Rejection, Desensitization, and Tissue Preservation in Male Urogenital Allotransplantation

Samuel Fidder

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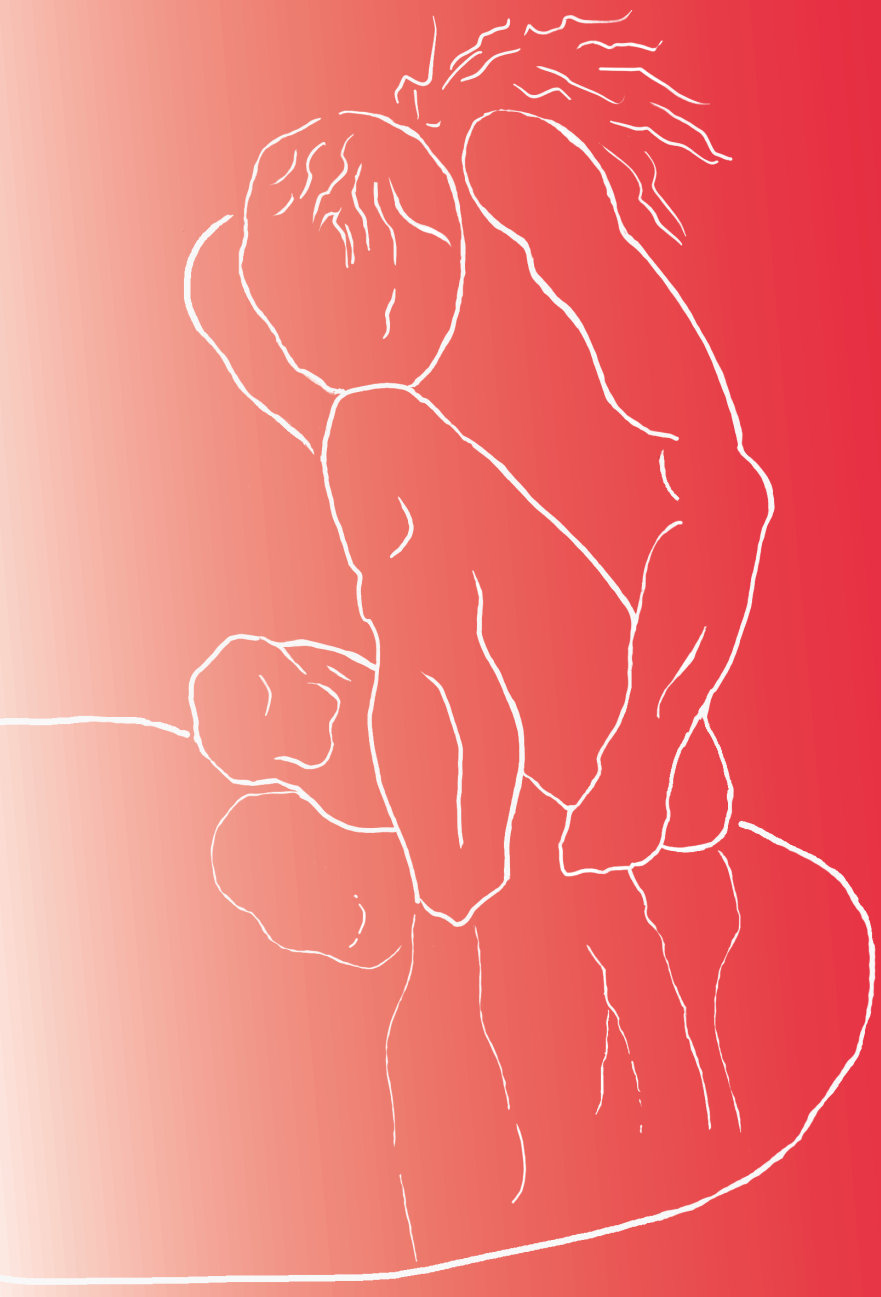

Chapter 1

Introduction

1

When thinking about medicine, the predominant image in the public's mind is that of doctors saving lives and curing disease. Saving lives by removing a tumor, inserting a breathing tube into a trachea, or infusing chemotherapy. Curing disease by administering antibiotics, starting an intravenous drip, and supplying vaccines. When not saving lives, doctors are mainly thought to help with optimizing the body's many functions: ensuring safe childbirth, opening clotted vessels, fixing broken bones. However, patients have more needs than mere survival and optimized bodily function. Humans are social animals and have a need for interaction and an urge to find romantic partners. People are self-aware and learn from an early age that the way they are perceived and perform socially is heavily influenced by another factor: appearance. Plastic & Reconstructive surgery is the specialty that incorporates considerations of function and form into the care that is delivered.

Reconstructive transplantation to regain form and function

Reconstructive transplantation is the most recent addition to the plastic surgeon's reconstructive elevator. In plastic surgery, reconstructing form and function is preferably done in the least invasive way possible, with options escalating from primary wound closure to local flaps and free flaps. In this context reconstructive transplants are the final reconstructive modality, only to be used when all other options are exhausted.[1] Reconstructive allotransplantation, commonly referred to as Vascularized Composite Allotransplantation (VCA) is thus most often indicated due to a lack of functional patient tissue available for autologous reconstruction. In reconstruction of facial musculature, fingers (including muscle, nerve and tendon), and erectile tissue, complete functional units are required. Such functional units consist of composite tissues that include vessels, nerves, tendons, bone and muscle; the human body does not have surplus tissue available that can truly replace these. All reconstructive transplants that have been performed to date [2–6] were undertaken in a quest for the restoration of form and function in the absence of available autologous tissue. Hand transplants allow a normal appearance and an independent life, face transplants allow for a normalized social life and can restore oral functions, and a larynx transplant enables oral communication and makes a patient's neck look natural again. The performance of these extraordinary medical procedures is built upon a long history of pioneering work by visionary scientists in both reconstructive surgery and the relatively new field of transplantation.

History of transplant surgery

The search for viable reconstructive options has been captured in writing throughout history with stories of actual transplants dating back to as early as 350 AD.[7] Interestingly, up until the twentieth century, most attempted transplants were of this reconstructive nature, as the concept of the vascularization of organs and the techniques to transplant them did not exist yet. More ancient folklore shows how xeno- and allotransplantation have captured the human imagination since antiquity. Descriptions of transplants are found in Babylonian writings and Chimera's made up of combined creatures appear regularly in Greek mythology and Hindu scripture. In the European medical history, the legend of the black leg stands out as a prime example of this fascination with allotransplantation. The legend goes that in the third century AD two patron saints of surgery named St Cosmas and St. Damian performed many miracle surgeries.[8] The transplant of the black leg was performed after Cosmas and Damian had to amputate the gangrenous leg of an older devout churchgoer. In the night they proceeded to successfully connect the leg of a recently deceased Ethiopian man to the patient's remaining stump.[9] Upon his awakening the patient was then surprised by a completely healthy (though not necessarily perfectly matching) leg in the place of his diseased one. Though highly unlikely to be based on historical fact given the current knowledge of neurological recovery and immunological challenges, the longevity of the story underlines the public's fascination with these procedures and introduced the concept of cadaveric transplantation. These and other stories are also seen as signs that experimentation with different forms of (mostly reconstructive) transplants went on for the following centuries.

The 16th century brought the first description of a true reconstructive transplant and is found in the writings of the surgeon Gaspare Tagliacozzi (1547-1599) reporting on the transplantation of noses.[10] As a part of the surgical innovation renaissance coinciding with the end of the Middle Ages, he described a transplant procedure in which a slave gave their nose to their owner. A true leap forward then occurred in the 19th century when more and more surgeons began to publish detailed descriptions of their experiments. Works by a doctor regarded a father of British surgery, John Hunter, reported on successful bone and Achilles tendon autografts as well as chicken testes allografts.[11] More surgeons followed and during the 19th century free grafting of nerves, cartilage, bone, cornea, skin, and tendons were described. The 20th century brought another leap forward in surgical technique pioneered by the French surgeon Alexis Carrel, who in the early 1900s described a vascular coaptation method that enabled kidney transplants.[12] It was however quickly discovered that successfully transplanted kidneys did not maintain function long enough to result in survival extension of patients, cementing the belief that some sort of incompatibility existed. Concurrently with Carrel, the Austrian surgeon Emerich Ullmann presented research on the successful autotransplantation of kidneys in dogs and even xenotransplanta-

tion of a dog kidney into a goat. He however ran into the same challenges related to graft rejection and abandoned his research after the unsuccessful outcome of a pig kidney xenotransplant into a woman with end-stage kidney failure.[13]

The twentieth century added insights into rejection and immunology and a momentous leap forward occurred when the American Surgeon Joseph Murray proved that kidney transplantation is possible between identical twins.[14] Like in many landmark surgical innovations, an approach that could have proven to be pure folly turned out to be highly successful, with the recipient surviving for 7 years after his transplant. Following in the footsteps of other surgical innovators such as William Stewart Halsted, Murray had performed many experiments in animals before attempting a human kidney transplant. Performing the surgery meant overcoming significant hurdles, including in dealing with the criticism of the public and medical contemporaries. 'Suddenly, the whole world was watching. The media quoted doctors who said the experiment was not only doomed to failure but also unethical.'[15] Murray had to consider the possibility of the procedure resulting in the accelerated death of the recipient Richard Herrick, but also of his donating brother Ronald, for it was not definitively proven that one's life expectancy was not negatively impacted by the removal of a kidney. Similarly, it was not established how long a human kidney could survive outside the body. It turned out that the ischemic time of 1 hour and 22 minutes was short enough. Sadly, subsequent allogeneic transplants performed by Murray and his team were rarely successful and he abandoned his transplant practice to focus on his work as a plastic surgeon.[15] Despite the setbacks faced by Murray after his landmark surgery, his kidney transplants were a catalyst for transplant surgery and transplant immunology and was followed by many successes in other organs, making transplantation a viable 'ultimate' option in the treatment of failing organs today.

War and plastic surgery

Most people justifiably view the atrocities of war as a pure negative. However, the destructive effects of war on the health of soldiers and civilians have served as a catalyst of medical innovation. The sense of responsibility governments have towards their soldiers has led researchers to find new ways to treat war injuries. In addition, inventions made with the purpose of destruction have later been used for curation: the deleterious effects of mustard gas on bone marrow have a direct link to the later development of chemotherapy through the work of Sidney Farber and his contemporaries in pediatric leukemia.[16]

For plastic surgery, World War 1 (WW1) served as a leap forward and laid the groundwork for plastic surgery as a separate surgical specialty,[17] though earlier conflicts also yielded notable surgical innovations. Even before the advent of modern weaponry, blood loss has been the main cause of death in war. In the siege of Turin

during the Italian War of 1536–1538 a French surgeon/barber called Ambroise Paré reintroduced the use of ligatures in response to the many deaths caused by massive blood loss. At that point this approach that was used by both Romans and Arabs had fallen out of favor and was supplanted by cauterization by means of boiling oil or hot iron rods. His implementation however stuck and is still in use for both civilian and military application.[18] The Napoleontic wars saw the first introduction of standardized triage through the pioneering work of Dominique Larrey, who worked as a battle surgeon for the French army. The battles from this war yielded wounded soldiers in numbers that overwhelmed the available medical teams. In response, Dr Larrey pioneered so called 'flying ambulances' and introduced a system where soldiers were not treated based on their rank, but divided into groups based on their care needs[19]. Injuries were deemed to be either treatable at the battlefield, requiring transportation to a medical center, or to be so severe that the soldier had to be considered beyond saving. This new approach was later widely adopted in battlefield medicine and is currently in use in emergency departments worldwide.[20]

In the American civil war, the surgeon Gordon Buck experimented with surgical reconstructive approaches in facial wounds and burn scars and was one of the first to publish papers with figures showing results before and after his procedures.[21–23] His lively descriptions of his cases and the outcomes inspired many surgeons that followed him. The trench warfare that defined many of the battlefields in WW1 led to many soldiers suffering from extensive facial injuries. Harold Gillies, a New Zealand born surgeon based in London, joined the Royal Army Medical Corps and saw the thousands of men suffering from gun inflicted facial wounds. His pioneering work in collaboration with French surgeon Hippolyte Morestin showed the feasibility of transplanting patients' own skin to cover facial defects. Gillies treated many such patients in London, establishing techniques that made plastic surgery a viable subspecialty. In the United States, similar developments spurred a movement establishing plastic surgery as a separate surgical specialty.[24] In 1921, a group of young American surgeons who pioneered reconstructive approaches in soldiers wounded on the WW1 battlefield established the first society of plastic surgeons.[25]

In continental Europe, plastic surgery saw a similar development kick started by the influx of young, deformed patients. Next to Morestin, surgeons such as Otto Lanz (meshed skin graft), Jaques Joseph (rhinoplasty), Vladimir Filatov (Filatov tubed pedicle), and Erich Lexer (rhytidectomy) were all intimately involved in the development of new techniques to alleviate the suffering of the wounded of WW1.[26] Dutchman Johannes Esser (1877-1946) studied with Morestin before WW1[27] and leaped at the opportunity to apply his skills in the war. His experiences at the Imperial and Reserve Hospital No. 2 in Brunn (presently Brno, Czech republic), which included performing over 700 facial plastic operations, helped him develop his landmark book[28] on plastic surgery. He described biological arterial flaps[29] and the bilobed nasal flap and spent his life operating on the most challenging plastic surgery cases around the world.

The widespread implementation of penicillin during WW2 meant that many soldiers survived extended injuries and returned home alive but maimed. The plastic surgeon Achibald McIndoe (coincidentally a cousin of Harold Gillies) pioneered different techniques treating aviators who suffered from extensive burn wounds because of burning fuels on downed aircraft. He focused not only on surgical reconstruction but also recognized the social rehabilitation needed to let these men resume satisfying lives after duty. He published on the walking-stalk skin graft and later was a founding member of the British Association of Plastic Surgeons. In Canada, similarly pioneering work in wounded airmen was performed by Dr. Ross Tilley.[30] As summarized by another pioneering plastic surgeon, John Staige Davis, both world wars led to significant innovations in wound debridement to prevent infection, the application of skin flaps and grafts, antibiotics, wound dressings and treatment of scar tissue.[31]

Innovations in vascular surgery were later described in the literature following the Korean war, with significant contributions coming from the army vascular surgeon Carl Hughes. Hughes showed that the then common practice of ligating damaged and heavily bleeding vessels often led to amputation, while direct vessel repair could save both life and limb, an insight that resulted in a major drop in limb amputation between the second world war and the Korean war. [32]

VCA: Combining plastic surgery and transplant surgery

Building on these innovations in plastic surgery and transplant surgery, the first reconstructive transplant of the modern era was reported in 1963. A hand transplant performed by Ecuadorian surgeons was surgically successful but was rejected within three weeks due to the inability of the drug combination of Hydrocortisone and Azathioprine to sufficiently suppress the recipient immune system.[33] Animal experiments from the 1980s testing these drugs and the newly discovered cyclosporine A in hand transplants found that the skin component was highly immunogenic and was not maintained long term with these approaches.[34–37] The use of Tacrolimus in combination with Mycophenolate Mofetil and Prednisone finally helped solve the puzzle of long term immunosuppression in skin containing grafts in the late 1990s. In short succession multiple teams performed successful hand transplants, most notably in Lyon, France[4] and Louisville, Kentucky, USA[38]. These first hand transplants were all found to have good clinical outcomes and enabled patients to resume many parts of their lives, including driving, preparing meals and supporting an independent life.[39]

The success of the first hand transplants was followed by the development of face transplant programs which, like hand transplants and kidney transplants before them, drew fierce criticism. VCA receives particular scrutiny due to the procedures not being considered life-saving, while the immunosuppressive drugs required for their maintenance do potentially limit patient's life expectancy through the development of kid-

ney failure, vascular disease, infections and malignancies.[40,41] Despite this criticism a French-Belgian team performed the first face transplant in 2005[3] in a woman who had lost her lips, nose and most of the tissue of her lower face. The transplant was a success, but partially due to the continued smoking of the patient, multiple flap revisions were needed in 2015 to replace rejected/necrotic parts of the graft.[42] The patient ultimately died in 2016 from complications of cancer.[43] Though this first case partially serves as a cautionary tale related to the risks of this procedure and highlights the importance of rigorous patient selection,[44] it was followed by many successful face transplants for different indications, making both hand and face transplants a clinical reality.

The Afghanistan & Iraq wars and reconstuctive transplantation

The most recent major conflicts involving western nations, namely the Gulf War (1990-1991) and the wars in Afghanistan (2001) Iraq (2003), led to new challenges for army medicine. Better gear allowed heavily injured soldiers to survive injuries that previously resulted in certain death, such as quadruple amputations. Such injuries also became more common, since improved body armor protected the torso and head, but left extremities relatively exposed. Changes in enemy tactics that relied on improvised explosive devices (IED) further increased the number of fighters returning with serious damage to extremities.[45] IED injuries often lead to more significant injuries than piercing rounds of ammunition, since blast injury also severely damages tissue that is further removed from any direct impact.[46] Though infectious complications of severe extremity trauma are still the most common complications to occur,[47] their mortality has been lowered immensely after WWII through administration of antibiotics and improvements in wound dressing and early debridement, with vacuum therapy adding to improvements in limb salvage.[48] An advanced global trauma care system set up by the allied forces also played a significant role in the increased survival rates amongst the wounded.[49] The combination of all these factors left western surgeons with a challenging population suffering from extended trauma to face, legs, arms and penis. In response, different countries, the United States in particular, invested heavily in research projects aimed at the optimization of reconstructive transplantation, fueling another surge of innovation in plastic surgery.

Penile loss, male identity and urogenital allotransplantation

With face and hand transplants established as reconstructive options, a report by the US Surgeon General of the Army's Dismounted Complex Blast Injury Task Force[50] in 2011 highlighted the need for reconstructive options for soldiers subjected to severe penile loss while on active duty. The incidence rose sharply over the years as insur-

gency fighters increased their use of IEDs and ground troops were used more often as the war in Afghanistan dragged on.[50–52] Other conflicts in the middle east similarly led to a significant number of penile injuries.[53] In South Africa meanwhile, many men suffer from penile loss due to complications of ritualistic circumcision, though exact numbers remain unknown through suspected underreporting.[54,55] In response, programs for penile transplantation were established in South Africa and the United States.

