be deemed a gift of life. Donor families and recipient families each have their own story to tell. Each story is as different as each individual. One aspect that they have in common, however, is the uncommon generosity of the human spirit during a time of great need and tragedy. Organ donation gives people the chance to rise above personal concern or gain by helping others in need of a life-saving transplantation. This opportunity is life-giving and life-changing to those who are at the end of the line for hope. It also affects the families, friends, colleagues, and acquaintances who love and support those in need of transplantation and who benefit from their renewed life and improved health after transplant. Organ donation is only termed the gift of life because of the role that altruism plays in its foundation; that term can be seen at its peak when grieving family members offer to donate the organs of a deceased loved one.

Good deeds and actions can result from tragedies, and organ donation is just one example. A family grieving over the loss of their child to an automobile accident can receive comfort in knowing that they are giving another human being a second chance at life when they are willing to make a donation. In some cases, more lives than one can benefit from the gift; a mother of five children receiving a heart; a widow with grandchildren receiving a new set of lungs; a teenager on dialysis receiving a kidney. Donor families embrace this sense of altruism when donating their loved one's organs and can in-turn take comfort in the idea that their loved one's legacy was one of life and giving. It is widely thought that there are benefits in the practice of altruistic donation. For donor families, specifically, they garner a feeling of consolation in knowing that their love one's death could ultimately be used for good. To multiply that altruism and have other good come from a tragic loss, that organ recipient could volunteer to participate

in life-saving research that may help in future transplantations. There are many aspects of a transplantation that — if done altruistically — can result in a rewarding and enriching outcome for both the donor, their family, and the recipient.

Altruism is desired for all aspects of organ donation; those situations involving donor families and even instances where transplant recipients desire to be included in a research study after their operation. The organ donation and transplantation system strives to honor the gift of donated organs by fully using those organs to save or improve the quality of the lives of transplant recipients. As a result of advances achieved through basic and clinical research over the past several decades, organ transplantation has become the optimal treatment for many end-stage organ-specific diseases. Furthermore, there are many aspects of organ transplantation that need to be improved upon, and one of the best ways to do that is by conducting research involving organ recipients. To date, organ transplantation research has focused almost exclusively on transplant recipients and on finding ways to improve transplantation processes and post-transplant health outcomes. With the help of altruistic transplant recipients, new methods can be identified that improve the quality and increase the quantity of organs for a successful transplantation. These people have been given a second chance at life, and research studies focused on improving organ transplantation could be their way of thanking that living or deceased donor for giving them the opportunity to lead a fulfilled and healthy life.

Deceased Donors and Cold Storage Solution

Living versus Deceased Donors

When it comes to the organ transplantation process, there are two ways that organs can be retrieved: from a living donor or from a deceased donor. The network has policies that regulate

how these donor organs are matched to patients on the waiting list, such as blood type, body size, and distance between the donor's hospital and the patient's hospital. The obvious difference between a living and a deceased donor is that one person is living and the other person is not. However, there is also a difference between what organs they are able to donate, the way those organs are prepared and transported, and even the overall outcome of the recipients. Living donors are few and far between when compared to the number of deceased donors, because they are essentially having to risk their own life and health to save that of another person. In 2018, however, the number of living donor transplants was the highest it had been since 2005, claiming almost 18% of more than 36,500 total transplants (2018 Living Donor Transplants Increase 11 Percent, 2019). The majority of the time, these donors are giving their organs or a part of their organs to either a family member or someone they know. For a lot of people in this case, the benefits of having their loved one for longer outweigh the risks of any short-term or long-term damage. Living donations, although offering more advantages for the recipient of an organ transplant, are harder to come by because they are limited on what organs they are able to give and they risk the health and life of not only one but two people.

When it comes to available organs for transplantation, it is obvious that deceased donors have less limitation than living donors on what they are able to give. Living donors are limited to giving one kidney, a lung, or a portion of the liver, pancreas or intestine. Deceased donors are able to give two kidneys, two lungs, liver, heart, pancreas, eyes, or various tissues. For both types of donation, the kidney is the most commonly transplanted organ. In 2019, renal transplantation accounted for 60% of the total transplants performed that year (Organ Donation Statistics, 2020). This large number is attributed to end-stage kidney disease which can be caused

by diabetes, high blood pressure, and anatomical problems of the urinary tract. Most people can undergo treatment with dialysis, but transplantation offers the closest representation of a normal life because the transplanted kidney can replace the failed kidney. This is the case for all other types of organ failure as well; transplantation is the ideal option. Furthermore, if living donation is available, it is preferred over deceased donation because they normally result in far better outcomes for the organ recipient.

Although deceased organ donation is much more common than living organ donation, the latter offers several advantages for the recipient of an organ transplant. Transplantation surgeries involving organs from a living donor can be scheduled in advance, ensuring that the transplant occurs at an optimal time. Many people with organ failure are still able to maintain a job if their health allows, so being able to schedule time off of work is an advantage. Also, living organ donations tend to become available before the recipient has to be placed on the waiting list or before they begin treatments, such as dialysis that accompanies renal failure. This not only prevents the patient's health from deteriorating further, but it also saves them from any stress that comes from anticipation. Finally, living donor organs tend to have greater longevity than those transplanted from a deceased donor; they may become stressed by having to spend a longer time in a cold storage solution while they are being transferred between hospitals. The use of these preserving compounds can temporarily reduce organ function, resulting in an organ that is not fully functional until days or weeks after the transplant.

Cold Storage Solution and Transplantation

Organ transplantation is the most effective therapy for patients with end-stage organ disease. Preservation solution and techniques are crucial for keeping the organ viable during

transportation, which is directly related to morbidity and survival rate of the patient after transplantation. By the start of clinical transplantation, there was already a significant amount of knowledge acquired by physiologists and anatomists on how to keep organs functioning outside of the body. Since the 1960s, static cold storage (SCS) has been the preferred method for sustaining these organs from the time they are taken out of the deceased donor and placed into the recipient. It is deemed as the "supply line" for organ transplantation by allowing time for surgery preparation, allocation and transit of the organ, and running of laboratory test to ensure fitness of the body part for a specific patient. However, prolonged time in this solution has been shown to increase the risk of damage to the organ that can result in more complications down the road (Jing et al, 2018, p. 845). Also, there have been difficulties with assessing donor organ function and viability after preservation in cold storage.