The loss of a penis is rare and not often considered. As such, patients refer to it as being a 'lonely injury'.[56] Reports of penile amputation are few and scarce literature is available on the subject. In cases where penile loss occurs it however is a devastating disruption of life, especially when suffered at an early age. Next to practical considerations such as an inability to void standing up, the absence of a penis has great implications for social life. For many men their sense of masculinity is connected to their penis. Even though it is almost always hidden from others, having a penis provides a certain level of pride and it can lead to severe psychological challenges if a penis does not function properly or is considered to be undersized.[57,58] The impact of penile loss might be even larger for younger patients who are still single and forming a sexual identity.[52] Finding a partner can be considerably more difficult without a penis, as conventional conception and sexual intercourse is impossible. The combination of these factors has meant that many men that lost their penis also feel like they lost their masculinity, greatly disturbing interactions with potential partners and reducing self-esteem. In light of this, penile transplantation has been developed and successfully performed in men that lost their penises due to surgical complications[59] and penis cancer.[60]

Challenges and recent delevopments in reconstructive transplantation

Though the first reported penile transplants were successful and showed promising results in terms of voiding function and erectile capability, challenges remain for penis transplantation and VCA in general.[59,61] The procedure is held to high ethical standards due to its position as a life-enhancing/normalizing procedure.[62] Risks related to treatment side effects and potential graft loss are less acceptable in VCA than in solid organ transplantation, which is often life-saving.

As successful transplants create an unnatural state by coercing the recipients' body into accepting tissue that it evolved to reject, it is understandable that many unwanted outcomes continue to challenge the field of transplantation. The main challenges specifically faced by the field of VCA are (1) immunosuppression side effects such as cancer, cardiovascular disease, opportunistic infections, kidney failure and neurotoxicity (2) sensitization of potential recipients resulting in accelerated rejection of transplants (3) poor graft motor function as a result of insufficient nerve regen-

eration (4) limitations in graft preservation time and associated limitations in donor matching and recipient pretreatment.

Immunosuppression side effects

Drugs have side effects. Immunosuppressive drugs required for successful transplantation are no exception. With these drugs being a necessity for patients' entire lives, their side effects can even be life-threatening. In the normal steady state of the body, the immune system is responsible for infection prevention and curbing of rogue cell proliferation. Meanwhile, it also influences blood clotting, gut health and nervous function.[63,64] With the immune system involved in so many processes, it is no surprise that suppressing immune function can have many unwanted effects. Immunosuppressive drugs currently in clinical use target different pathways in the cell lines that populate the immune system. With the establishment of specific tolerance to a transplants' foreign DNA still elusive, these drugs work through an overall weakening of the immunological response to all cells and tissues considered foreign or out of order. This weakening comes with heightened susceptibility to infections that normally do not pose a threat to healthy individuals (influenza, COVID-19, fungal infections, bacterial pneumonia)[65–67] and a higher likelihood of developing cancers,[68–70] particularly of the skin.[71] Next to the effects of a weakened immune system the drugs also have an unwanted influence on vascular tissue (leading to increased incidences of peripheral artery disease[72] and coronary artery disease)[73] and kidney function[74] (in particular with the widely used calcineurin inhibitor Tacrolimus). Lastly, years-long use of immunosuppressive drugs affects sensory function of peripheral nerves[75,76] (often through de development of diabetes)[77] and can give patients inhibited awareness of wounds on hands and feet, which can result in problematic chronic wounds.

The current gold standard in organ transplantation, a combination of Mofetil Mycophenolate, Prednisone and Tacrolimus is also the most widely used in reconstructive transplantation.[78] With the novelty of these transplants, few efforts have been made to use a less severe regimen in the clinical setting. The most extensively reported regimen has been the one used by the Pittsburgh/Johns Hopkins University group that employs an approach combining a bone marrow infusion with the use of Tacrolimus mono therapy.[79] Though feasible in animal models, tolerance induction (and the lessened need for immunosuppression associated with it) is yet to be successfully applied in a clinical setting. With few novel immunosuppressive agents having found their way into the clinical practice since the introduction of tacrolimus, an unmet need remains for treatments that allow for transplant tolerance without the use of immunosuppressive drugs that come with serious side effects.[79]

Recipient sensitization

Recipient sensitization occurs when patients have had previous exposure to foreign tissue. This can occur through blood transfusions, skin transplants, previous organ

transplants and even through pregnancy.[80] It is extensively researched in kidney transplants in particular and often confirmed through the existence of donor-specific antibodies in the recipient's blood.[81] Though sensitization is the product of the normally desirable development of immunological memory to foreign HLA, it is highly problematic in transplantation, as it can lead to accelerated rejection of a graft. In VCA, sensitization is a common problem, since many patients that are VCA candidates become sensitized through treatments of their initial injuries.[82] Clinically, rituximab, eculizumab, plasmapheresis, tocilizumab and intravenous immunoglobulins are used to prevent accelerated rejection in sensitized recipients, with often limited success.[83,84] As in solid organ transplants, elevated levels of donor specific antibodies are associated with early rejection in VCA.[85] The treatments that apply monoclonal antibodies or involve plasmapheresis currently rely on mitigation of the direct effects of donor-specific antibodies and the accelerated cellular response to foreign tissue. Few studies aim to address the donor-specific memory that plays a central role in the accelerated rejection caused by sensitization. Promising results of studies aimed at eliminating this memory in rat kidney transplants[86] leave room for desensitization research aimed at B-cell memory in VCA.

Nerve regeneration

Human nerves can grow at a pace of about 1mm/day.[87] In the clinical setting this means that a face transplant can recover sensibility and motor function within several months. Hand transplants can also regain adequate function when a transplant is performed close to the wrist.[5] With arm transplants proximal of the elbow, hand function can be limited as the motor plates on intrinsic hand musculature have already atrophied before new nerve ingrowth can reach them.[88] Currently, no treatments exist that can effectively speed up nerve regeneration, though the application of polyethylene glycol has shown promising results in preclinical studies in enabling fusion between donor and recipient nerves.[89] Current studies aim to improve on such strategies enabling nerve fusion that could potentially lead to accelerated growth of sufficient nervous tissue and improve survival rates of distal nervous endplates.

Graft preservation

In the current clinical practice, reconstructive transplants that include muscle can be maintained for several hours outside the body before they need to be connected to a recipient's blood supply. Though this ischemia tolerance is enough to allow for such transplants to occur, the ischemic time constraint means that a clinical transplant can only be performed at a limited distance from the location of a donor.[90] This limitation results in scarce availability of donor tissue. Longer preservation time would enable care providers to more carefully match donors and recipients and improve graft survival.[91] An extended preservation time would also open up opportunities to pre-treat

recipients with innovative immunomodulatory regimens to lessen the often severe initial immune response and rejection episodes in the first weeks of treatment, which currently are common in VCA.[78] Low-subzero ice-free preservation, high-subzero ice-free preservation, and normothermic[92] or high-subzero machine perfusion are currently studied with the aim to extend preservation time.[91] For solid organ transplants improvements in these approaches have been reported recently in rat livers (using normothermic machine perfusion),[93] human livers (using supercooling paired with machine perfusion),[94] and human kidneys (using normothermic machine perfusion).[95] For VCA, such techniques have recently been applied in porcine musculocutaneous flaps (using mid-thermic machine perfusion)[96] and porcine limbs (using mid-thermic machine perfusion).[97]

Aims of this thesis

VCA and penile transplantation are a clinical reality but significant barriers still exist to wide clinical application. Limited donor availability, limits to tolerated ischemic time, toxicity of immunosuppressive regimens, poor nerve function, and chronic graft rejection are a few of the many aspects that require improvement before these procedures can be considered a standard option for patients with major tissue loss. This thesis consists of clinical and preclinical studies aimed at addressing these barriers.

The aims of this thesis are:

1. To study the rejection patterns observed in hand, face and penile transplants and elicit the potential role of graft skin as a sentinel for rejection of the entire graft.
2. To understand treatment options for patients who developed immunological sensitivity to donor cells and test if an autologous bone marrow transplant can effectively remove such donor specific memory, allowing for successful reconstructive transplantation in these patients.
3. To test the feasibility of expanding permitted graft ischemic time through high subzero ice-free graft preservation. Can such an approach result in genitourinary graft survival after days-long storage?
4. To provide an ethical framework for the application of urogenital transplantation in a clinical setting and establish the feasibility of functional urogenital transplantation in a patient suffering from penile loss due to extensive blast trauma.

Outline of this thesis

Part 1: Objectifying clinical and histological graft rejection in VCA
The first part of this thesis is dedicated to the development of standardized systems for graft rejection in VCA. For this purpose, **Chapter 1** is a detailed description of the development of a rat penis transplant model that aims to provide a standardized vehicle for immunological studies. This model is then employed to develop a rejection classification for penis transplants in rats that aims to discern between the different tissue types in such grafts. Namely, **Chapter 2** describes an effort to make a distinction between rejection patterns in skin, corpora, vasculature, and urethral lining tissue. Using this data an assessment is made of the role graft skin can potentially play as a sentinel for graft rejection. A large set of histology samples and clinical photography of pig hind limb transplants is then used in an attempt to establish a clinical and histological rejection classification in swine VCA described in **Chapter 3.**

***Part 2: Improving transplant candidate access: desensitization strategies in* VCA**
The problem of recipient sensitization is well known in solid organ transplantation and is particularly prevalent in VCA. With trauma often being the cause of existing defects requiring a transplant, many patients are sensitized to foreign DNA through (temporary) skin grafts and donor blood. With current strategies using plasmapheresis and immunoglobulin therapy lacking in efficacy in highly sensitized patients, an effort to successfully mitigate the effects of sensitization with a treatment regimen that uses a syngeneic bone marrow transplant to desensitize recipients is described in **Chapter 4.**

Part 3: Expanding graft availability: high subzero ice-free graft storage
A major challenge in VCA is rapid graft deterioration in the absence of perfusion and oxygenation. This limited tolerance for ischemia warrants the rapid transportation of grafts and swift reperfusion in the recipient. For VCA it has been established that current techniques in clinical use only allow for mere hours of ischemic time. These time constraints make it impossible to transport tissues across large distances and do not allow for pre-treatment of recipients to optimize immunological outcomes. Extending tolerated ischemic time would enable such improvements in matching and immunological optimization. **Chapter 5** studies how engineered peptoids modeled after peptides found in arctic fish could be used to maintain rat penile grafts at high subzero temperatures before being transplanted.

Part 4: Ethical clinical application of penile transplantation
Though now proven to be feasible from a technical standpoint, penile transplantation is a procedure that has major implications and risks. Grafts may reject sooner than the benchmark set by solid organ transplants, patients may suffer from severe side effects of immunosuppression, prove to be unable to accept a graft psychologically

or a partner can reject the whole notion of a penile transplant. Keeping in mind that performing a penis transplant means that lifelong care for the graft and the patient is required **Chapter 6** describes a suggested ethical framework surrounding penile transplantation as established by a single center. **Chapter 7** reports on the operative and immunological approach used in the first human transplantation of a graft that includes the complete penis, scrotum and partial abdominal wall in a wounded US soldier.

Part 5: General discussion and future perspectives

Finally, **Chapter 8** consists of a discussion of the achieved aims of this thesis and the future perspectives concerning rejection, desensitization, and tissue preservation in urogenital allotransplantation.

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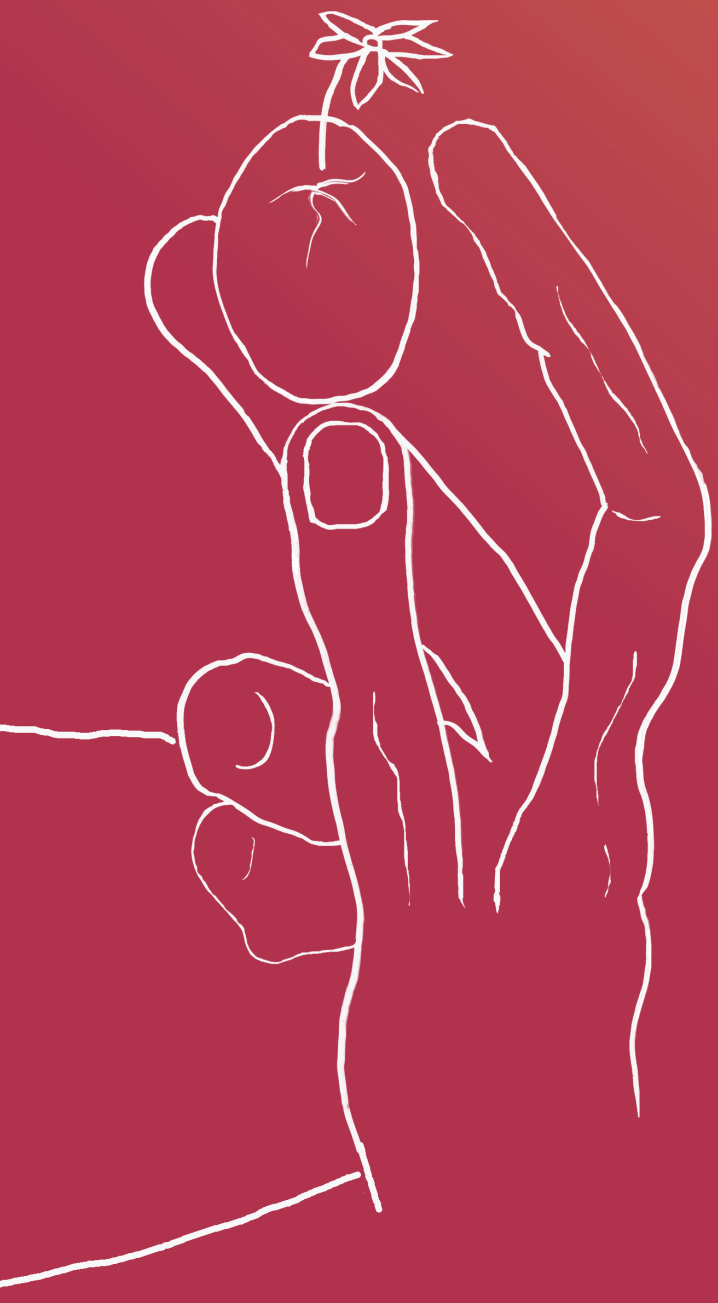
Part I

Objectifying clinical and histological graft rejection in VCA



Chapter 2

A Novel Rat Microsurgical Model to Study the Immunological Characteristics of Male Genital Tissue in the Context of Penile Transplantation

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Samuel A.J. Fidder, MD[1,2], Georg J. Furtmüller, MD[1], Andres Matoso, MD[3,4,5], Joanna W. Etra, MD[1], Kara Lombardo, MSc[5], Maria Chicco, MBBS[1], Byoung Chol Oh, DVM, PhD[1], Dalibor Vasilic, MD[2], WP Andrew Lee, MD[1], Richard J. Redett III, MD[1], Damon S. Cooney, MD, PhD[1], Gerald Brandacher, MD[1]

1. Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
2. Department of Plastic and Reconstructive Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands.
3. Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
4. Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
5. Department of Urology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Abbreviations: VCA Vascularized Composite Allotransplantation, **LEW** Lewis, **BN** Brown Norway, **NIH** National Institutes of Health, **DPV** Dorsal Penile Vein, **DPN** Dorsal Penile Nerve, **DPA** Dorsal Penile Artery, **IPA** Internal Pudendal Artery, **SEA** Superficial Epigastric Artery, **SEV** Superficial Epigastric Vein, SFA Superficial Femoral Artery

Abstract

Penis transplantation represents an exciting new avenue for restoration of male genitalia and function after devastating tissue loss. This animal model is designed to fill a critical void to study immunologic aspects related to reconstructive transplantation of male genitalia. A rat penile graft dissection was designed based on the internal pudendal arteries and dorsal penile vein and includes the skin of the prepuce. A non-suture cuff technique was used to anastomose the graft vessels to the recipient superficial epigastric and femoral vessels. 77 penile transplantations were performed. Graft design yields suitable caliber and length of vessels at the radix of the penis. Anastomosis of the dorsal penile vein and the internal pudendal arteries insures optimal graft perfusion. The non-suture cuff technique allows for successful microvascular anastomosis by a single surgeon with an average overall operative time of 2.5 hours. Long-term graft survival (>30 days) was observed in syngeneic transplants. We have established a robust murine model with ideal vascular perfusion of penile tissue to study the unique immunobiology of male genitourinary allotransplantation. Heterotopic inset further allows for visual monitoring of graft viability, while the native penis serves as an optimal control.

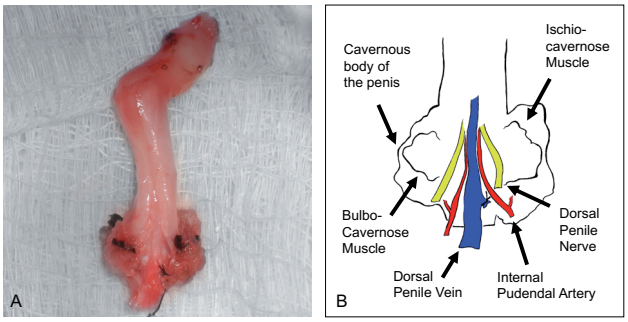

Figure 1: Graft design. The graft is designed to use the dorsal penile vein (DPV) and the bilateral internal pudendal arteries. Both dorsal penile nerves are included in the graft.