Many early, pivotal discoveries in medicine have allowed for the organ preservation system that we see today. Starting with the first closed artificial circulation system that was constructed by Max von Frey and Max Gruber (Jing et al, 2018, p. 846) This discovery allowed for organ perfusion to continue for several hours without having to be interrupted to resupply oxygen to the blood that was flowing out of the tissues. Historically, blood was used as a perfusate in early apparatuses, but because this required a large supply of blood to operate, scientists began to experiment with altered or new perfusates. They attempted to use a different animal's blood but the use of cross species was toxic to the graft. Scientists then diluted the animal's own blood using saline but this led to severe edema in the lungs. These discoveries led to the transition of using blood as a perfusate to a more successful, chemically defined perfusion solution. Along with these findings, scientists also realized that colder temperatures, as opposed

to room temperature perfusion, might lessen organ damage by mitigating cellular metabolism. Considerably lowering the temperatures of the solution, in fact, extended organ preservation from hours to days. All of these experiments and findings lead to a simple method for organ preservation that was more cost-efficient and favorable for organ transportation than its predecessors. The birth of SCS replaced dynamic perfusion methods and became the standard method of organ preservation that hospitals still use to this day.

Since the 1960s, SCS has gradually become the preferred method of organ preservation for transplantation surgeries. With the shortage of organs available for transplant, it is crucial that the organs that are donated are functioning at their highest possible capacity, and organ preservation is a crucial step in producing that outcome. The main goal in organ preservation is to maintain function of the organ and tissue during storage so that the graft will function at re-perfusion, when the blood flow is restored. The process of SCS involves flushing the procured organ with preservation solution at 0–4 °C, then immersing it into preservation solution at the same temperature until transplantation (Jing et al, 2018, p. 846). The hypothermic environment is responsible for abating the rate of cellular metabolism, and the preservation solution provides protection for the tissue against harmful agents. These cold storage solutions also contain antioxidants and nutrients that sustain the cells and slow inflammation, but doctors are beginning to realize that is not always enough. In 2016, there were a total of almost 5000 recovered organs that were not transplanted, and a large percentage of that number was due to prolonged cold storage of those organs in the preservation solution (Kindy et al, 2018). The majority of organs can only tolerate 24 to 48 hours of cold ischemia before they are no longer functional, but every hour spent in storage solution results in more damage to the tissue. This prolonged state of

hypothermia is supposed to limit ischemia-reperfusion injury, but it actually results in cellular edema and eventually cell death. With the already limited amount of deceased donor organs available, it is absolutely crucial that all available organs are used for transplantation. Although the use of SCS has been proven to cause damage to the tissue, it is still essential for preservation of the organs during transplantation. Many research groups have focused on targeting the methods of destruction of this cold storage solution so that they can protect organs from further tissue damage, and also allot more time — when needed — for transplantation. In fact, a research lab at Arkansas Children's Research Institute in Little Rock, Arkansas is doing just that.

The Parajuli Lab

Identifying pathways related to renal cold storage that lead to renal damage after transplantation will help design novel pathway-specific therapies to improve graft outcome. The focus of the Parajuli Lab at Arkansas Children's Research Institute is centered around that goal of improving transplant outcomes by identifying cold storage-mediated renal damage and by acquiring targeted therapies during cold storage. Leading this research team is Dr. Nirmala Parajuli who also serves as assistant professor of Pharmacology and Toxicology at the University of Arkansas for Medical Sciences. Using male Lewis rats as transplant donors and recipients, her team has been successful in emulating transplant conditions for further research. So far, their lab has shown that mitochondrial function of the kidneys was compromised after cold storage alone, and this was exacerbated when cold storage was combined with transplantation. A recent report showed in a rat model of renal transplantation that mitochondrial dysfunction precedes compromised proteasome function and this results in a vicious cycle of mitochondrial injury and proteasome dysfunction. Also, they have come to realize that there are therapeutics, such as

Mitoquinone, that preserve mitochondrial and proteasome function during cold storage that may provide beneficial outcomes following transplantation. In addition, the researchers at the Parajuli Lab have found that proteasome inhibition/down-regulation increases reactive oxygen species, which then impairs proteasome subunits in renal proximal tubular cells. All in all, they have established that oxidant production increases during cold renal preservation, and mitochondria are a key target for injury. Cold preservation has greatly facilitated the use of cadaveric kidneys for transplantation but damage occurs during the preservation episode. In the summer of 2019, I was given the great opportunity to serve as a research intern in the Parajuli Lab, researching the effects of renal cold storage and transplantation on immunoproteasome and the complement system.

Introduction to Research

Renal transplantation is the preferred method of treatment for end stage kidney disease. The majority of donor kidneys come from deceased donors and have to be stored in cold storage solution (CS) until the recipient is identified. However, prolonged CS is associated with poor long-term outcome. Unfortunately, the mechanisms of CS-related damage are largely unknown. Our laboratory recently reported that the proteasome and renal function were significantly decreased in rat kidney transplants that involved CS combined with transplantation (CS/Tx), as opposed to those that did not undergo CS (auto-transplantation/ATx). The long-term goal is to improve the transplant outcome by identifying CS-mediated renal damage and by acquiring targeted therapies during CS. This study contributes to that objective by characterizing immunoproteasome (a proteasome variant) and the complement system (a group of serum proteins that participates in eliminating pathogens and debris) activation within the kidneys after

CS/Tx. Our hypothesis is that CS/Tx will exacerbate the function of immunoproteasome and complement systems. Lewis rat kidneys exposed to 18 hours of cold storage were used for transplantation (CS/Tx). Kidneys with no CS exposure were transplanted (ATx) and used as a transplant control. The sham (Sh) kidneys with right nephrectomy were used as a control. Using paraffin embedded kidney sections and immunohistochemistry/immunofluorescence, immunoproteasome and complement levels/function were evaluated. Immunoproteasome function was significantly increased only after CS/Tx when compared to Sh and ATx. Immunohistochemistry of kidney sections revealed a modest increase of immunoproteasome catalytic subunits, LMP2 and LMP10, after ATx when compared to Sh, but a profound increase of these subunits was detected after CS/Tx. Similarly, complement proteins C3 (an upstream component) and C5b-9 (a cytolytic terminal activation product) were increased in kidneys after ATx (detected by immunofluorescence), but an excessive increase of these proteins was observed after CS/Tx. Furthermore, TUNEL assay revealed exacerbated cell death in kidney sections after CS/Tx, whereas ATx showed a slight increase of cell death. These results suggest that the prolonged CS worsens activation of the immunoproteasome and complement system, further leading to renal damage and dysfunction.