Introduction

The field of vascularized composite allotransplantation (VCA) saw its first breakthrough surgery in 1998[1] when a team led by J.M. Dubernard performed a successful hand transplant. Hand transplants were followed by facial transplants, the first of which was reported in 2005.[2] Uterine[3] and penile transplants were more recent additions to the expanding field of VCA. These are grafts that enable their recipients to conceive and bear children, breaking new ground for reconstructive and restorative allotransplantation.[4,5] Penile transplantation has become a clinical reality with successful efforts reported in South Africa and the USA in 2014,[6] 2016,[7] 2017, and 2018.

Due to the success of the procedure in the short term, penile transplantation has garnered support as a viable option for reconstruction of the penis, groin, and perineum in patients with extensive injuries that cannot be reconstructed with conventional operative techniques.[8] Early reports demonstrate that penile transplants can result in restoration of normal urinary and a satisfying level of sexual function.[6] Furthermore, they show that immunosuppression protocols used in other types of VCA sufficiently prevent rejection in the first year post-transplant.[6] However, many questions regarding the immunogenicity of penile grafts remain, as data discussing the immunological aspects specific to penile transplants remain limited.

To study the immunological characteristics of the unique components of penile transplants – namely, urethral lining tissue and the corporal bodies – animal models are crucial. Several rodent penile transplantation models have been developed, each with relevant limitations when applied for immunological research.[9-12] Building on the extensive experience of our group in rodent microsurgery and surgical model development,[13-19] we present a novel, highly reproducible technique for a heterotopic penile transplantation in rats with a >90% success rate using a non-suture cuff technique for revascularization (Figure 1). The method is specifically designed to accommodate immunological research pertaining to transplantation of the male genitalia and has been used as such.[20] A key component of the study design is the placement of the graft on the dorsal aspect of the thigh, facilitating simple visual monitoring of the graft.

Materials and Methods

male, 8- to10-week-old animals with fully grown genitalia were used for this model. For the study of transplant rejection, a fully mismatched strain combination (Brown Norway [BN] to Lewis [LEW]) was utilized.

Animals were housed in pathogen free facilities and were cared for in accordance to the Johns Hopkins University Animal Care and Use Committee (Protocol no. RA16M178), in compliance with the guidelines published by the National Institutes of Health (NIH Publication no. 86-23, revised 1985). All surgeries were performed using microsurgical instruments (S&T AG, Neuhausen, Switzerland).

Donor penis procurement

The donor is sedated with 4% isoflurane gas anesthesia and the animal is maintained on 2% inhaled isoflurane throughout the procedure. The surgical area in the anterior groin is shaved and the operative field is scrubbed once with 70% alcohol and once with 10% povidone-iodine. The rat is positioned in a stable, supine position to expose the operative field using a sterile field drape, sterile instruments, and a high magnification surgical microscope (40X). Toe pinch withdrawal reflex is tested to monitor adequate depth of anesthesia prior to starting the procedure.

A midline incision (~2cm) is made in the pubic skin cranial and caudal to the penis (Figure 2A). The hairless prepuce is separated from hair-bearing skin using scissors, cutting the skin proximal to the orifice of the bilateral preputial sebaceous glands (Figure 2B). Micro-forceps and micro-cautery are used to proximally dissect the penis down to the pubic bone, and the venous plexus that covers the base of the penis is exposed (Figure 2C). Cautery is used to divide bilaterally the venous pudendal plexus and the inferior external pudendal vein. A symphysiotomy is then performed using a Mayo scissor (Figure 2D).

Using micro-forceps, the dorsal penile vein (DPV), dorsal penile nerves (DPN) and the dorsal penile arteries (DPA) are dissected at the base of the penis (Figure 3A). Starting 5 mm distal from the pubic bone, the DPAs are dissected distally distally to the point where DPV and the DPAs disappear beneath the tunica albuginea and enter the corpus cavernosus. A 6-0 silk suture is used to manipulate both vessels and nerves while dissecting (Figure 3B).

The DPV is released from underlying tissue with proximal dissection using a 6-0 silk suture to manipulate the vein in a no-touch fashion. Beneath the vein's bifurcation into the pudendal plexus, the DPV is closely integrated in the cavernous body. Micro-scissors are used to dissect beneath the vein and one of the branches of the venous pudendal plexus is ligated with four 8-0 silk ligatures. The tissue between the ligatures is coagulated and then transected with scissors (Figure 3C).

The vein is then dissected proximally into the pelvis to obtain additional length (2 mm), and both DPAs are dissected proximally into the pelvis. Sparing the deep

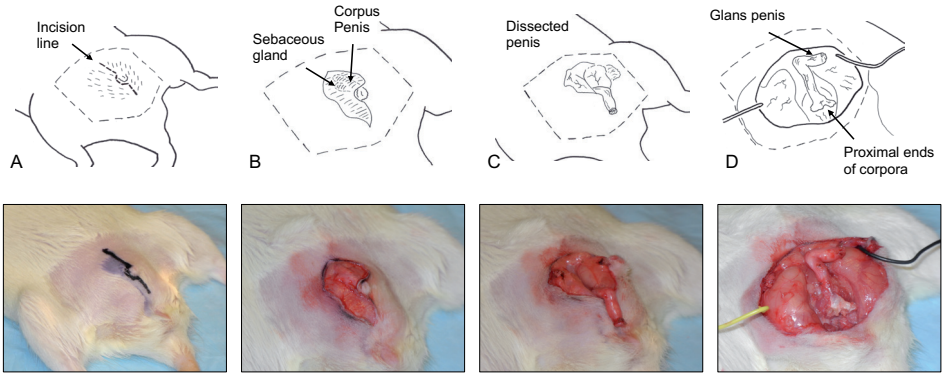

Figure 2: Surgical Approach. *Donor procedure:* (A) Ventral view of the incision in the midline across the prepuce. (B) Ventral view after superficial dissection. (C) View after dissection of the entire penis. (D) Graft appearance after ligation of the proximal ends of the corpora.

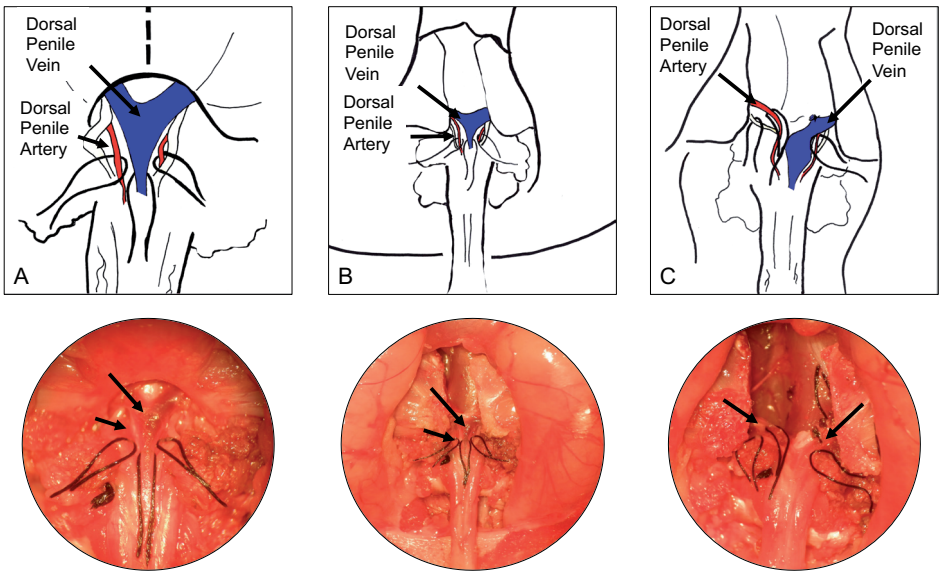

Figure 3: Crucial steps in vascular dissection. (A) Dissected dorsal penile vein, arteries and nerves. (B) Surgical appearance after cleaving of the symphysis pubis. (C) Surgical appearance after ligation of the right branch of the dorsal penile vein.

penile artery, all other branches, including the bilateral arteries of the urethral bulb (that tissue is not part of the graft), are coagulated and cut, finishing the dissection at the level of the internal pudendal artery (IPA). The penile nerves that accompany the DPA are also disssected.

Then, the cavernous bodies are dissected, ligated with a single 2-0 silk ligature at the bifurcation, and cut proximally. The ischiocavernosus and bulbocavernosus muscles are ligated bilaterally using 2-0 silk ligatures and transected with the micro-

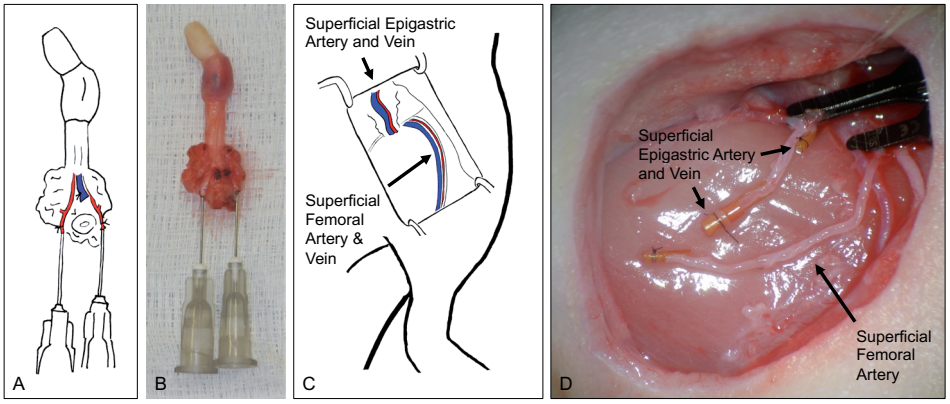

Figure 4: Graft and recipient vessel preparation. (A ,B) Explanted graft during perfusion. Blue: dorsal penile vein. Red: bilateral internal pudendal arteries and dorsal penile arteries. (C) Appearance of groin after skin incision. The superficial epigastric artery and vein and the superficial femoral artery and vein are visible. (D) Superficial epigastric artery and vein and superficial femoral artery after dissection and placement of cuffs.

cautery, carefully avoiding the vessels and nerves. The urethra is ligated proximal to the base of the penis with a 2-0 silk ligature and transected proximal to the ligature using the micro-cautery. Both the IPAs as well as the DPV are ligated with 8-0 silk ligatures and the vessels and nerves are transected with scissors (Figure 1).

The graft is then flushed through both IPAs with 5 mL of cold (4°C), heparinized (30 IU) saline and stored at 4°C wrapped in saline soaked gauze (Figure 4A and 4B).

Recipient Preparation

The recipient animal is sedated, prepared, and positioned per the donor procedure. The hair in the operative field in the groin region as well as the dorsal aspect of the entire leg is shaved.

Parallel and immediately superficial to the inguinal ligament, a 2 cm incision is made in the groin skin of the animal using scissors and the groin is dissected to expose the inferior external epigastric pedicle. The inferior superficial epigastric artery (SEA) and vein (SEV) are carefully dissected from their origin at the femoral pedicle to the bifurcation in the inguinal fat (Figure 4C). The superficial femoral artery (SFA) is dissected from the origin of the SEA and SEV to about 2 cm down the leg, and the distal 3 mm of each vessel is skeletonized. The SEA and SEV are then ligated at the level of their respective bifurcations in the distal fat using an 8-0 silk ligature, and the SFA is ligated at the distal end of the dissection (Figure 4D).

The SEA and SEV are clamped at their proximal origin with a single micro-clamp and the SFA proximally with a second clamp. All vessels are cut proximal to their ligatures. A 27-gauge polymide cuff is placed on both arteries and a 21-gauge cuff

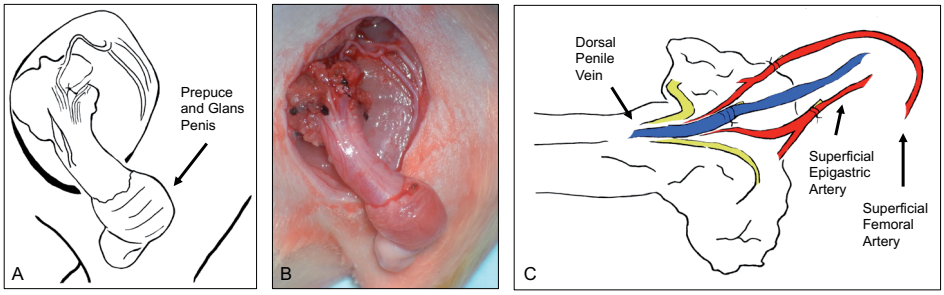

Figure 5: Vascular anastomosis and graft inset. (A, B) Overview of graft placement in the groin. Surgical field and graft after reperfusion. Note the complete return of color to the graft. (C) Graft vessels anastomosed to recipient vessels through non-suture cuff technique. Blue: Dorsal penile vein anastomosed to the superficial epigastric vein. Red: Top; superficial femoral artery anastomosed to the medial internal pudendal artery. Bottom; superficial epigastric artery anastomosed to the lateral internal pudendal artery. Yellow: penile nerves.

on the vein, after which the vessels are covered and protected with a moist gauze (Figure 4D).

Graft implantation

The recipient groin incision is spread with a retractor, a moist gauze is placed in the space between the recipient's tail and leg, and the graft is placed in the groin with the glans resting on the gauze. The SEA is then clamped with a single micro clamp, which is stabilized in a horizontal position using a mosquito forceps and a stabilizing base. The lateral IPA of the graft is then anastomosed to the recipient SEA using the cuff (Figure 5A and 5B). The recipient vein is similarly clamped and stabilized and anastomose the dorsal penile vein to the SEV using the cuff technique.[18] The medial IPA of the graft is then anastomosed to the recipient SFA with the cuff (Figure 5C).

All vessels are then unclamped at their origin. The entire graft should be perfused within 30 seconds. Note: Sufficient perfusion is confirmed by (1) oozing from the prepuce, (2) bright pink coloration of the glans and corpora, (3) venous return though the recipient vein. Once perfusion is confirmed and hemostasis is obtained, the base of the graft is sutured to the abdominal wall. The stumps of the ischiocavernosus and bulbocavernosus muscles, as well as stumps of the cavernosus bodies, can be used for this purpose.

The animal is then turned over on its side so that the dorsal aspect of the leg on the recipient's side is exposed. A 5mm-diameter skin defect is created on the caudal dorsal aspect of the thigh followed by a subcutaneous tunnel from the defect to the groin using forceps and curved scissors. The glans penis is gently guided out of the dorsal incision through the subcutaneous tunnel. The edges of the prepuce are sutured into the incision with 6-8 standing 6-0 nylon sutures (Figure 6). Hemostasis is confirmed, the subcutaneous (fat) layer is closed with a running 4-0 Polysorb suture, and the groin incision is closed with 6-8 standing 4-0 nylon sutures.

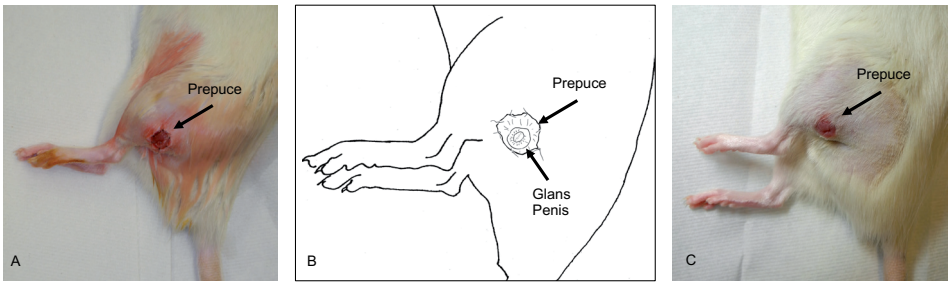

Figure 6: Graft placement on dorsal aspect of the thigh. (A) Syngeneic transplant, graft placement immediately post-surgery. (B) Schematic overview of graft placement. (C) Syngeneic transplant at POD7.

Postoperative care

Post-operative analgesia is provided with buprenorphine (0.2 mg/kg subq) every 12 h for the first 7 days. The animals are administered up to 5 mL of normal saline subcutaneous to compensate for perioperative fluid loss and placed in a preheated cage under a heating lamp or on a heating pad to completely recover from anesthesia. For antibiotic coverage, Enrofloxacin 10 mg/kg subq is administered daily for 10 days. The surgical site is monitored for infections and the weight of each recipient animal is obtained every day post-surgery. Weight loss greater than 15% percent must be considered an endpoint.

Results

Using this method, a total of 80 grafts were transplanted with a >90% surgical success rate for the underlying studies. Surgical failures resulted from postoperative bleeding (n=4) and graft thrombosis (n=3). Surgical site infections are fully prevented with antibiotics (Enrofloxacin). Total graft ischemia time is limited to 45-70 minutes. Clinical allograft rejection can be monitored easily due to the heterotopic inset location at the dorsal aspect of the thigh in both rejecting and non-rejecting grafts (Figure 6 and 7). After successful surgery, graft viability was monitored by daily visual inspection. All syngeneic grafts remained viable until their respective endpoints of POD 14 – POD 90 (Figure 7). Histological samples of all syngeneic grafts showed viable tissue and no signs of necrosis. (Figure 8)

This advanced rat penile transplant model was designed for the assessment of immunobiologic features of tissues specific to the male genitalia, such as urethra and corpora, in the setting of vascularized composite allotransplantation. The design enables transplantation of the complete penis on a pedicle that ensures optimal perfusion of both superficial and deep graft tissues through both penile arteries (Figure 1). The used technique results in successful donor and recipient procedures, including microvascular anastomosis by a single surgeon with an average operative time of 2.5 hours.

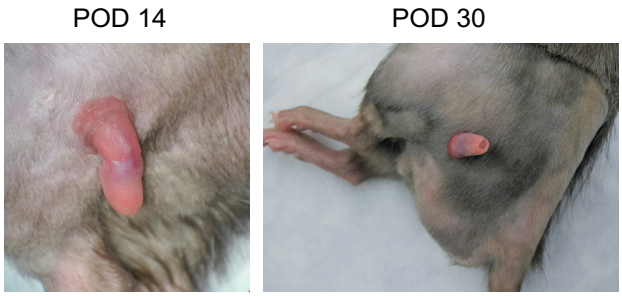

Figure 7: Clinical images of syngeneic grafts 14- and 30-days post-transplant.
Graft color is indicative of ample perfusion at all timepoints. No sign of rejection is visible at any timepoint.