Background

My research was focused on researching the effects that renal cold storage and transplantation had on immunoproteasome, which is a variant of the constitutive proteasome (as shown by Figure 1), and the complement system, specifically the pathways C3 and C5b-9. The constitutive proteasome is a protein degradation missionary that maintains homeostasis in the cell. It has already been shown by the Parajuli Lab that proteasome function is compromised in

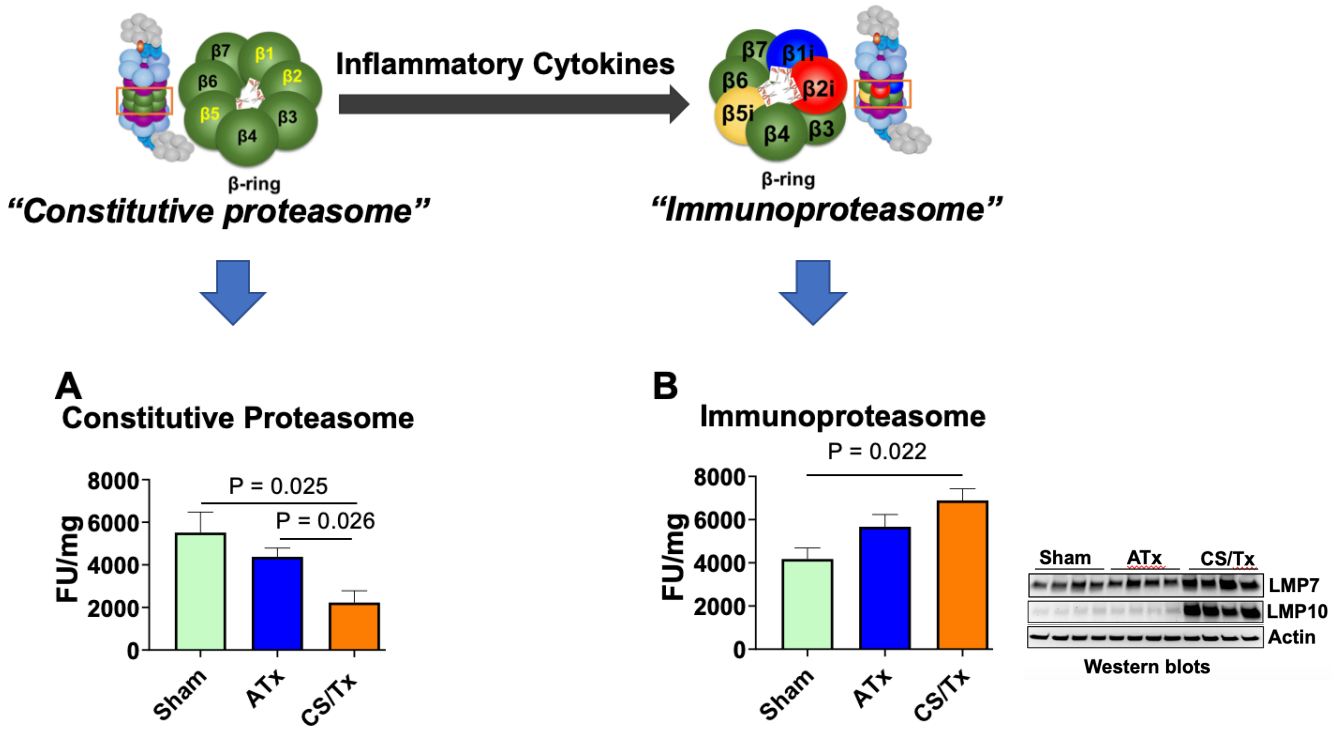

Figure 1. Immunoproteasome

CS/Tx. In Figure 1, the bar graph shows that the constitutive proteasome saw more dysfunction and less activation in the CS/Tx kidney as opposed to the Sh kidney. During stress, inflammatory cytokines are released, triggering activation of the immunoproteasome. Using a western blot, the lab had already started researching the effects of CS/Tx on the B2i (LMP10) and B5i (LMP7) components within the immunoproteasome beta ring. Their results in the graph in Figure 1 show that there was a higher amount of immunoproteasome activation in the CS/TX kidney as opposed to the Sh kidney. This means that there was a higher number of inflammatory cytokines released due to stress. I was given the task of researching the effects of CS/TX on B1i (LMP2) and B2i (LMP10) within the immunoproteasome beta ring.

My research was also focused on studying the effects that renal cold storage and transplantation had on the complement system, specifically the pathways C3 and C5b-9.