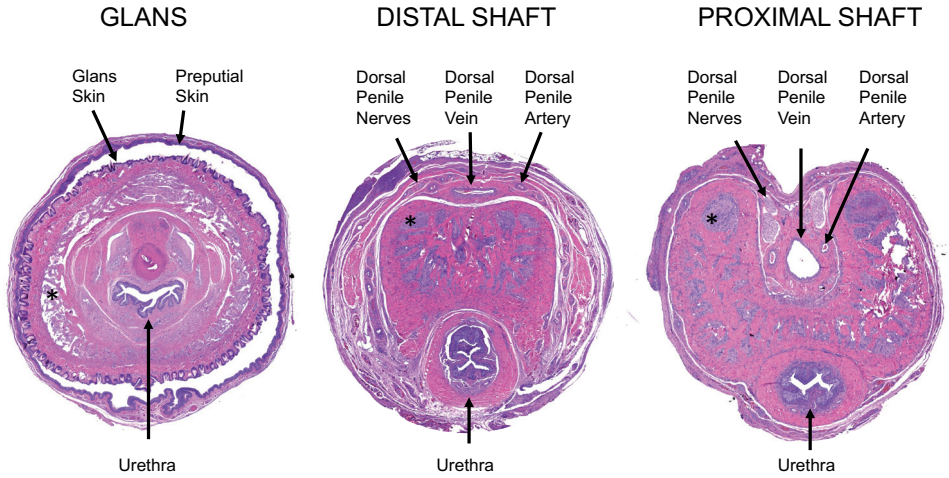

Figure 8: Histology images of a syngeneic penile graft at postoperative day 14. *Left.* Cross section of the penis at the level of the glans features double layer of squamous epithelium including glans (inner) and preputial skin (outer). The vascular channels of the glans are open (*). *Center.* Cross section of the penis at the level of the distal shaft allows visualization of the dorsal neurovascular bundle. Vascular channels of the corpora cavernosa show fibrous obliteration (*). *Right.* Cross section of the penis at the levels of the proximal shaft. Dorsal vessels and nerves are of larger diameter are visible and patent. As in the distal shaft, vascular spaces of the corpora cavernosa show fibrous obliteration of the lumen, associated with impaired corporal outflow that is associated with the model design (*).
All tissues appear fully viable on histology.

Discussion

For the reconstruction of devastating injuries with extensive tissue loss, hand and face transplants have evolved as valid treatment options for cases not amenable to conventional reconstructive methods. More recently, penile transplantation has proven to be clinically viable in the short term with the use of conventional immunosuppressive protocols.[6,7]

The goal of penile tissue transplantation is trifold: to restore body image, regain voiding function, and enable sexual intercourse. All of these functions can only be regained when the patient's immunological response to the graft is successfully

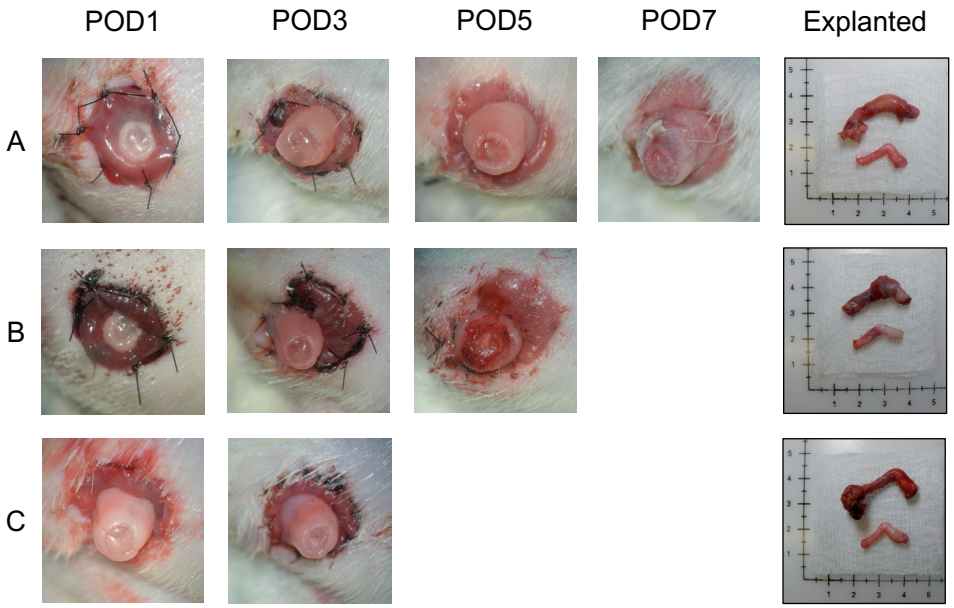

Figure 9: Allogeneic (BN into LEW) penis transplants POD1-7 using a doube artery model. Rows A-C. POD1: Normal graft appearance. POD3: Erythema and edema of glans and preputial skin. POD5: Increased edema and erythema. POD7: Erythema, edema, generalized epidermal sloughing. Explanted column: grafts are compared to native penises. Marked edema is visible after explantation at all time-points (A: POD7, B: POD5, C: POD3).

controlled. The primary, overarching goal in penile transplantation and (reconstructive) transplantation as a whole is thus a state of immune quiescence that allows for acceptance of the transplant with reasonable amounts of maintenance immunosuppression. Despite highly encouraging early results in four human recipients, little is known about the long-term outcomes of penile transplantation and the accompanying immunosuppressive treatments. Currently, we are unaware of any animal studies that address penile transplant outcomes or the effectiveness of immunosuppressive treatments in the setting of penile transplantation. To enable researchers that aim to expand the limited knowledge, our group sought to design a male genital transplant model. Considering that rodent studies are currently the main in vivo model for transplant immunology research and that rat models supply fully mismatched rat strains and combine relative affordability with sufficient penile vessel size, our group used the rat for this penile transplant model. An earlier, single-artery model developed by our group has been used in a previously published study on rat penile rejection in fully mismatched strain combinations.[20] In this study, 25 allogeneic and 6 syngeneic transplants were clinically and histologically monitored at post-operative days (POD) 3-30. Allogeneic grafts were found to reject in a 4-stage clinical progression. Epidermolysis clinically started at POD7, and full rejection and necrosis was found to occur between POD14-16. Histological analysis showed that skin and urethral lining tissue were first

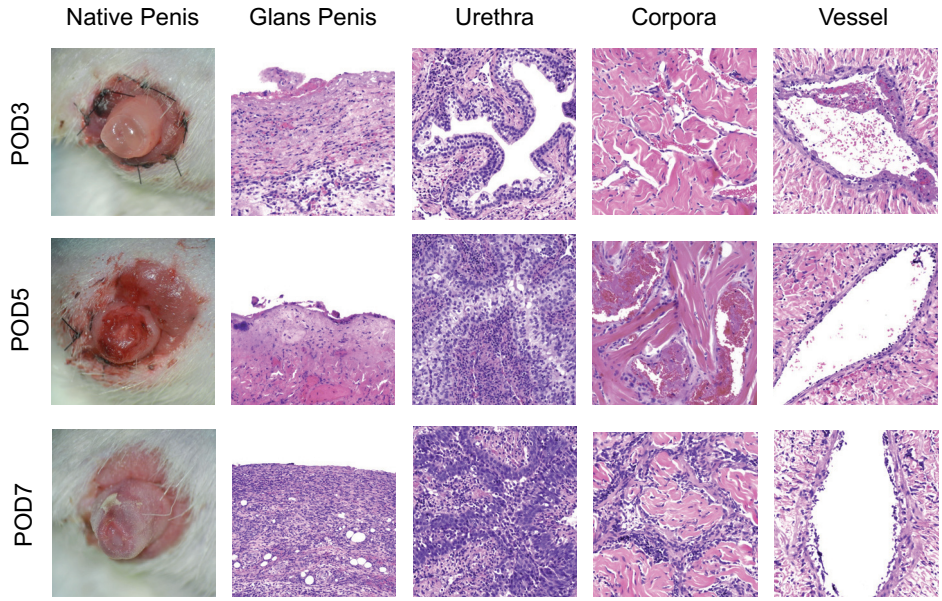

Figure 10: Clinical and histological images of allogeneic rat penile transplants. POD3: Grade II rejection; histological sloughing of epidermis, mild-moderate inflammation of the skin. POD5: Grade III rejection; dense inflammation of the skin, moderate urethritis, minimal inflammation of corpora. POD7: Grade III rejection; dense severe inflammation of the skin, severe urethritis, increased inflammation of corpora.

rejection targets followed by tunica albuginea and corpora cavernosa in a distal to proximal pattern.

Subsequent experiments that involveld greatly extended ischemic times demonstrated a high rate of vascular complications using the single artery inflow model. These complications necessitated improvements to the graft's blood supply and were resolved by the modification of the model to include a second arterial anastomosis, the technique that is demonstrated in this manuscript. To confirm that the addition of a second arterial anastomosis did not significantly alter the immunological properties of the model, three additional allogeneic transplants were performed and histologically analyzed at POD 3, 5, and 7. These transplants had a clinical (Figure 9) and histological (Figure 10) rejection pattern that was the same as the pattern found in the previously published study using the single-artery model.[20] As the rejection pattern was unaltered by the addition of vascular inflow, we conclude that this model provides improvements in risk of tissue ischemia but is similar to the previous model when applied in immunological research. As it involves a second anastomosis, the double-artery surgery is more elaborate than the single-artery approach, but only minimally adds to the surgical time (approximately 5-10 min for harvest and implant combined).

Although various small[16–19] and large[21] animal models have been described for penile replantation and transplantation, they are limited in their application for immu-

nological research of the vascularized penile graft. The first reported penile transplantation model, described by Koga et al., was a non-vascularized model, with the graft placed in a pouch created within the recipient's omentum.[10] Though the graft was reported to revascularize in the omentum, graft monitoring could only be achieved via laparotomy. The model proposed by our group connects the superficial epigastric artery and vein and the superficial femoral artery to the dorsal penile vein and pudendal arteries, using all the existing physiological graft vasculature, which closely resembles clinical graft design and perfusion. The model leads to adequate perfusion and perfect graft survival in a successful syngeneic transplant.

Karamürsel et al. designed an autologous transplantation model: they anastomosed the graft's right IPA and IPV to either the femoral or the saphenous artery and vein.[9] The graft was implanted on the ventral aspect of the thigh or rerouted to the pubic region. In this model there is a considerable size mismatch between recipient and donor vessels, which complicates surgical anastomosis. More importantly, in our experience, graft placement on accessible areas such as the ventral aspect of the thigh allows animals to auto-mutilate the transplanted tissue. Our model is a heterotopic model in the rat groin, which tunnels the glans penis to the dorsal aspect of the thigh, making the graft visible at the dorsal aspect of the hind limb (Figure 6 and 7). This vascularized design facilitates daily graft inspection in the conscious animal and obviates the need for repeated anesthesia while keeping the animal from damaging the graft.

Sonmez et al. described a heterotopic allogeneic penile transplantation model;[12] the authors anastomosed the graft's corpus spongiosum and dorsal penile vein to the saphenous artery and vein. The graft was placed in the pubic region, after rerouting of the recipient's native penis into the scrotum. While the size match between corpus spongiosum and saphenous artery may be more adequate, non-physiological arterial perfusion via the corpus spongiosum may lead to non-descript histological changes and may further alter the rejection process, thus limiting the value of results obtained. The model as described by our group uses physiological bilateral vascular perfusion, which is enabled by the use of the earlier described cuff technique (Figure 1 and 5). With the cuff technique, the 0.1-0.2 mm vessels of the penile pedicle can be anastomosed reliably with minimal to no blood loss.

Zhao et al. described an orthotopic penile transplantation model in the Beagle dog;[21] anastomoses of the deep dorsal vein, dorsal arteries and nerves, as well as the corpora cavernosa and urethra were performed. The recipients were catheterized. Eight grafts were lost early after surgery; in the twelve remaining recipients, urinary catheters were removed at POD 7 and the authors reported physiological urination with a linear stream. This model shows promise for translation, but is very resource intensive and could be limited by the large bone marrow-containing baculum in the dog penis. As a first platform for immunology research in penis transplantation, we believe the rat is the best model system.

Given our model's intended use for immunological research, our group deemed orthotopic placement too great a burden on the recipient animal. Orthotopic placement requires recipient penile amputation and carries a significantly increased risk for surgical failure. In addition to vascular anastomosis, orthotopic placement requires coaptation of erectile tissue and the urethra. These added surgical procedures create considerable risks of urinary retention and hematoma. This is illustrated by the experience of Seyam et al., who transected and replanted rat penises immediately distal to the bulb with anastomoses of the dorsal vein, dorsal nerves, and tunica albuginea.[11] They report that initial attempts to anastomose the urethra resulted in animal death from urinary retention and describe that anastomoses of the dorsal penile arteries could not be performed, resulting in compromised graft viability.

Limitations

Like every heterotopic transplant model, the one described in this article has certain limitations regarding its functionality: there is no voiding through the urethra, nor is there erectile function. Fibrosis of the erectile bodies and minor signs of inflammation of urethral tissue is observed in syngeneic controls, which can possibly be attributed to the heterotopic design. Finally, it is important to note that the bone marrow-containing baculum in the rat penis can possibly be a confounding factor in the rejection process. These findings need to be taken into account when interpreting the outcomes in studies using our model.

Conclusions

In summary, we developed a method for penile transplantation in rats using a non-suture cuff technique, which has proven to be a feasible model with a high success rate. Given its heterotopic placement, the model is best suited for immunological or tissue preservation research. This manuscript is intended to enable future research into the specific immunological aspects of penile transplantation.

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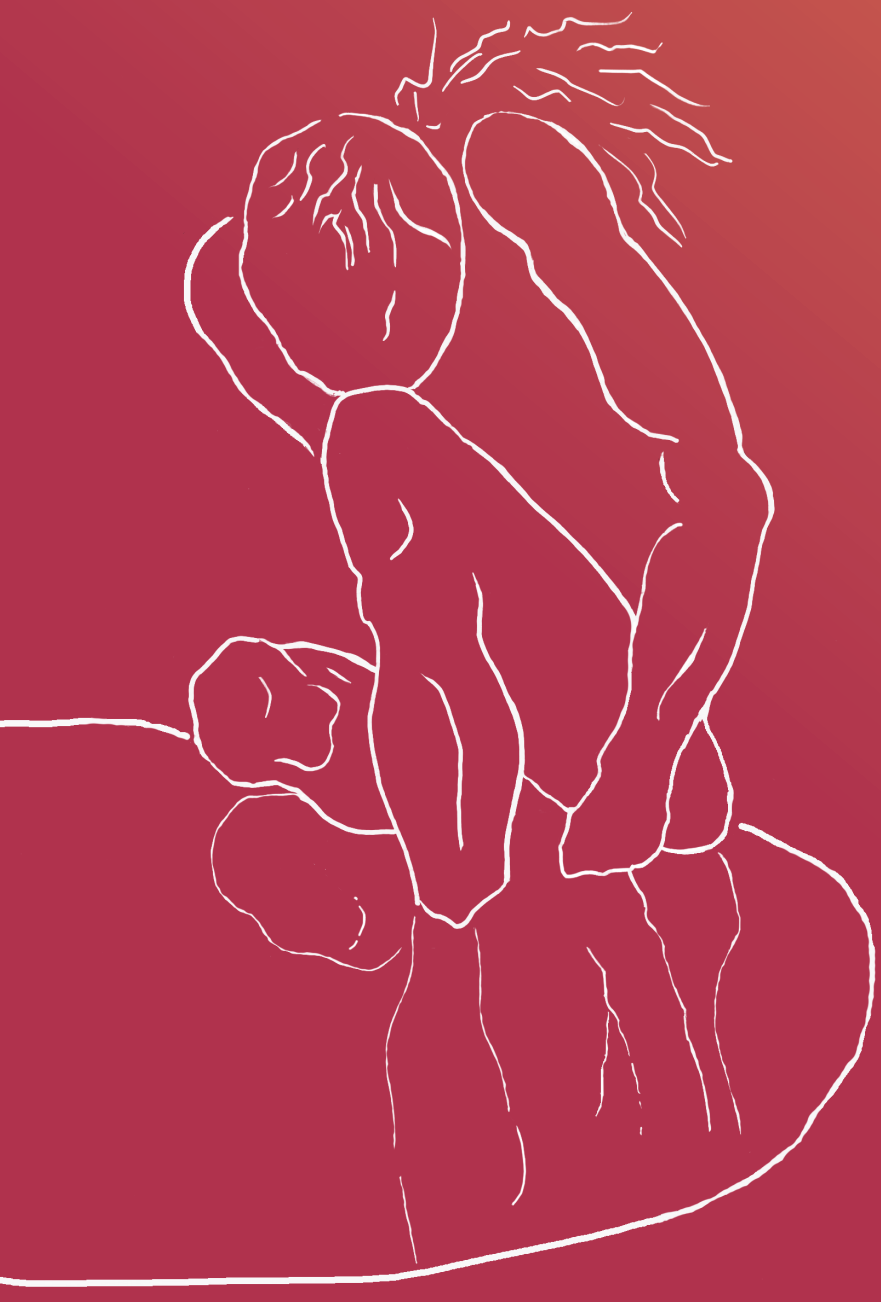

Chapter 3

Characterization of Clinical and Histological Rejection of Male Genital Tissues using a Novel Microsurgical Rat Penile Transplantation Model

Transplantation 2019

Samuel A.J. Fidder, MD[1,4*], Georg J. Furtmüller, MD[1*], Brian Simons, DVM, PhD[2], Byoung Chol Oh, DVM, PhD[1], Maria Chicco, MBBS,[1] Joanna W. Etra, MD[1], Cory Brayton, DVM, DACVP[3], Carisa M. Cooney, MPH[1], Dalibor Vasilic, MD[4], Barbara Kern, MD[1], Denver Lough, MD, PhD[1], WP Andrew Lee, MD[1], Richard J. Redett, MD[1], Gerald Brandacher, MD1° Damon S. Cooney, MD, PhD[1°]

1. Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA
2. Department of Urology, Johns Hopkins University School of Medicine, Baltimore, MD, USA
3. The Department of Molecular & Comparative Pathobiology; Johns Hopkins University School of Medicine, Baltimore, MD, USA
4. Department of Plastic and Reconstructive Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands

*These authors contributed equally to this work °Co-senior authors

Abbreviations: BN Brown Norway, **CD3** Cluster of Differentiation 3, **H&E** Hematoxylin and Eosin staining, **LEW** Lewis, **N** Number, **POD** Post-operative day, **VCA** Vascularized Composite Allotransplantation

Abstract

Background: Penis transplantation represents an exciting new avenue for restoration of male urogenitalia. However, little is known about the specific immunological features of penile transplants, limiting their application in complex urogenital reconstruction. To properly study this emerging form of transplantation, adequate preclinical models are a necessity. The purpose of this study is to establish a clinical and histological rejection classification of urogenital tissue transplants using a new rat heterotopic penile transplant model that includes preputial skin.