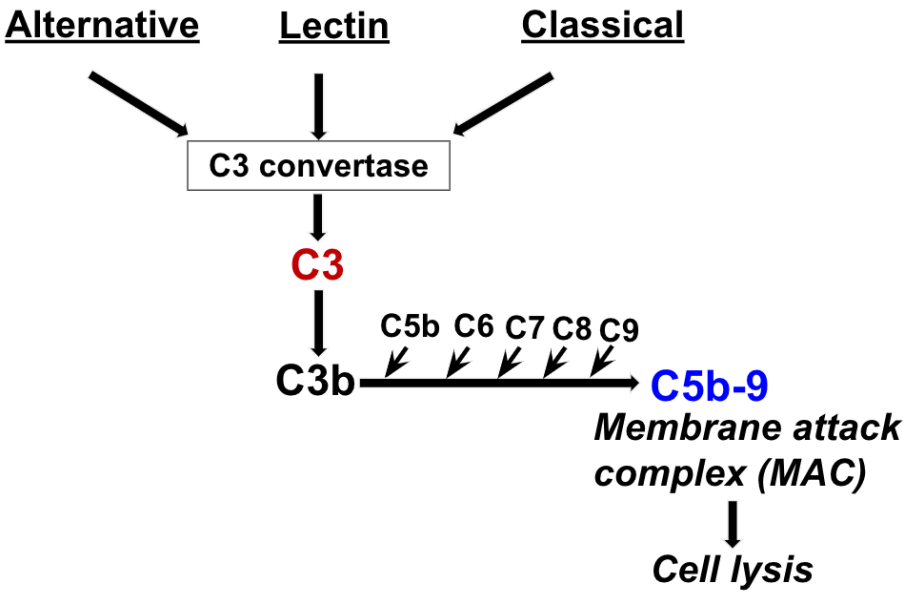

Figure 2. Complement system

The complement system is a group of serum proteins that detects and gets rid of pathogens within the tissue. It specifically functions as a part of the immune system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promote inflammation, and attack the pathogen's cell membrane. There are three biochemical pathways that activate the complement system (alternative, lectin, and classical) as shown in Figure 2. The focus of my research, however, was not on those three pathways specifically, but what they all merge on: C3, an immune system protein. When stimulated by one of several triggers, C3 is activated and a cascade begins that further triggers C5b-9. This final point in the complement cascade serves as a cytolytic membrane attack complex that if deposited in the cell leads to cell lysis or cell death, as shown in Figure 2. The end result is stimulation of phagocytes to clear foreign and damaged material.

Materials and Methods

All protocols in this section were replicated from research conducted by Dr. Nirmala Parajuli (Parajuli et al, 2011; Parajuli et al, 2017).

Animals: Male Lewis rats (200 –250 g) were used as transplant donors and recipients. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences (UAMS), and all animal experiments were performed in compliance with institutional and National Institutes of Health guidelines.

Orthotropic Renal Transplant Surgery: For donor surgeries, rats were anesthetized with isoflurane, and the left and right kidneys were removed and flushed with and stored in UW solution at 4°C for 18 h. The right kidneys of donor rats were referred to as the CS group (as shown in Figure 3). For recipient surgeries, rats were anesthetized with isoflurane, the native left kidney was removed, and the donor left kidney (exposed to CS) was transplanted. The native right kidney was immediately removed so that renal function was entirely dependent on the transplanted left kidney. After 24 h of reperfusion, the transplanted left kidney and blood were collected under anesthesia and saved as the 18-h CS/Tx group (as shown in Figure 3).

Autotransplant surgery: Autotransplant (ATx) surgery was included in these studies so that the impact of CS could be isolated from the impact of transplant surgery alone. ATx was performed (as shown in Figure 3); the left kidney was removed, flushed with saline, and immediately transplanted back into the same rat without CS exposure. After 24 hours, the transplanted kidney was harvested under anesthesia; these kidneys were referred to as the ATx group.

Sham surgery: Rats underwent the same procedure for right nephrectomy but without renal transplantation (sham operation in Figure 3); the right kidney was saved as a control kidney.

The left kidney and blood were harvested 24 h later and saved as the sham group.

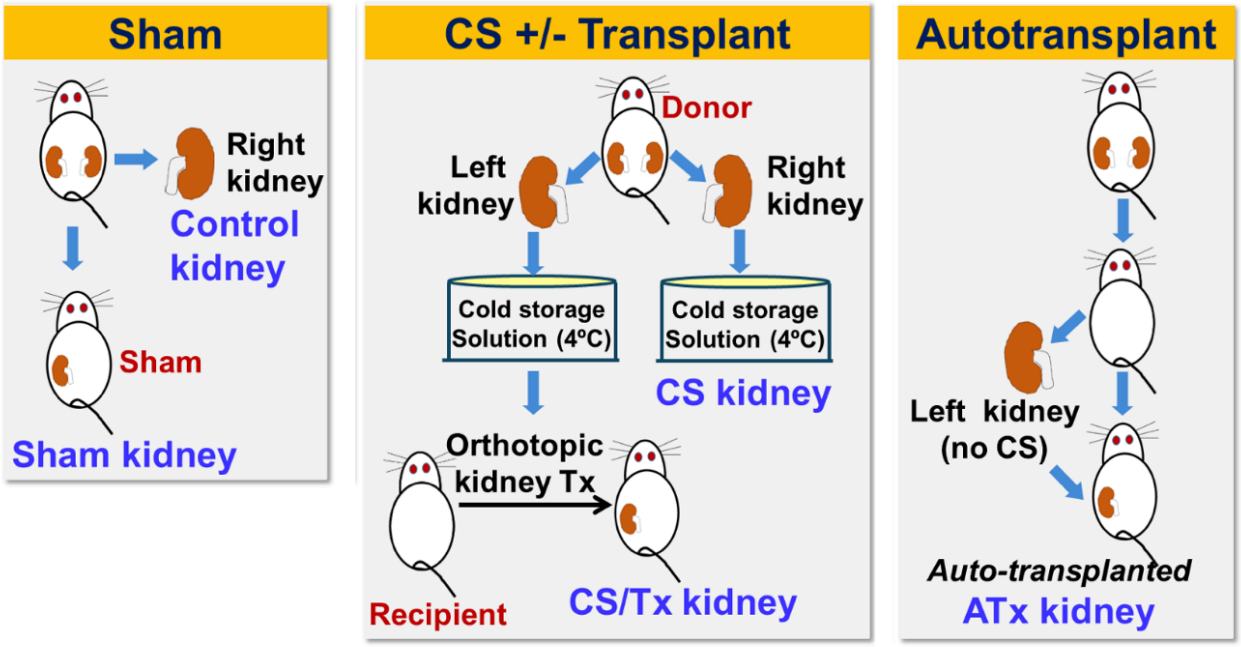

Figure 3: Rat Renal Transplantation Model

Immunohistochemistry: For immunohistochemical analysis, antigens were retrieved by heating sections in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase was quenched by incubating the sections with Peroxidase Suppressor (Thermo Scientific, Rockford, IL, USA) for 15 min at RT. The slides were blocked with Non Serum Protein Block (Dako, Carpinteria, CA, USA) for 20 min at RT. Primary antibodies were prepared in antibody diluent solution (0.5% non fat dry milk and 1% BSA in TBS) and incubated for 1 hour at room temperature. Counterstaining was performed with Mayer's Hematoxylin (Electron Microscopy Science). All images were taken using a Nikon Eclipse E800 microscope (Q Capture imaging and Nikons Elements software) Semi-quantitative evaluation of nitrotyrosine staining was performed based on the percentage of positive tubules in 10 high power fields (200×) from

cortex and medulla using the following scores: 0 - null/negative; 1 - 10% positivity; 2 - 25% positivity; 3 - 50% positivity; 4 - 75% positivity; 5 - 100% positivity.

Immunofluorescence: For immunofluorescent analysis, antigens were retrieved by heating sections in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase was quenched by incubating the sections with Peroxidase Suppressor (Thermo Scientific, Rockford, IL, USA) for 15 min at RT. The slides were blocked with Non Serum Protein Block (Dako, Carpinteria, CA, USA) for 20 min at RT. Primary fluorescent antibodies were prepared in antibody diluent solution (0.5% non-fat dry milk and 1% BSA in TBS) in the dark and incubated overnight at 4°C. Counterstaining was performed with DAPI (4′,6-diamidino-2-phenylindole). All images were taken using a Nikon Eclipse E800 microscope (Q Capture imaging and Nikons elements software). Semi-quantitative evaluation of the cortex and medulla staining was performed based on fluorescent levels detected by the ImageJ software.

TUNEL Assay: For visualization of apoptotic cells *in situ,* terminal transferase-mediated dUTP nick-end labeling (TUNEL) method was utilized according to the protocol provided by the manufacturer (TACS™ TdT Kit, R&D Systems, MN, USA). Counterstaining was performed using methyl green solution. Seven different fields (3 cortex, 2 outer medulla, 2 inner medulla) from each mouse kidney section were considered for evaluation. TUNEL-positive cells from each field were grouped in two nephron segments, namely proximal tubule (glomerulus and proximal tubules) and distal nephrons (distal tubule, loops of Henle, and collecting ducts) and the average was reported.

Results

The activities of LMP2 and LMP10 within the immunoproteasome beta-ring were assessed using immunohistochemical analysis, which has the advantage of determining the tissue distribution of these antigens of interest. Rat kidneys exposed to CS (18 hr) followed by transplantation in a new recipient rat (CS/Tx) resulted in a greater activation of both LMP2 and LMP10 when compared to sham kidneys (rats with removal of the right kidney, but no CS or Tx) and ATx kidneys (rats with removal of the left kidney with Tx, but no CS). Compared to kidneys from sham rats, kidneys subjected to CS/Tx showed impaired renal function 24 hr after transplantation with an increased number of inflammatory cytokines due to high levels of homeostatic stress. Semi-quantitative evaluation of the staining was based on the percentage of positive tubules in 10 high power fields (200×) from cortex and medulla (see Figure 4).