Methods: Syngeneic and allogeneic heterotopic penile transplantations were performed on Lewis and Brown Norway rats using a new model designed by our group. Grafts were clinically and histologically monitored at post-operative days (POD) 3-30.

Results: Six syngeneic and 25 allogeneic transplants were performed. All syngeneic and tacrolimus-treated grafts survived until endpoint. Allogeneic graft rejection is shown to follow a 4-stage clinical progression with all untreated allografts developing epidermal sloughing at POD7 and fully rejecting between POD14-16. Histological samples were used to develop a specific 4-grade rejection classification analogous to the 2007 Banff Criteria for skin-containing allografts.

Conclusions: Graft skin and urethral lining tissue are first rejection targets followed by tunica albuginea and corpora cavernosa in a distal to proximal pattern. We established a robust and reproducible murine model to study the immunobiology of male genital tissue in the context of transplantation and developed a novel 4-grade clinical and histological rejection scale based on graft skin and urethral lining as the main targets of rejection.

Introduction

Vascularized composite allotransplantation (VCA) started with the first successful hand transplant in 1998[1] and the first successful face transplant in 2005.[2] Since then, the field has expanded to include other forms of transplantation such as the uterus and penis. The first uterine transplant program, launched by a Swedish team, employed translational research in both small and large animal models, which ultimately resulted in successful clinical trials.[3,4] This group reported the first live birth from a transplanted uterus in 2015.[5]

Penile transplantation was first performed as an isolated attempt in 2006 when a team in China transplanted a middle-aged man who had suffered traumatic amputation of the penis. Despite encouraging early results, the graft was explanted on post-operative day (POD) 15 for reasons of psychological rejection.[6] Between 2014 and 2018, four further cases of penile transplantation were performed in South Africa (N=2) and the United States (N=2) with favorable short-term outcomes reported on the 2014 case from Johannesburg[7] and the 2016 case from Boston.[8,9]

Urogenital injuries have devastating physical and psychological consequences.[10,11] During Operations Iraqi Freedom and Enduring Freedom, improvements in body armor and battlefield medicine resulted in an increased number of veterans surviving blast-inflicted injuries affecting the groin. Suddenly, hundreds of wounded warriors with extensive defects, for which no fully restorative treatment existed, entered the US military hospitals and the VA.[12] In other parts of the world, complications resulting from ritual circumcision constitute a frequent cause of full or partial penile amputation.[13] Conventional reconstructive methods use autologous tissue and implants to form a neophallus, but these techniques have high complication rates such as urethral stricture, fistula formation, and implant extrusion.[14,15] Military victims of blast-related pelvic injuries are commonly found to have extensive combined urogenital, pelvic floor, and extremity injuries, posing a further challenge to conventional techniques as donor sites for reconstructive flaps are often unavailable. Though the ethics of penile transplantation need careful attention, especially in the very early stages of this novel clinical application of VCA strategies, the operation is becoming more and more accepted as an important reconstructive technique.[15,16]

Several experimental studies of penile transplantation have been performed in rats and dogs. With these models primarily demonstrating technical feasibility of the surgery,[17–20] limited experimental data are available regarding the immunological characteristics of urogenital tissue. In this study, we address this critical deficiency through the design of a robust and easily reproducible surgical model, which was then used to establish a clinical and histological rejection scale in penile transplantation based on the 2007 Banff Criteria.[21]

Materials and Methods

All research was approved by the Johns Hopkins Animal Care and Use Committee (#RA16M178); animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Surgical care: All surgeries were performed in the lab surgical suite using micro-surgical instruments (S&T AG, Neuhausen, Switzerland). Anesthesia was administered via inhaled 2% isoflurane; intra- and post-operative heat loss was prevented using heat lamps and heating pads (Braintree Scientific Inc., MA). Analgesia with 0.02 mg/kg buprenorphine was administered via subcutaneous injection one hour prior to surgery and 2 times per day postoperatively for 72 hours. After surgery, rats were checked upon twice a day for 5 days, then daily. Antibiotic prophylaxis was given with subcutaneous 0.10 mg/kg/day enrofloxacin. Animals were housed individually in rat laboratory housing facilities. Euthanasia was performed by cervical dislocation under isoflurane induced anesthesia.

Rat strains: Six- to eight-week-old male Brown Norway (BN) and Lewis (LEW) rats were acquired from Envigo Inc. Lewis rats served as recipients and BN rats as donors in the allogeneic penile transplantation groups. Syngeneic transplants were performed in a BN to BN combination.

Study design: Animals were assigned to a total of 11 experimental groups (Table 1). Syngeneic transplants, BN into BN, were performed to validate the surgical model for long term survival (Group 1). Untreated syngeneic and tacrolimus-treated allogeneic (0.5 mg/kg/day through intraperitoneal injection) transplant combinations with a POD14 endpoint served as negative controls (Groups 2&3). To obtain histopathological data for the male genital rejection classification, 21 transplants were performed with predetermined endpoints ranging between POD3–POD18 (Group 4).

Novel penile transplantation model

Donor procedure: (Figure 1A-D): A 2-cm skin incision is made over the ventral aspect of the penis (Figure 1A). The prepuce is released from the surrounding skin and the preputial sebaceous glands. Penile dissection is continued to the level of the pubic bone and symphysiotomy is performed (Figure 1B). The dorsal penile vein and dorsal penile arteries are released from the surrounding tissue. One branch of the dorsal penile vein is sacrificed, enabling dissection of a further 3mm of vein on the opposing side. Arteries are dissected to the level of the internal pudendal artery and then cut. Penile corporal bodies, as well as ischiocarvernosus and bulbocarvernosus muscles, are ligated and transected, releasing the graft (Figure 1C,D) (Figure 2). The transplant is then flushed with heparinized saline and stored on ice.

Recipient procedure (Figure 1E-H)*:* A 2cm incision is made in the recipient's left groin and the left superficial epigastric artery and vein are released from the surrounding tissue (Figure 1E,F). The left superficial epigastric artery and vein are connected to

Table 1: Experimental groups, stratified by day of graft harvest.

Group	Donor	Recipient	Transplant	Treatment	N	Harvest POD
Syngeneic control 1	BN	BN	Penis	None	4	>45
Syngeneic control 2	BN	BN	Penis	None	2	14
Allogeneic Tacrolimus	BN	Lewis	Penis	Tacrolimus	3	14
Allogeneic untreated 1	BN	Lewis	Penis	None	3	3
Allogeneic untreated 2	BN	Lewis	Penis	None	3	5
Allogeneic untreated 3	BN	Lewis	Penis	None	3	7
Allogeneic untreated 4	BN	Lewis	Penis	None	1	9
Allogeneic untreated 5	BN	Lewis	Penis	None	3	11
Allogeneic untreated 6	BN	Lewis	Penis	None	3	14
Allogeneic untreated 7	BN	Lewis	Penis	None	3	16
Allogeneic untreated 8	BN	Lewis	Penis	None	3	18

BN, Brown Norway; **POD**, Post-operative Day

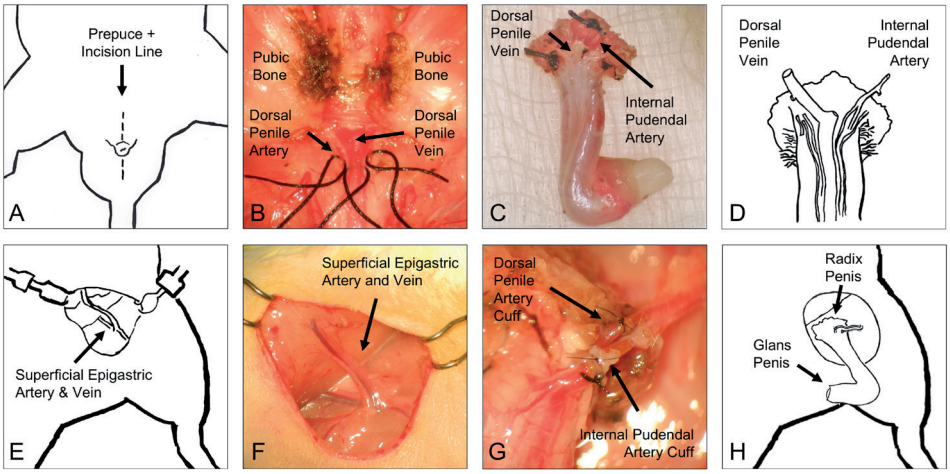

Figure 1 Key surgical steps. *Donor procedure:* (A) Ventral view of the midline incision across the prepuce. (B) Surgical view after opening of the rat pelvis. (C) Clinical view of penile graft, flushed with heparinized saline. Dorsal penile vein and internal pudendal artery are visible. (D) Schematic overview of graft radix penis. *Recipient procedure:* (E,F) Overview and surgical view of groin incision and Superficial Epigastric Artery and Vein. (G) Graft vessels anastomosed to recipient vessels through non-suture cuff technique. (H) Schematic overview of graft placement in the groin.

the right internal pudendal artery and the dorsal penile vein of the graft, respectively, using a non-suture cuff technique (Figure 1G,H; Figure 3A,B). Graft is inset at the posterior aspect of the thigh (Figure 3C).

Monitoring of graft rejection: Standardized clinical photos were taken at pre-determined post-operative time points (POD 3,5,7,9,11,13,14,15,16,18). Explanted grafts were photographed at study endpoints. Photographs were obtained in standardized lighting conditions and at a fixed aperture.

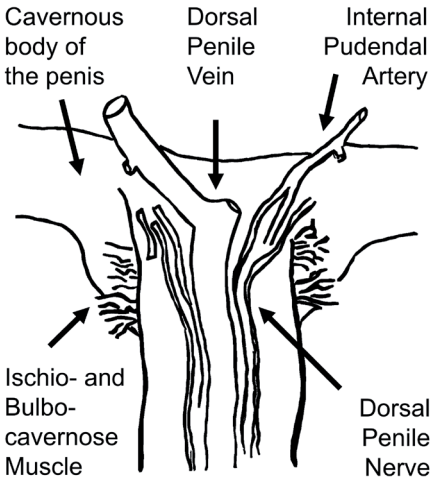

Figure 2: Graft design. Graft pedicle consists of the internal pudendal artery and the dorsal penile vein.

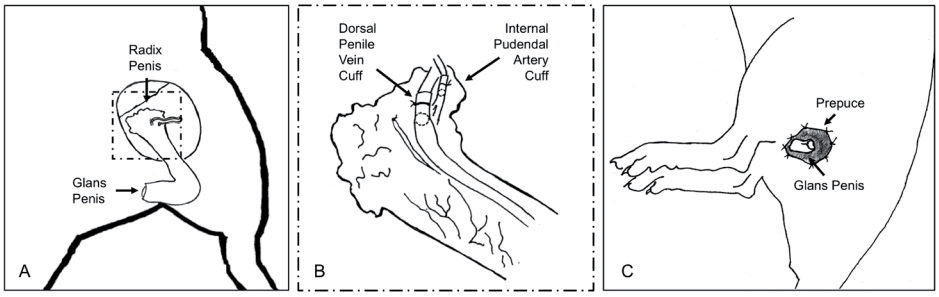

Figure 3: Graft inset. (A) Placement of penis in the groin. (B) Close up view of vascular anastomosis including cuff placement on internal pudendal artery and dorsal penile vein. (C) Placement of the graft on the dorsal side of the leg after tunneling of the graft beneath the skin.

Tissue procurement and processing: Animals were sacrificed at pre-determined post-operative timepoints and perfused with 10% buffered formalin; both transplanted and native penises were procured from each recipient. Shaft and glans specimens were sectioned at standardized distances from the glans edge, fixed with 10% buffered formalin, and embedded in paraffin.

Paraffin embedded tissue samples were sectioned and stained with hematoxylin and eosin (H&E) or processed for immunohistochemistry. Immunostains were performed using antibodies directed against CD3 (Abcam).[22] In brief, sections were deparaffinized, rehydrated, and heat retrieval was performed using Target Retrieval Solution (Dako). After incubation with primary antibodies, sections were incubated with ImmPRESS HRP secondary antibodies (Vector Laboratories) and developed with IMPACT DAB (Vector Laboratories).

Histological analysis: To determine the baseline histologic changes associated with surgery and transient cold ischemia, syngeneic (BN to BN) allografts and tacrolimus-treated allogeneic transplants were compared to the native penis at POD14.

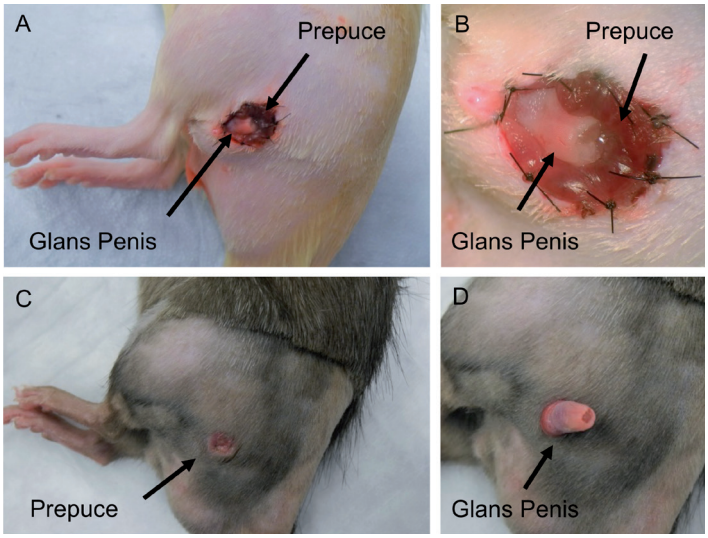

Figure 4: Transplant design in syngeneic and allogeneic recipients. (A) Allogeneic POD3 graft placement overview. (B) Allogeneic POD3 graft detail. (C) Syngeneic POD30 graft placement overview, graft hidden. (D) Syngeneic POD30 detail, graft exposed. POD, postoperative days.

Allogeneic transplants procured at the predetermined time points were analyzed by H&E and by immunohistochemistry for T cells (CD3). Pattern, severity, and timing of acute rejection was compared to typical pathology in other VCA rat models and to the 2007 Banff Criteria for human VCA rejection.[21,23,24]

Results

Viability of surgical model

The graft design developed for this study yielded optimal perfusion of the entire graft. A 91% surgical success rate was achieved with an average operative time of 2.5 hours for a single surgeon. Cold ischemic time was limited to 45-70 minutes. Clinical allograft rejection was monitored with ease as a result of the heterotopic inset location at the dorsal aspect of the recipient thigh (Figure 4). Survival and tissue viability of syngeneic penile grafts up to 90 days demonstrated feasibility and applicability of this surgical model as a research tool for VCA (Figure 5; Figure 6A).

Clinical Rejection

Syngeneic transplants: All syngeneic transplants showed no signs of rejection over the course of the study (Figure 5). All transplants reached the endpoint of 14 (N=2), 30 (N=2), or >45 days (N=2) with rejection Grade 0 (Figure 4C,D; Figure 6A).

Allogeneic transplants, tacrolimus-treated: Animals treated with tacrolimus (0.5 mg/kg/day; trough levels 3.3-3.7 ng/mL, N=3) did not show clinical rejection signs (Grade 0) up to and including the study endpoint of POD14 (Figure 6B).

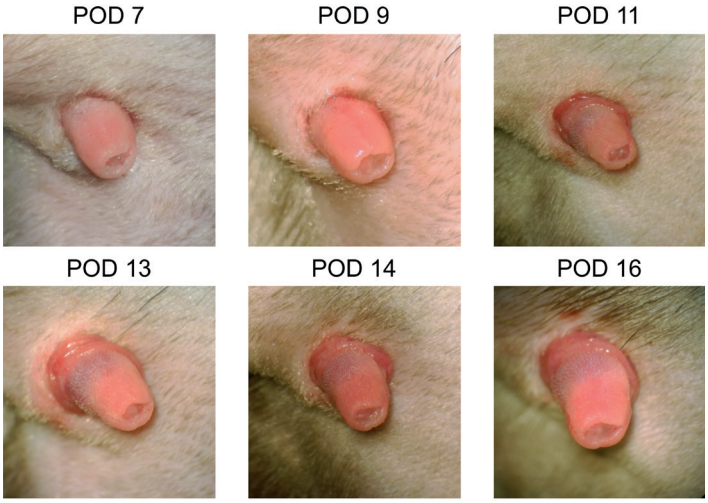

Figure 5: Clinical images of syngeneic graft POD7-16. Graft color is indicative of ample perfusion at all timepoints. No sign of rejection is visible at any time point. POD, postoperative days.