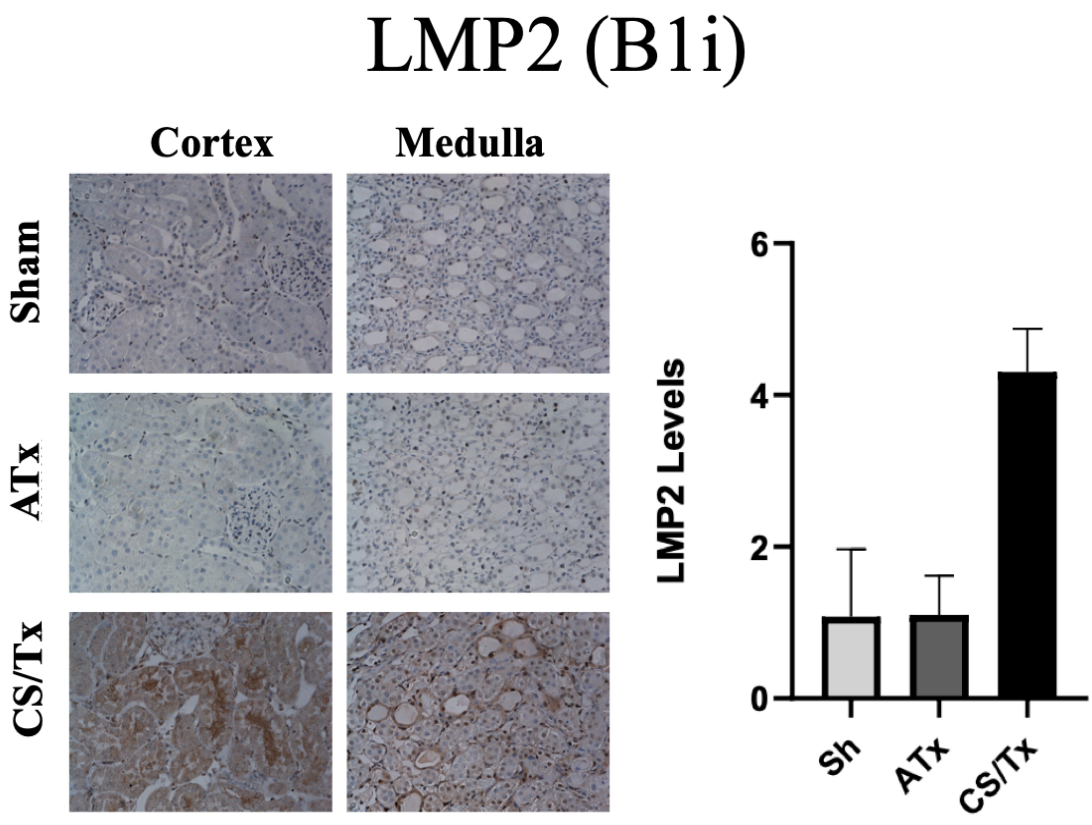

Figure 4. IHC results with LMP2 antibody

As already mentioned, within the immunoproteasome beta-ring there are seven proteasome subunits (refer to Figure 1). This research focused on the LMP2 (B1i) subunit. These pictures taken with a Nikon Eclipse E800 microscope (Q Capture imaging and Nikons elements software) show the localization of immune cells and proteasome at the cellular level. Positive brown staining in these tubular cells indicates greater activation of LMP2 during CS/Tx in both the cortex and medulla region of the renal tissue. As shown, there is hardly any positive brown staining in the Sh and ATx conditions, indicating that CS/Tx causes renal damage and dysfunction. Around twenty images were taken for each renal transplant condition and were given a score of 1 to 5 (0 - null/negative; 1 - 10% positivity; 2 - 25% positivity; 3 - 50% positivity; 4 - 75% positivity; 5 - 100% positivity) to quantify the number of positive cells, and then they were averaged. The graph in Figure 4 shows that between about 85% of the tubular cells in the CS/Tx condition had a positive brown staining, and only about 10% of the tubular cells in the Sh and ATx condition had a positive brown staining. These results show that immunoproteasome function was significantly increased when the kidneys were exposed to CS/Tx.

This research also focused on the LMP10 (B2i) subunit within the immunoproteasome beta-ring. These pictures taken using a Nikon Eclipse E800 microscope (Q Capture imaging and Nikons elements software) show the localization of immune cells and proteasome at the cellular level. Positive brown staining in these tubular cells indicates greater activation of LMP10 during CS/Tx in both the cortex and medulla region of the renal tissue. As you can see, there is hardly any positive brown staining in the ATx conditions, indicating that CS/Tx causes renal damage

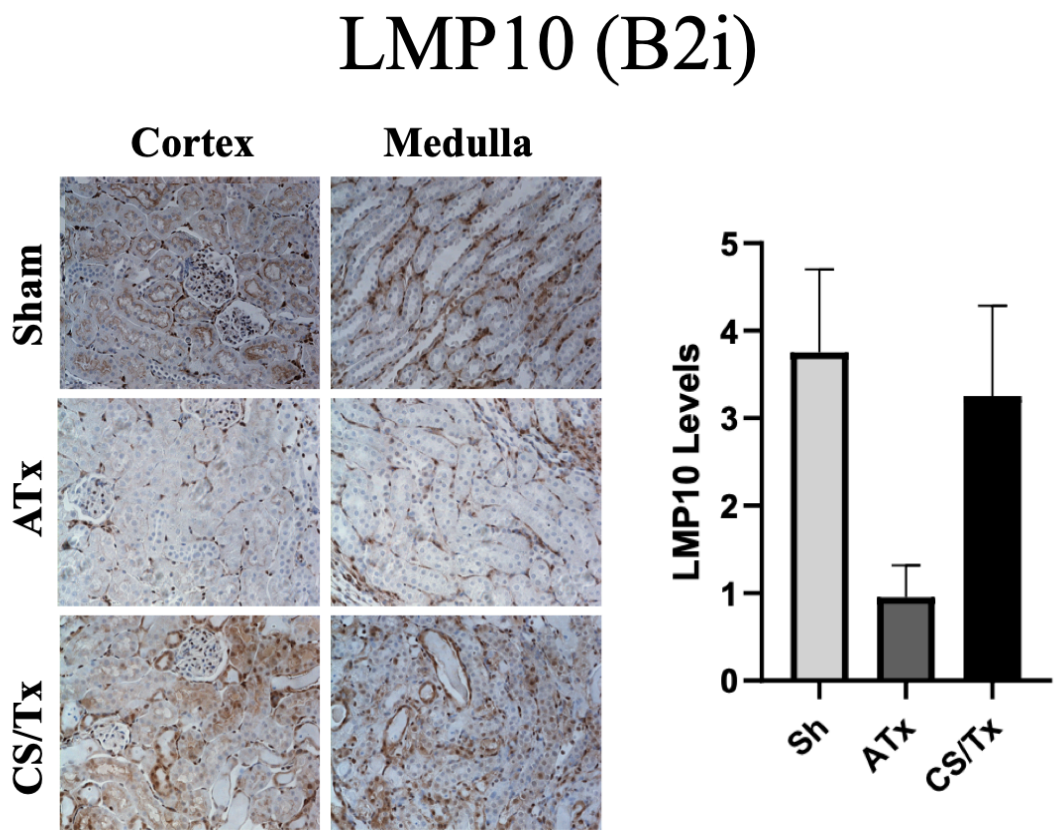

Figure 5. IHC results with LMP10 antibody

and disfunction. However, in the Sh images there is also a brown-staining within the tubular cells, which was not expected. It was concluded that the LMP10 sham sections were compromised; further testing with an increased section number would be necessary to determine if sham sections were flawed or if this particular antibody did not work. However, the data in Figure 5, with CS/Tx levels reaching almost 60%, still shows that overall immunoproteasome function is up-regulated in renal cells exposed to cold storage.

The activities of C3, an immune protein that plays a central role within the complement system, were assessed using immunohistochemical analysis to quantify the amount of positive brown-stating within the cortex and medullar regions of the renal tissue. Immunofluorescent

analysis was used to measure the levels of C5b-9, the membrane attack complex within the complement system, which allows not only for the detection of the antigen in a specific location within the renal cells, but also gives a level of expression based on fluorescence. Rat kidneys exposed to CS (18 hr) followed by transplantation in a new recipient rat (CS/Tx) resulted in a greater activation of both C3 and C5b-9 when compared to sham kidneys (rats with removal of the right kidney, but no CS or Tx) and ATx kidneys (rats with removal of the left kidney with Tx, but no CS). Compared to kidneys from sham rats, kidneys subjected to CS/Tx showed impaired renal function 24 hours after transplantation with an increased number of phagocytic cells, which clear foreign and damaged material. Semi-quantitative evaluation of the cortex and medulla C3 staining was performed based on fluorescent levels detected by the ImageJ software.

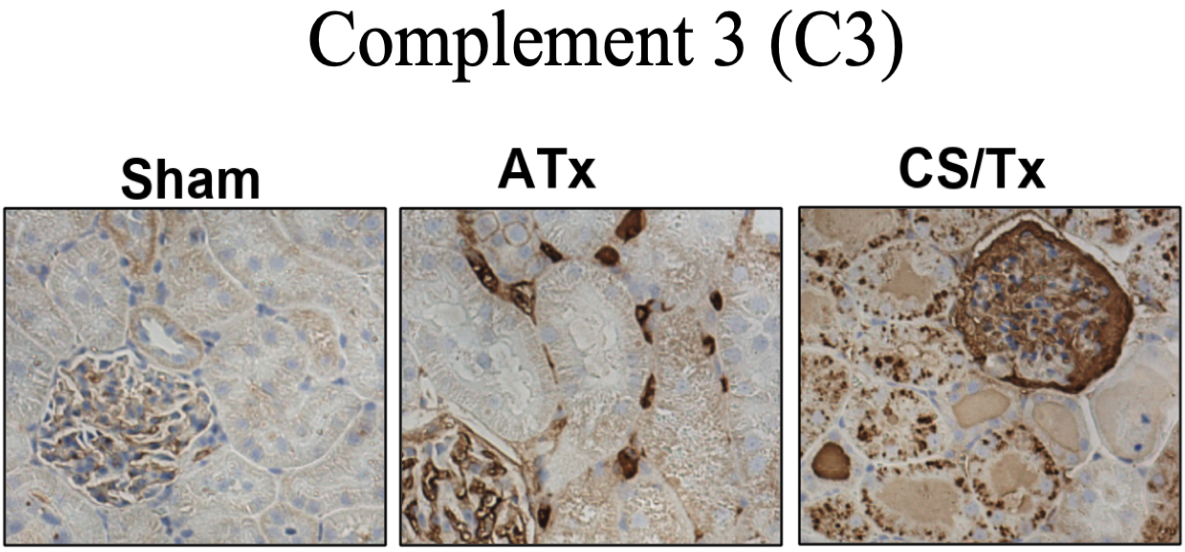

Figure 6. IHC results with C3 antibody

As mentioned, the complement system, also known as the complement cascade (refer to Figure 2), is a group of serum proteins within the immune system that detects and gets rid of pathogens within the tissue. C3, specifically, is an immure protein that, when activated,

stimulates the rest of the cascade to completion. These pictures taken with a Nikon Eclipse E800 microscope (Q Capture imaging and Nikons elements software) show the localization of this immune protein at the cellular level. Positive brown staining in these tubular cells indicates greater activation of C3 during CS/Tx in the cortex region of the renal tissue. As you can see in Figure 6, there is hardly any positive brown staining in the Sh and ATx conditions, indicating that CS/Tx causes renal damage and dysfunction. The small amount of brown staining that there is in the ATx image is not within the nuclear region of the cells but along the outer membrane, whereas, in the CS/Tx image the same thing cannot be said; there is a large amount of brown staining in the nuclear region of those cells. No semi-quantitative evaluation was done with the C3 antibody images because of time constraints during the research process.