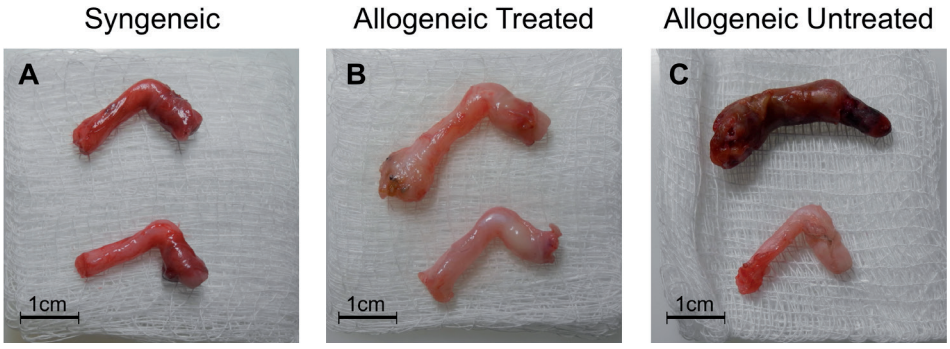

Figure 6: Transplants at POD14. Top grafts: (A) Syngeneic, (B) Untreated allogeneic and (C) Treated allogeneic. Bottom grafts: native penis controls. Syngeneic grafts and grafts treated with 0.5 mg/kg FK show no signs of rejection at POD14. POD, postoperative days.

Allogeneic transplants, untreated: All grafts followed a clinical rejection pattern analogous to rat hind limb transplantation (Figure 6C, Figure 7, clinical photos). Erythema and edema of the prepuce was present starting POD2. Edema of the corpora and glans penis was visible on POD3. Edema and erythema of glans and prepuce reached a plateau at POD7. On POD7, epidermal sloughing of prepuce and glans skin was observed. Starting POD9, scarring and skin loss of the distal end of the glans penis was visible with proximal progression. Black necrosis of the glans penis occurred at POD14-16 and followed a progressive distal to proximal pattern (Figure 7, Figure 8).

Histological analysis

Syngeneic Transplants: Histology of VCA tissues was viable and similar to native tissues in most areas. Grafts were consistently found to have fibrous connective tissue filling existing cavernous vascular spaces (Figure 9B). Mild urethritis was also present in all

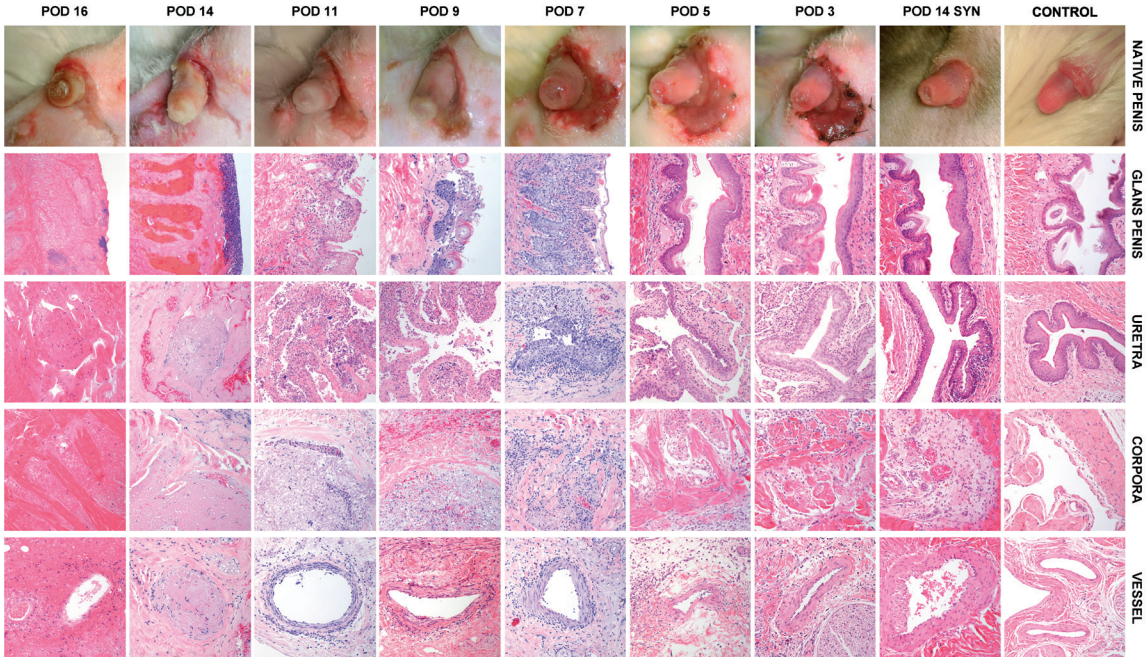

Figure 7: Clinical and histological rejection of untreated penile allograft. *Clinical images*. Native control: no signs of rejection. Syngeneic control, POD14: no signs of rejection. Allograft POD3: moderate edema of entire graft; erythema of prepuce. POD5: Increased erythema and edema of entire graft. POD7: Severe erythema and epidermal sloughing of prepuce and distal glans skin. POD9: Necrosis of prepuce and distal glans skin. Epidermal sloughing of proximal glans skin. POD11: Progression of necrosis proximally. POD14: fully necrotic skin on entire graft. POD16: black necrosis of distal glans. *Histological images*. Representative images of glans, urethra, corpora, and major vessel (dorsal artery). Native control, no signs of rejection. Syngeneic control, POD14: mild urethritis and fibrosis of corpora; no signs of rejection. POD3: mild dermal and submucosal infiltrate of glans and urethra (Grade I), minimal changes in corpora or vessels. POD5: Mononuclear cell infiltrate involving epidermis and urothelium (Grade II). Corporal inflammation is limited to tunica albuginea; minimal to mild vascular inflammation. POD7: full-thickness epidermal and urothelial inflammation (Grade III), with inflammation throughout the corpora and intimal arteritis. POD9: worsening inflammation; some epidermal and urethral necrosis and ulceration (Grade IV). POD11: epidermal and urothelial necrosis and loss, increasing corporal inflammation and transmural arteritis. POD14-16: large areas of necrosis, loss of epidermis and urothelium, and with vessel-occluding thrombi. POD, postoperative days.

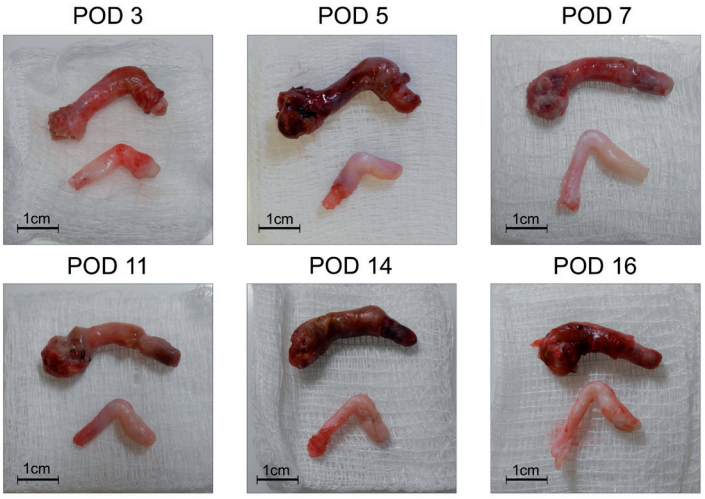

Figure 8: Allogeneic graft (top) vs. native penis (bottom) POD3 – 16. Procured grafts show progressive signs of rejection. Edema is present at all time points. POD, postoperative days.

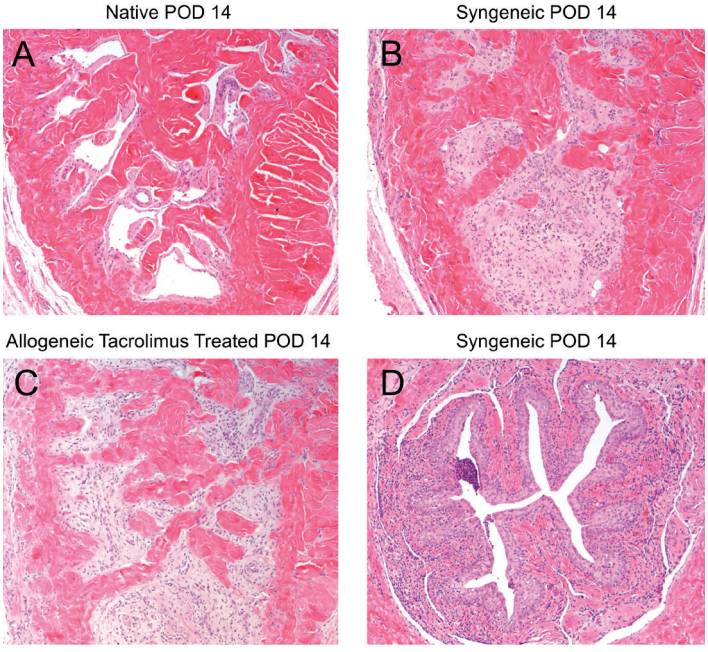

Figure 9: H&E stained sections of corpora cavernosa from POD14 allografts (B-D) and native penis (A). Compared to native penis (A), cavernous spaces in syngeneic (B) and tacrolimus treated allogeneic grafts (C) are filled with fibrous connective tissue. Mild urethritis (D) was present in most grafts, with infiltration of inflammatory cells and accumulation of neutrophils in urethral lumen. POD, postoperative days.

cases, with scattered infiltration of inflammatory cells into the mucosa and occasional neutrophil accumulation in the distal urethral lumen (Figure 9D). Tacrolimus-treated allografts were similar to syngeneic grafts, with fibrosis of corporal vascular spaces and mild urethritis (Figure 9C).

Allogeneic Transplants: Histologic analysis of untreated allogeneic grafts mirrored the clinical rejection pattern (Figure 7, histological images POD3-16). At POD3, mild to

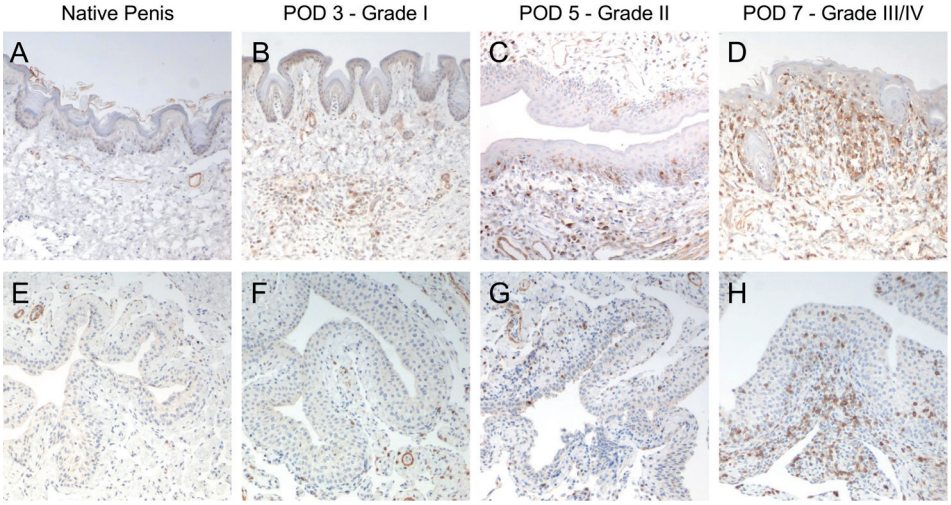

Figure 10: Immunohistochemistry of glans skin and urethra. Samples stained for CD3+ T-cell infiltrate. Native controls for skin (A) and urethra (E) show rare CD3+ T-cells. Skin samples of graft show progressive T cell infiltrates from dermis (B, Grade I), epidermis (C, Grade II) and full thickness infiltrates (D, Grade III) T-cell infiltrate. Urethra shows a similar progression (F,G,H).

moderate interstitial edema was present between the tunica albuginea and epithelium with a mixed inflammatory infiltrate (macrophages, neutrophils, lymphocytes). Mild, diffuse dermal and subcutaneous inflammation was present with perivascular cuffing but without epidermal involvement (Banff Grade I, mild). Urethral inflammation was similar to that of the skin and prepuce but also included neutrophils within the urothelium and lumen, akin to syngeneic and tacrolimus-treated grafts. Tunica albuginea and corpora were relatively spared with minimal inflammatory infiltration. Immunohistochemistry for T cells (CD3) confirmed perivascular and dermal infiltration without epidermal involvement (Figure 10B,F). At POD5, subcutaneous edema and interstitial inflammation were more prominent, and inflammation and T cell infiltration began to involve the epidermis (Banff Grade II, moderate). Inflammation at this timepoint involved the tunica albuginea but not the tissue deeper into the corpora. At POD7, full-thickness epidermal inflammation with apoptosis was present with focal areas of epidermal necrosis (Banff Grade III/IV). Urethral inflammation surpassed any inflammation seen in syngeneic grafts and was severe with areas of ulceration. Inflammation extended throughout the corpora at this timepoint. At POD9 and 11, inflammation progressively increased with widespread epidermal and urethral necrosis and ulceration (Banff Grade IV). Transmural arteritis was common. At POD14 and later, the graft was non-viable, with widespread areas of necrosis and thrombi within vessels.

Discussion

In this study, we describe the development of a novel surgical penile transplant model designed to study the unique immunologic features of male genital vascularized composite allografts. We further report on specific rejection patterns and associated classification systems of penile allografts on the rat. Our proposed histological classification system complements the 2007 Banff rejection classification of skin-containing allografts.[21]

Though several models of penile transplantation have been reported, specific limitations restrict their application in immunological studies of male genital tissues. Koga et al.[18] report a heterotopic model transplanted into the omentum without vascular anastomosis and perfusion. Karamürsel et al.[17] describe a heterotopic replantation model that places the graft on the ventral side of the recipient and does not report a rate for surgical success or automutilation. A heterotopic model by Sonmez et al.[19] anastomosed the graft's corpus spongiosum and dorsal penile vein to the saphenous artery and vein, constituting a non-physiological use of the corpus spongiosum as an arterial pedicle. Zhao et al.[20] reported an orthotopic penile transplantation model in Beagles. They report a 40% surgical failure rate with good functional outcomes in the successful surgeries. Interpretation of this study is hampered by the presence of a large bone-marrow containing baculum in the graft.

Our model poses several advantages compared to these previously reported methods, including reliable perfusion, high surgical success rate, limited morbidity, and enabling easy and standardized rejection monitoring. Limitations due to heterotopic placement, however, remain. The development of an orthotopic transplant model with a focus on anatomic reconstruction may better enable functional studies of micturition in a fully neurotized graft.

In our in-vivo study, we characterized the clinical and histological appearances of different tissues within the graft during various stages of rejection. We observed that clinical rejection of penile transplants is comparable to other VCA grafts, such as the hindlimb.[25] This may indicate comparable antigenicity of the penile and hind limb grafts, an observation that is further supported by the efficacy of 0.5 mg/kg/day tacrolimus in maintaining both graft types at POD14[26] (Figure 6). Graft skin appears to be the first target of rejection, followed by edema of the entire graft. Epidermal sloughing and graft necrosis appear to follow a distal to proximal pattern. To reflect this clinical progression, we propose a 4-stage rejection classification (Table 2, Column A) that focuses on erythema, edema, epidermal sloughing, and necrosis. Glans necrosis was chosen as end-stage rejection considering the paramount importance of the glans penis in a penile transplant.

Overall, histology follows a pattern of acute rejection similar to allografts from other organs. Rejection progressed consistently in all animals: the severity of pathology of grafts within each group was similar and increased at each time point. From

Table 2: (A) Clinical rejection classification for penile transplantation, and (B) proposed histologic rejection scale for rat penile allografts, (C) 2007 Banff Criteria for histologic skin rejection grading in VCA.

	A. Clinical Rejection Grading	**B. Histologic Rejection Grading**	**C. Banff Criteria for Skin Rejection in VCA[21]**
Grade 0	No signs of rejection, pink non-swollen graft	No or rare inflammatory infiltrates. Minimal to mild urethritis may be present.	No or rare inflammatory infiltrates
Grade I	Erythema of prepuce	Mild perivascular inflammation with no involvement of epidermis. Minimal to mild urethritis may be present. No to minimal infiltration of tunica albuginea or corpora cavernosa.	Mild. Mild perivascular infiltration. No involvement of the overlying epidermis.
Grade II	Erythema + edema of prepuce and glans	Moderate to severe perivascular inflammation with mild epidermal involvement and mild to moderate urethritis. Mild to moderate Infiltration of tunica albuginea without involvement of corpora cavernosa.	Moderate. Moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement (limited to spongiosis and exocytosis). No epidermal dyskeratosis or apoptosis.
Grade III	Erythema + edema + epidermal sloughing of glans and prepuce skin	Dense inflammation and epidermal involvement with apoptosis, dyskeratosis, or keratinolysis. Moderate to severe urethritis with focal urothelial ulceration. Moderate to severe inflammation of tunica albuginea and corpora cavernosa.	Severe. Dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis and/or keratinolysis.
Grade IV	Necrosis of glans penis	Severe inflammation with necrosis of epidermis, urothelium, tunica, and/or corpora.	Necrotizing acute rejection. Frank necrosis of epidermis or other skin structures.

these observations, we developed criteria for a histologic grading scale to allow quantitative comparison of allograft rejection in future studies (Table 2, Column B) by modifying the 2007 Banff Criteria[21] to reflect the pattern seen in our model and to include structures specific to penile allografts. The 2007 Banff Criteria for grading skin rejection in VCA describes Grade 0 as no or rare inflammatory infiltrates, Grade I with mild perivascular infiltration without epidermal involvement, Grade II with moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement but without epidermal dyskeratosis or apoptosis, Grade III as dense inflammation and epidermal involvement with epithelial apoptosis, dyskerato-

sis, and/or keratinolysis, and Grade IV as frank necrosis of the epidermis or other skin structures (Table 2C).[21] Using these criteria as a guideline, the rat penile grafts were evaluated for rejection.

Based on the degree of inflammation described in the Banff Criteria, a grading system was adapted to involve the specific features of histologic changes seen in this model. Notably, the presence of mucosa is not fully accounted for by the classic grading system, nor is the different distribution of inflammation. Grade I and II lesions remain similar to Banff Criteria for skin lesions and were expanded to include urethral mucosa and submucosa. Inflammation involving the tunica albuginea and corpora cavernosa was delayed compared to epidermal tissues, so inflammation of these structures is described in higher grades of rejection. In untreated allografts, Grade I lesions were present at POD3, and Grade II lesions were first present at POD5. Grade III lesions included more severe epidermal inflammation with evidence of epidermal damage – such as apoptosis or keratinolysis – and focal ulceration of urethral mucosa, first observed at POD7. Severe inflammation with necrosis of any structure is considered a Grade IV lesion; these lesions were prominent at POD9 and progressed until the graft was non-viable. Other auto-transplant or syngeneic allograft rat penis transplant models report either normal histology or moderate corporal fibrosis in the implanted penis at POD30 and POD90.[18,19,27]

In our study, two findings were noted during examination of syngeneic and tacrolimus-treated allografts that should not be considered a feature of immune rejection: by POD14, the vascular channels in the corpora were filled with fibrous connective tissue. Similarly, mild urethritis was present in syngeneic grafts, likely due to ascending bacterial colonization facilitated by lack of urine flow. Background inflammation of the urethra may complicate scoring of Grade I and II lesions, but inflammation was composed primarily of neutrophils with exudate in the urethral lumen and should be distinguished from the mononuclear inflammation associated with rejection. Inflammation of the skin of the glans and prepuce during rejection progressed in parallel with the remainder of the graft. This suggests that clinical examination or small skin biopsies could be sufficient to monitor the progress of rejection longitudinally without sacrificing the animal and examining the entire graft.

To our knowledge, this study represents the first histopathological analysis of allogeneic vascularized composite male genital tissue rejection in a rodent model. Several limitations, such as moderate sample size and possible differences in immunological behavior between rat and human penile tissue, remain. While anti-CD3 staining and H&E staining analysis does not address the entirety of humoral and cellular immune responses towards the penile allograft, the histological analyses performed in this study provide a solid reference for translational research in penile transplantation. Future studies may use this model and the rejection scale in rat penile allotransplantation specifically to elucidate the effects of different immunosuppressive regimens on penile grafts and the effectiveness of graft (cryo)preservation strategies.

In conclusion, we designed a robust and easily-reproducible heterotopic surgical model of penile allotransplantation in order to enable future research in a preclinical setting. Furthermore, we established a baseline clinical and histological rejection scale, which we hope will facilitate further research efforts in urogenital transplantation.

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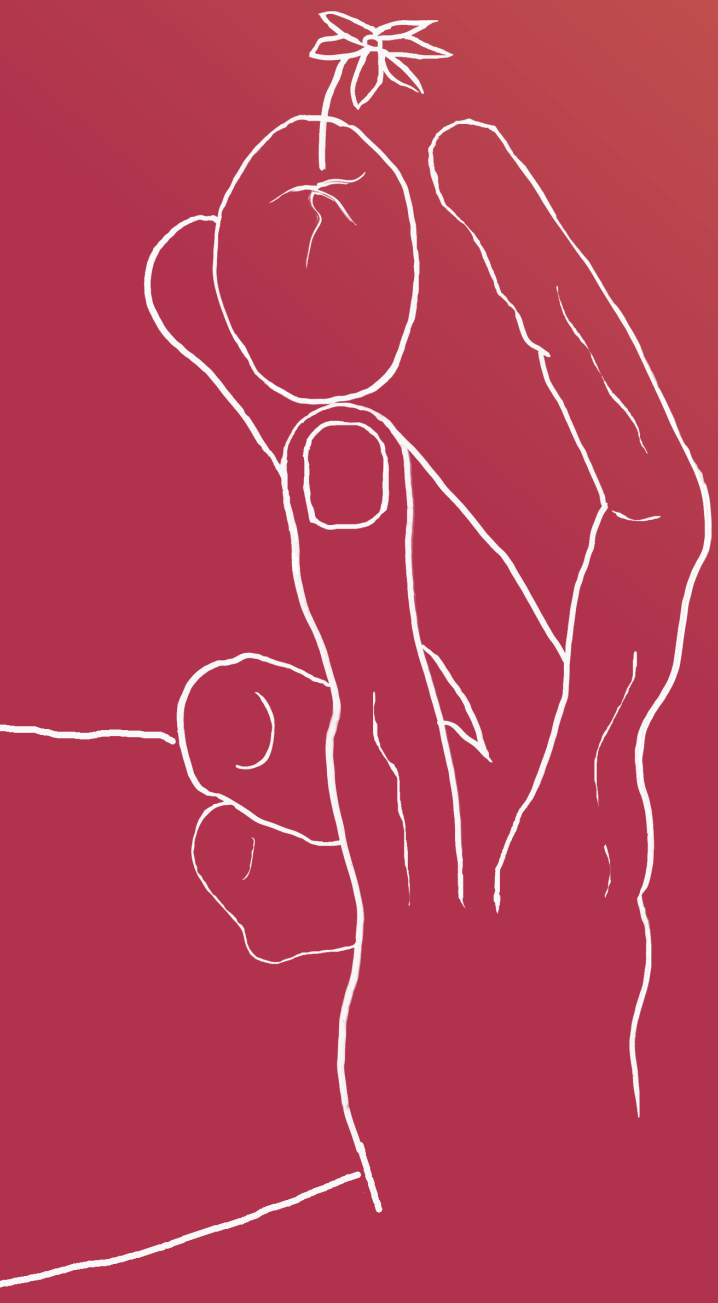

Chapter 4

A Skin Rejection Grading System for Vascularized Composite Allotransplantation in a Preclinical Large Animal Model

Transplantation 2019

Joanna W. Etra, MD[1,4*], Michael J. Grzelak, BS[2,4*], Samuel A. J. Fidder, MD[4], Keli Kolegraff, MD PhD[4], Steven Bonawitz, MD[5], Jaimie Shores, MD[4], Byoungchol Oh, DVM PhD[4], Damon S. Cooney, MD PhD[4], Sarah E. Beck, DVM, PhD, DACVP[3•], Gerald Brandacher, MD, FAST[4•]

1. Department of Surgery, Johns Hopkins University School of Medicine, Baltimore MD
2. Johns Hopkins University School of Medicine, Baltimore MD
3. Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore MD
4. Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory, Johns Hopkins University School of Medicine, Baltimore MD
5. Department of Surgery, Cooper Medical School of Rowan University, Camden NJ

*These authors contributed equally to this report. •These authors contributed equally to this report.

Abbreviations: VCA, vascularized composite allotransplantation; **H&E**, Hematoxylin & Eosin

Abstract

Background: The Banff Criteria have been accepted as a system for grading histological rejection in graft skin in human Vascularized Composite Allotransplantation (VCA). Preclinical swine hindlimb transplantation models have an important role in translational studies in VCA. However, unified grading criteria for rejection in swine skin have not yet been established.

Methods: 214 swine skin biopsy specimens were reviewed, including 88 native skin biopsies and 126 specimens from the skin component of heterotopic swine hindlimb transplants. Thorough review was performed in a blinded fashion by an expert veterinary pathologist with attention paid to the applicability of the Banff criteria as well as specific histologic characteristics and trends. Clinical and histopathologic rejection scores were then directly compared.

Results: 214 specimens reviewed showed significant similarities between swine and human skin, as previously published. Notable swine-specific characteristics, including pauci-cellular infiltration with rare epidermal cell infiltration or necrosis, were accounted for in a proposed grading system that parallels the Banff Criteria.

Conclusion: This comprehensive grading system, based on the Banff Classification for skin rejection in VCA, provides a standardized system for more accurate comparison of rejection in preclinical swine VCA models.

Table 1: The Banff 2007 working classification of skin-containing composite tissue allograft pathology7CTA can undergo immune-mediated rejection; therefore standardized criteria are required for characterizing and reporting severity and types of rejection. This article documents the conclusions of a symposium on CTA rejection held at the Ninth Banff Conference on Allograft Pathology in La-Coruna, Spain, on 26 June 2007, and proposes a working classification, the Banff CTA-07, for the categorization of CTA rejection. This classification was derived from a consensus discussion session attended by the first authors of three published classification systems, pathologists and researchers from international centers where clinical CTA has been performed. It was open to all attendees to the Banff conference. To the extent possible, the format followed the established National Institutes of Health (NIH).

Grade	Findings
Grade 0	No or rare inflammatory infiltrates
Grade 1	Mild. Mild perivascular infiltration. No involvement of the overlying epidermis.
Grade 2	Moderate. Moderate-to-severe perivascular inflammation with or without mild epidermal and/or adnexal involvement (limited to spongiosis and exocytosis). No epidermal dyskeratosis or apoptosis.
Grade 3	Severe. Dense inflammation and epidermal involvement with epithelial apoptosis, dyskeratosis and/or keratinolysis.
Grade 4	Necrotizing acute rejection. Frank necrosis of epidermis or other skin structures.

Introduction

Vascularized Composite Allotransplantation (VCA) is an increasingly utilized reconstructive procedure for patients with upper extremity amputation or devastating facial tissue defects. While the skin component has been considered an obstacle for widespread application of VCA due to its high antigenicity and thus requiring the use of high-dose multi-drug maintenance immunosuppression,[1–3] it also offers a unique opportunity for rejection monitoring as clinical visualization and biopsy collection are considerably more facile than in solid organ transplantation.[4] As of now, along with clinical assessment of the graft, biopsy and histologic evaluation of the skin component is the gold standard in monitoring for episodes of acute rejection[5–8]. Thus, the ability to grade rejection histologically is of great importance in VCA treatment, monitoring, and maintenance. For VCA patients, the Banff 2007 Working Classification was formalized to make uniform the pathologic grading of rejection in skin biopsies.[7] This system provides a structure and guideline to human skin pathologic diagnosis. Based on a grade of 0 to 4, these criteria outline the histopathologic findings through different stages of rejection, as summarized in Table 1.

As VCA is a relatively young field with few human patients and studies, preclinical and translational models are especially important in evaluating outcomes and improvements in treatment regimens as well as immunological monitoring.[9] It is well established that swine skin is comparable to human skin in clinical and histopathological settings.[10–18] Anatomically, both pig and human skin have similar thickness ratios of dermis to epidermis, density of hair follicles, pigmentation (breed dependent), and dermal connective tissue composition.[18] Pig skin, like human skin, is also tightly adher-

ent to the subcutaneous layer, in contrast to rodent skin.[18] Furthermore, pigs are easy to work with as they are easily trained to human contact, and their large size, which could be an obstacle in housing and care, can be mitigated through the use of minipig breeds rather than standard-sized breeds. Specifically, the swine hindlimb allotransplantation model is a well-described large animal model that can be used to adequately assess the immunologic aspects of VCA as comparable to human allografts.[19] Despite common use of swine for VCA research, there is a need for more detailed histopathologic characterization of the unique characteristics of VCA rejection in the skin of minipigs compared to humans.[20] Given the importance of an analogous model, it is vital that we accurately and reproducibly classify histologic findings in skin samples from swine VCA. Thus, we here present a modified grading system, based on the Banff Classification, for acute skin rejection in VCA in a preclinical swine model.

Methods

Study Cohort

All studies were performed with approval from the Johns Hopkins University Institutional Animal Care and Use Committee (IACUC). Hindlimb transplants were performed as previously described by our group[19] across full and partial SLA-mismatched Massachusetts General Hospital (MGH) minipigs from 2011 to 2018 under multiple different study protocols. 137 animals were evaluated for inclusion into the study, which is, to our knowledge, the largest cohort of VCA-model minipigs reported. Biopsies included into the review were those with episodes of rejection with concurrent biopsy and clinical photograph available. Control specimens evaluated were native skin samples and ischemic skin without rejection (spontaneous vascular thrombosis in the first postoperative week with subsequent ischemic graft failure). This ischemia can be differentiated from rejection clinically, as they have notably different natural history. Animals that we allow to reject immediately postoperatively (no treatment) follow a reproducible pattern of severe edema with erythema, significant graft warmth, purple discoloration of the graft increasing in hue starting postoperative day 4 or 5, and subsequent bullae formation with epidermal sloughing. The grafts lost due to ischemia all had immediate pallor and cool temperature (both on clinical exam and infrared thermography), light blue discoloration of the graft with moderate edema beginning around postoperative day 5, and subsequent blackening/necrosis of the graft to full eschar. Examination of graft vasculature for patency was performed at animal euthanasia. Biopsies were evaluated at different stages of clinical rejection, and the treatment and timing of biopsies were specific to the different studies into which the animals were enrolled.

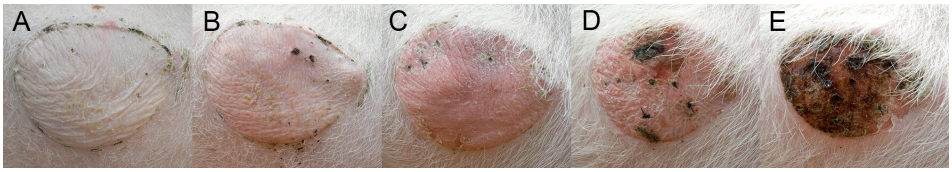

Figure 1: Examples of each clinical rejection grade in a swine hindlimb transplant performed in a full SLA-mismatch. Grade 0 (A) shows no difference between graft skin and native skin; Grade 1 (B) has mild erythema; Grade 2 (C) has moderate erythema with mild scaling and scabbing; Grade 3 (D) has severe erythema and scabbing with areas of epidermal sloughing; Grade 4 (E) has full graft epidermolysis and necrosis.

Sample preparation

Cutaneous biopsies were obtained from either the skin paddle of a heterotopic hindlimb transplant of a swine or native animal skin. Procedures were performed using a 5 mm punch biopsy. Specimens were immediately fixed in formalin for a minimum of 24 hours. The more recent samples were transitioned into ethanol after 24 hours, but the initial samples in the cohort remained in formalin until embedding. All of the specimens were embedded in paraffin and then stained using a standard Hematoxylin and Eosin (H&E) staining protocol. Immunohistochemical analysis of the samples was performed to identify global trends in infiltrating cellular phenotype. Specimens were evaluated for presence of T cells, B cells, regulatory T cells, and macrophages (CD3: Dako A0452; CD20: Biocare ACR3004B; FoxP3: eBioscience 14-5773-82; AntiS100A9: Thermo MA1-80446). Neutrophils and eosinophils were distinguished by morphologic appearance on H&E.

Clinical grading criteria

Clinical rejection scores of the VCA allografts were assigned based on the clinical features of the graft skin (Figure 1): Grade 0 shows no difference between graft skin and native skin; Grade 1 has mild erythema; Grade 2 has moderate erythema with the beginning of scaling and scabbing; Grade 3 has severe erythema and scabbing with areas of epidermolysis; Grade 4 has full-thickness graft epidermolysis with areas of necrosis.

Histopathologic grading criteria

Clinical rejection grading was given at timepoints corresponding to each biopsy based on review of prior clinical assessment and photodocumentation. All graft skin biopsies were reviewed retrospectively in a blinded fashion by a board-certified veterinary pathologist (S.E.B.) and assigned a rejection score (Table 4). This rejection score takes into account both the amount of dermal inflammation (Figure 2) and the presence of epidermal inflammatory infiltration and/or necrosis (Figure 3). The full grading system is described in detail in the Results section.

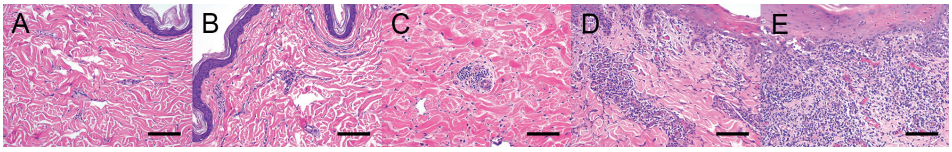

Figure 2: Examples of dermal inflammation scoring (a component of the proposed swine skin rejection scoring system). Inflammation scores are based on the following criteria: "none" (A) = no perivascular cuffs of lymphocytes; "minimal" (B) = <5 cuffs, <2 cells thick in any direction; "mild" (C) = <5 cuffs, <2 cells thick in any direction; "moderate (D) = 5-15 cuffs, any thickness; and "severe" (E) = no distinct cuffs with diffuse infiltration, any thickness. The number of cuffs is determined by the average of the number of inflammatory cuffs counted over three 20X fields in the dermis. All images are 200X with 100mm scale bars.

Statistical Analysis

Data were collected and maintained in a database created using Microsoft® Excel (v 16.16.2). All categorical variables were described as count (percent). Statistical analyses were performed using Stata/IC 15.1 (StataCorp LLC). A mid-p McNemar test was utilized for data analysis.

Results

Of the swine skin samples included in this study, a cohort of 214 samples were evaluated in a blinded fashion by a board-certified veterinary pathologist with extensive experience with swine histology. The cohort included samples of VCA graft skin over multiple timepoints and treatment regimens, ranging from post-transplant day 0 to post-transplant day 509. Within this group of tissue samples, 88 were native skin biopsies taken at the same time as the samples biopsied from graft skin. The cohort also included 6 samples from ischemic controls to account for differences in non-rejection inflammatory states (Table 2). The clinical rejection scores, based on presence of severe erythema, scaling/scabbing, epidermolysis, or necrosis (Figure 1), were assigned to the graft at the timepoint the biopsy was taken. Out of the graft biopsies with associated available photograph on the corresponding day (N=126), 37 were assigned clinical Grade 0 rejection, 54 assigned Grade 1, 16 Grade 2, 6 Grade 3, and 13 Grade 4 (Table 3).