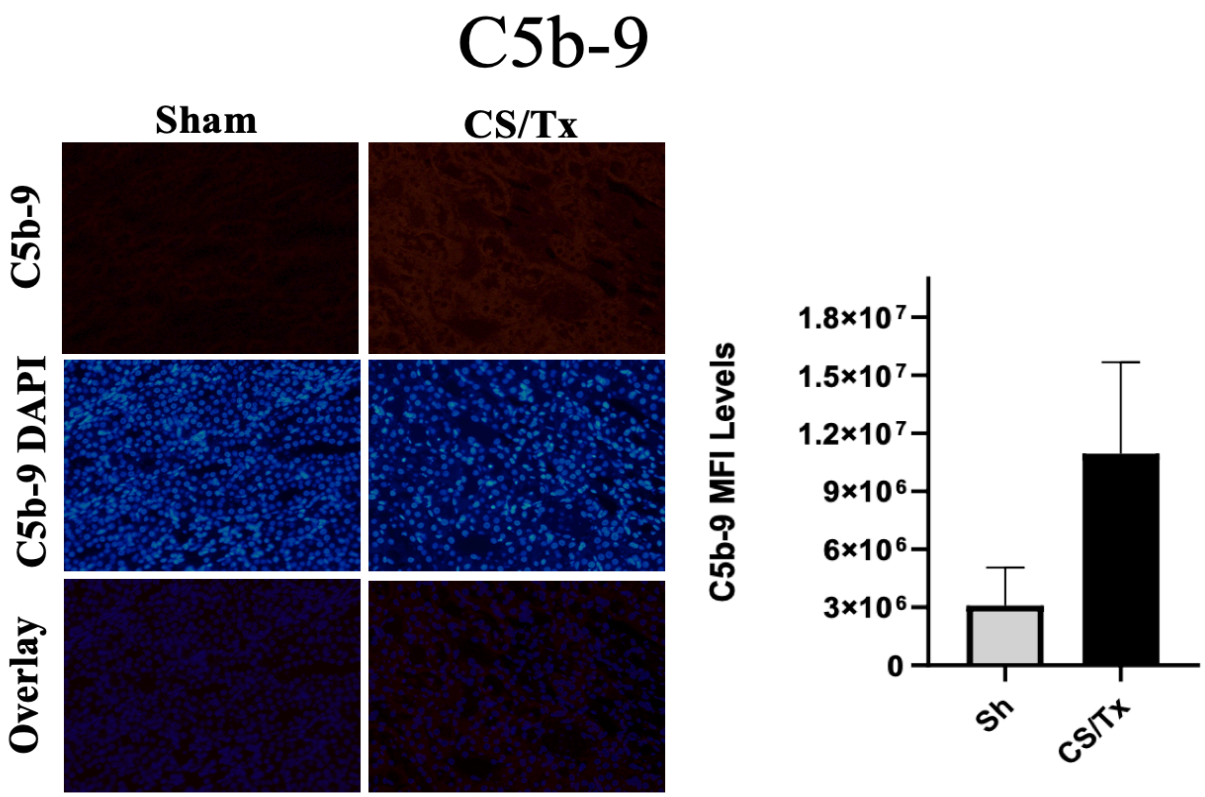

Figure 7. IFC results with C5b-9 antibody

At the end of the complement cascade is C5b-9, the membrane attack complex, that when activated leads to cell lysis or cell death. These pictures taken with a Nikon Eclipse E800 microscope (Q Capture imaging and Nikons elements software) show the fluorescent activation levels of this immune protein at the cellular level. Brighter fluorescent staining in these tubular cells indicates greater activation of C5b-9 during CS/Tx in the cortex and medullar regions of the renal tissue. As you can see in Figure 7, the red fluorescent color is much stronger and defined in the CS/Tx condition as compared to the Sh. Using the DAPI counterstain, the images showed much brighter, almost neon green, nuclei in the CS/Tx condition that cannot be seen in the Sh image. The C5b-9 antibody image and the C5b-9 with DAPI image for both conditions were overlaid using ImageJ software to produce two contrasting photos. In the CS/Tx overlay image you are able to see both the red fluorescence of the tubular cells and the DAPI counterstain of the nuclei, whereas, in the Sh overlay image you can only see the DAPI counterstain. The graph in Figure 7, with data produced from the ImageJ software, affirms that C5b-9 levels were much greater in CS/Tx kidneys than in Sh kidneys. All of this data allows us to conclude that CS/Tx exacerbates the function of C5b-9, leading to a greater amount of cell lysis or cell death.

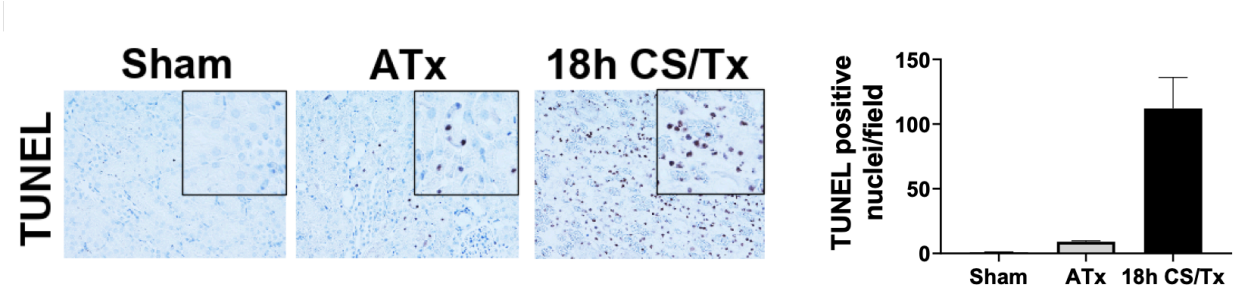

Figure 8. TUNEL assay results

A terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed to further examine the cell death that was seen with both the immunohistochemical analysis of

C3 and the immunofluorescent analysis of C5b-9. Rat kidneys exposed to CS (18 hr) followed by transplantation in a new recipient rat (CS/Tx) resulted in a larger number of apoptotic cells when compared to sham kidneys (rats with removal of the right kidney, but no CS or Tx) and ATx kidneys (rats with removal of the left kidney with Tx, but no CS). As you can see in Figure 8, the dark purple spots are the apoptotic cells, and there is a much larger amount in the CS/Tx image than there is in both the Sh and ATx images. TUNEL-positive cells were quantified by separating the images for each condition into four quadrants, counting the number of apoptotic cells in each quadrant, and then taking an average of all the images. The graph in Figure 8 validates what is seen in the images next to it; there were more than 100 positive nuclei per field in the CS/Tx condition, close to 15 positive nuclei per field in the ATx, and nearly zero in the Sh condition. This data of apoptotic cells from a TUNAL assay substantiates the data of cell lysis that was obtained from the immunohistochemical analysis of C3 and the immunofluorescent analysis of C5b-9. All of this data allows us to conclude that CS/Tx not only activates the complement cascade, which enhances the ability of phagocytic cells to clear harmful material, but also leads to large amounts of apoptosis within renal cells.

Conclusions

The availability of human kidneys for clinical transplantation is limited and the need of transplantable, good quality human organs is increasing by the hour. Therefore, it is crucial to improve organ preservation quality to maximize procured renal allograft for clinical transplantation, and that includes associating prolonged CS with a better long-term outcome. The long-term goal of the Parajuli Lab is to improve the transplant outcome by identifying CS-mediated renal damage and by acquiring targeted therapies during CS. This study contributed to

that objective by characterizing immunoproteasome (a proteasome variant) and the complement system (a group of serum proteins that participates in eliminating pathogens and debris) activation within the kidneys after CS/Tx. Lewis rat kidneys exposed to 18 hours of cold storage were used for transplantation (CS/Tx). Kidneys with no CS exposure were transplanted (ATx) and used as a transplant control. The sham (Sh) kidneys with right nephrectomy were used as a control. Using paraffin embedded kidney sections and immunohistochemistry/ immunofluorescence, the levels and function of immunoproteasome and the complement system were evaluated. Our hypothesis was that CS/Tx would exacerbate the function of immunoproteasome and the complement system. This research study supported our hypothesis by clearly demonstrating that CS/Tx increases immunoproteasome expression and function, and CS/Tx increases activation of the complement system. Furthermore, increased immunoproteasome and complement activation after CS/Tx correlates with increased renal damage and dysfunction. These results highlight the importance of studying how transplant conditions, specifically cold storage solution, affect renal tissue, so the preservation of renal allograft can be prolonged and the quality of transplant organs can be improved.

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