The pathologist assessing each of these samples assigned in a blinded fashion a grade of histologic rejection to the sample based on the Banff grading system with attention to the degree of inflammation present. To score inflammation, the number of dermal lymphocytic perivascular cuffs was averaged over at least three 20X fields. Perivascular cuffs were defined as circumferential inflammatory cells immediately surrounding a blood vessel. If perivascular cuffs were present in the sample, the cuff thickness was estimated based on the number of lymphocytes from the blood vessel to the outer edge, for which the number was also averaged over at least three 20X fields. As perivascular cuffs are often not completely symmetrical in nature, the thickest portion of the cuff was used to define the degree of inflammation present (Table 5). Samples

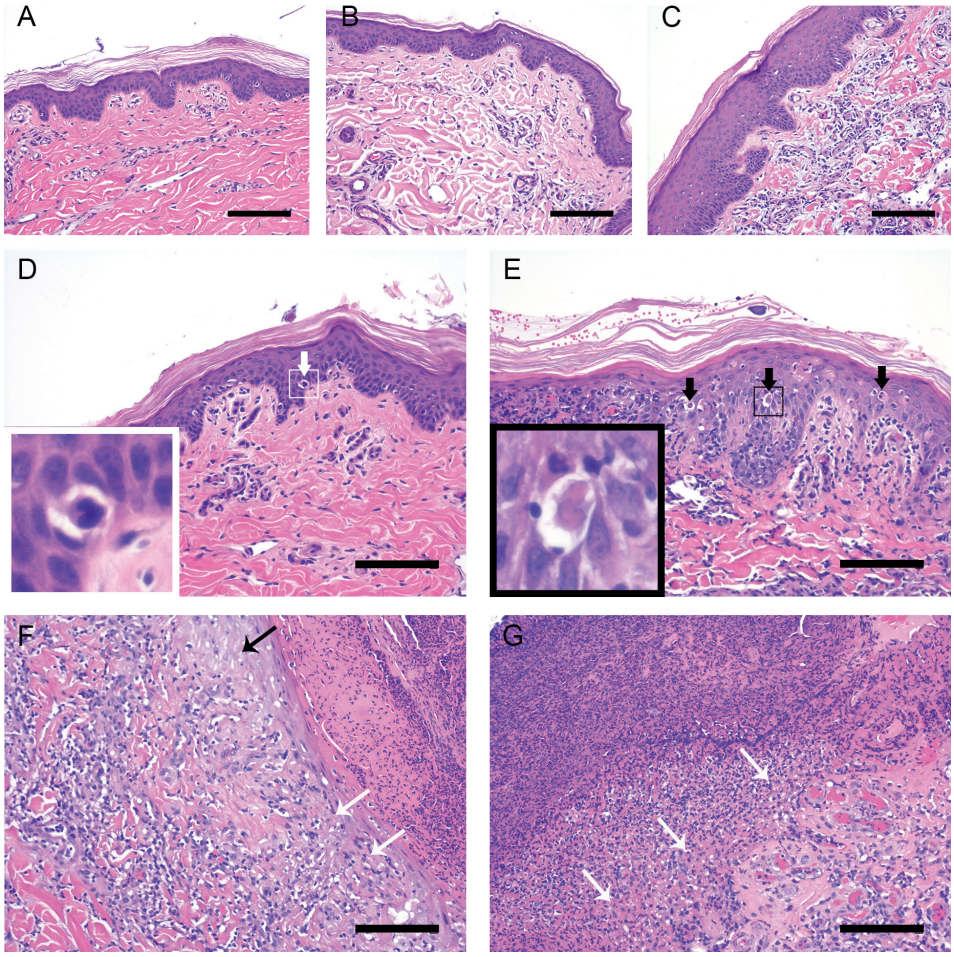

Figure 3: Examples of the proposed swine VCA skin rejection classification. Grade 0 rejection (A) and Grade 1 rejection (B) are characterized by none/minimal or mild inflammation (respectively) with no epidermal involvement. For the swine rejection classification, Grade 2 is split into 2A (C), characterized by dermal inflammation but no epidermal involvement, and 2B (D), characterized by variable inflammation with epidermal infiltrating inflammatory cells (white arrow, inset 600X). Grade 3 rejection is split into 3A (E), characterized by variable inflammation with single cell keratinocyte necrosis (black arrows, inset central arrow 600X), and 3B (F), characterized by multifocal or segmental full-thickness epidermal necrosis (white skinny arrows) with areas of intact epidermis (black skinny arrow). Grade 4 rejection (G), like in the original Banff classification, is characterized by diffuse full-thickness epidermal necrosis (white skinny arrows). All images are 200X with 100**m**m scale bars.

were then given an overall inflammation score based on the following criteria: "none" = no perivascular cuffs; "minimal" = less than 5 cuffs, no more than 2 cells thick; "mild" = <5 cuffs, 3 cells thick or more; "moderate" = 5-15 cuffs, any thickness; "severe" = greater than 15 cuffs, any thickness (Figure 2). Swine-specific histologic findings were correlated with the level of clinical rejection in a revised histological grading system.

Table 2: Specimens reviewed.

Specimen	Number
Ischemic Controls	6
Native Skin Biopsies	88
Graft Skin Biopsies	120
Total Samples Reviewed	214

Table 3: Characteristics of graft skin rejection assessments.

	Histologic Grade	Clinical Grade
Grade 0	15	37
Grade 1	59	54
Grade 2	28	16
Grade 3	14	6
Grade 4	10	13
Totals	126	126

Table 4: Swine VCA skin rejection classification (modified Banff criteria).

Grade	Dermal Inflammation	Epidermal involvement
0	None to minimal	None
1	Mild	None
2A	Moderate	None
2B	Mild to moderate (may be paucicellular)	Infiltrating inflammatory cells (may be few) without keratinocyte necrosis
3A	Moderate or severe	Multifocal single cell epidermal necrosis, variable infiltrating inflammatory cells
3B	Mild to severe (may be paucicellular)	Multifocal epidermal necrosis (may be full thickness, not diffuse), infiltrating inflammatory cells
4	Mild to severe (may be paucicellular)	Diffuse full thickness necrosis (entire epidermis is necrotic and/or sloughed off)

Table 5: Inflammation scoring rubric.

Grade	Defining criteria = # of perivascular cuffs of dermal lymphocytes +/- macrophages and neutrophils/eosinophils (average over at least three 20X fields)
None	No perivascular cuffs
Minimal	<5 cuffs, no more than 2 cells thick in any direction
Mild	<5, more than 2 cells thick in any direction
Moderate	5-15, any thickness
Severe	>15, any thickness

Of the graft skin sections evaluated (N=126), 15 were given Grade 0, 59 given Grade 1, 28 given Grade 2, 14 given Grade 3, and 10 given Grade 4 (Table 3). Along with inflammation, epidermal inflammatory cell infiltration and keratinocyte necrosis were recorded for each sample. With review of the samples, it was noted that not

all specimens fit into the grading system outlined by the Banff criteria. Specifically, there were samples with significant inflammation but without epidermal infiltrates, and conversely, there were samples without significant inflammation but that did have epidermal infiltration. These characteristics were considered and stratified into subcategories within Grade 2 and Grade 3 of the proposed criteria. After full analysis of all of the samples, swine-specific trends and particular cellular characteristics were compiled to construct a new grading system for the skin component in swine VCA (Table 4).

Rejection Grade 0 consists of normal dermal and epidermal skin without evidence of inflammation. In swine (as well as human) skin, there are always a small amount of perivascular lymphocytic infiltrates present in normal skin biopsies, which must be accounted for in giving rejection grades to allografts.[21] However, in Grade 0 rejection (Figure 3A), no epidermal changes are seen. Grade 1 (Figure 3B) also does not have epidermal changes; however, there is a mild perivascular lymphocytic infiltrate present, increased from the sparse lymphocytic infiltrate seen in normal porcine skin histology.

As previously mentioned, we have stratified Grade 2 rejection into two subcategories: Grade 2A (Figure 3C) and 2B (Figure 3D). This subdividing accounts for specimens that contain paucicellular perivascular inflammation but do have some epidermal infiltration without keratinocyte necrosis. Grade 2A is defined as moderate perivascular infiltrate based on cuff characteristics (Table 5) without epidermal involvement. The defining characteristic of Grade 2B rejection is the presence of epidermal inflammation; although there is often perivascular dermal lymphocytic inflammation, it can range from very few lymphocytes to moderate lymphocytic cuffing and accounts for up to but not necessarily moderate perivascular inflammatory cell presence with the aforementioned epidermal infiltration of inflammatory cells. Similarly, Grade 3 has been partitioned into 3A (Figure 3E) and 3B (Figure 3F). Rejection Grade 3A is characterized by moderate or severe inflammation with multifocal single cell epidermal necrosis. Grade 3B is characterized by variable dermal inflammation (up to severe) with multifocal, full-thickness, epidermal necrosis. Although both 3A and 3B feature epidermal necrosis, the key difference between the grades is that 3A has only single cell keratinocyte necrosis that does not affect the entire thickness of the epidermis (Figure 3E), while 3B has larger, multifocal areas of necrosis that involves the entire thickness of the epidermis, resulting in large areas of ulceration. However, in Grade 3B there are still areas of intact epidermis, while in Grade 4 the rejection is defined by diffuse, full-thickness, epidermal necrosis affecting the entire site.

For internal validation, all samples were also scored by a trained second independent, blinded party (M.G.), using the proposed porcine VCA skin rejection grades. A statistical analysis was performed to evaluate for discordance between the histological and clinical assessments of each sample. Given the subjectivity and lack of accepted standardization in grading of clinical rejection, association was evaluated in a dichotomous fashion using low-grade rejection, defined as Grades 0, 1, and 2, and high-

grade rejection, defined as Grade 3 or Grade 4. A McNemar test was used to evaluate relationship between the low- and high- grade histologic and clinical rejection scores for each sample. Because the paired nominal data had few discordant pairs, a mid-p McNemar test was used.[22] The analysis resulted in a p-value of 0.3, showing no evidence of discordance between the histologic and clinical grading systems.

Through the review of all of the specimens, graft capillary thrombosis was not appreciated. However, occasional occurrences of graft arteriopathy was noted, which were retrospectively found to be more frequent in those grafts that had been allowed multiple episodes of rejection (Supplemental Figure 1).

Discussion

Experimental studies using swine models have been a staple in the preclinical study of VCA, due in part to the similarities between swine and human skin as well as the ease in operating on and assessing progress in this particular large animal model.[9] The Banff 2007 Working Classification for Vascularized Composite Tissue Allografts provided the first unified criteria for the grading of skin rejection in VCA in humans.[7] This classification greatly improved our ability as a field to compare and learn from other patients in this relatively rare procedure as well as to provide an objective measure to follow individual graft progression, assisting in both graft monitoring and titration of immunosuppressive treatment. However, while these criteria were also considered to be fairly applicable to the experimental swine models, as the skin is largely similar, there has not been an in-depth analysis of grading criteria as they pertain to histologic findings in swine skin. Given the importance of an accurate comparative model, we created these swine-specific grading criteria for skin rejection.

By retrospectively studying rejection in a large number of VCA transplants in MGH minipigs, we have proposed new, more refined rejection criteria specific to the MGH minipig based on the original Banff criteria. Although we have highlighted many aspects of the striking similarity of pig skin anatomy and healing compared to that of human skin,[10–18] pigs are different than humans both in their behavior and in some aspects of their inflammatory response. Pigs are more likely to traumatize skin post transplantation, so small superficial pustules are not uncommon incidental findings. Anecdotally, pigs also have a more heavily eosinophilic component to their granulocytic inflammatory response compared to humans. However, the features of skin rejection, namely lymphocytic perivascular dermal inflammation and epidermal inflammation and necrosis, are strikingly similar.

The Banff 2007 Classification of skin rejection in VCA stratify the rejection grades by amount of inflammatory infiltrate present. Specifically, Grades 0-4 histologic rejection are defined in part by no or rare inflammation, mild inflammation, moderate inflammation, severe inflammation, and necrosis, respectively.[7] In our proposed

new grading criteria, we have subdivided rejection Grades 2 and 3 into 2A/2B and 3A/3B. Within Grade 2 rejection, we have distinguished between rejection characterized by moderate dermal inflammation without epidermal involvement and rejection characterized by variable dermal inflammation but inflammatory cell infiltration of the epidermis. This delineation is important, as in our experience, epidermal cellular involvement tends to correlate better with worse clinical rejection when compared to strictly dermal perivascular inflammation despite pauci-cellular inflammation that may not correlate with the moderately cellular inflammation assigned to Grade 2. For Grade 3, we have distinguished between epidermal necrosis that is single cell (3A) versus numerous (3B), accounting for the possibility of multifocal epidermal necrosis with different levels of inflammatory infiltration. Where the Banff 2007 Criteria defines Grade 3 histologic rejection by dense inflammation with epidermal involvement, we have noted in swine rejection that fairly severe epidermal necrosis may be associated with relatively few inflammatory cells in the dermis, and yet still quickly progress to Grade 4 rejection. This necessitated a subdivision of Grade 3 rejection that included more severe epidermal necrosis with or without large perivascular lymphocytic cuffs in the dermis. With this new definition of Grade 2 and 3 rejection in VCA skin in a swine model, we can accurately place histopathological grades by biopsy including the details that might otherwise have assigned other grades to these specimens.

Using our revised, swine-specific rejection criteria, we have drawn several conclusions from acutely rejecting animals both in general pattern and in specific details. Although granulocytes (neutrophils and eosinophils) as well as macrophages were present in rejecting skin samples, the vast majority of infiltrating inflammatory cells were lymphocytes. Of these, most were T cells, with fewer B cells (Supplemental Figure 2), consistent with previous findings.[5,23,24] While overall inflammation is a major component of our modified grading criteria, the most clinically relevant factor seems to be the extent of inflammatory infiltration into the epidermis. Similar to human VCA rejection, our group found that swine grafts could be rescued up to but not including Grade 4 histopathologic rejection, which is characterized by diffuse epidermal necrosis.[7] Notably, even those that had histologic Grade 3B rejection – with multifocal epidermal necrosis – were able to be rescued using standard immunosuppressive treatment (steroid bolus treatment and calcineurin inhibitor) due to the ability of the graft to re-epithelialize. We also found that dermal inflammation could be quite significant, but if the epidermis was not involved, the clinical appearance was much less severe with a relatively low clinical rejection score (Grade 2 or lower) (Figures 1, 3). We also did not include inflammation in the subcutis in the rejection scoring system, as subcuticular inflammation does not reflect the appearance or behavior of the graft; clinically important inflammation is restricted to the dermis and epidermis. Neutrophilic inflammation was significantly correlated with Grade 4 rejection (Figures 2, 3). However, neutrophilic dermatitis is not considered specific to the pathogenesis of rejection; rather, neutrophils are a generic response to tissue damage (in this case, epidermal necrosis).[25,26]

Through this extensive review of pathologic specimens, it became increasingly evident that the accurate assessment of rejection and grading relies not only on a good grading system, but also on the technical aspects of obtaining, preserving, and staining the biopsy as well. When evaluating graft rejection, it is important to interpret the histologic appearance in the context of the gross appearance. Ideally, multiple biopsies should be obtained from multiple sites. Significant differences in histologic appearance can occur within the same graft even millimeters apart. The clinical rejection of an experimental graft should not necessarily be predicted based on one punch biopsy taken from a focal area of epidermal necrosis, as re-epithelialization of the necrotic area may occur if the rest of the graft survives and the necrotic area is small. Specimen preparation is also of importance, as the maintenance of tissue architecture is relevant to enable slide staining and get high quality, consistent specimens to evaluate pathologically. Though we did have some excluded samples for which the biopsy and/or fixing or embedding provided slides with insufficient tissue to adequately assess, the rest of our samples were uniform enough that they could be adequately compared. However, in our experience, 24 hours of formalin fixation followed by placing the sample in ethanol before paraffin embedding provided the optimal preparation.

As mentioned previously, it can be difficult to ensure that these grafts remain without scratching or traumatic injury, as this can cause inflammation unrelated to rejection that can confound histologic appearance. The grafts are insensate, so preventing the animal from injuring the graft requires diligence and attention. For this purpose, our included animals were all maintained in single-animal runs after transplantation to avoid graft damage from another pig. The cohort was also housed in specialized runs with protective polyethylene paneling that provides smooth walls to the enclosure. The animals are seen at least once a day to assess graft condition. This prevents the majority of animal scratching of the graft in our studies and largely mitigates the concern for inflammation unrelated to rejection. Furthermore, we do not currently have complete knowledge on the effect of the experimental treatment regimens on the skin and histological outcomes. Most of the animals received tacrolimus therapy either for a set time period or in pulsed dosing, though a few had costimulation blockade or cellular therapy. None of the regimens correlated with any particular rejection grade, but as our study evaluated skin samples only in the context of whether or not they were rejecting and independent of the individual treatment regimens, we cannot exclude confounding of the different experimental treatments on the skin rejection grade.

The importance of this proposed grading system lies in its implications for future studies. As the histologic grading system shows correlation to the clinical grading system, it can be used in the setting of an acute rejection episode to delineate the severity of the episode, often not homogenous throughout the graft. However, with accurate grading of acute rejection episodes, we can also evaluate the relationship between

acute rejection clinical appearance, the grade of acute rejection, and the development of chronic rejection changes and other long-term outcomes. Graft arteriopathy was noted in several specimens, particularly in those that experienced multiple episodes of acute rejection (Supplemental Figure 1), possibly representing chronic changes. It has been shown that increased number of acute rejection episodes is associated with increased risk of chronic rejection;[27,28] while this has not been studied in depth in translational models, an accepted and reproducible grading system for the acute episodes will prove important in a thorough investigation into this topic.

In the current era of rapid medical and surgical advancements, adequate pre-clinical models are crucial to continued medical research and patient safety. Due to the limited patient population in the relatively young field of VCA, pre-clinical models are even more critical to our understanding of the relevant immunomodulatory processes and our discovery of less toxic and more effective treatment regimens. These new criteria here defined for histologic grading of skin rejection in swine – with the grading criteria paralleling those of the Banff Classification – provide a uniformity in histopathological assessment and contribute to the ability to analyze findings in swine preclinical models in the evaluation of VCA rejection.

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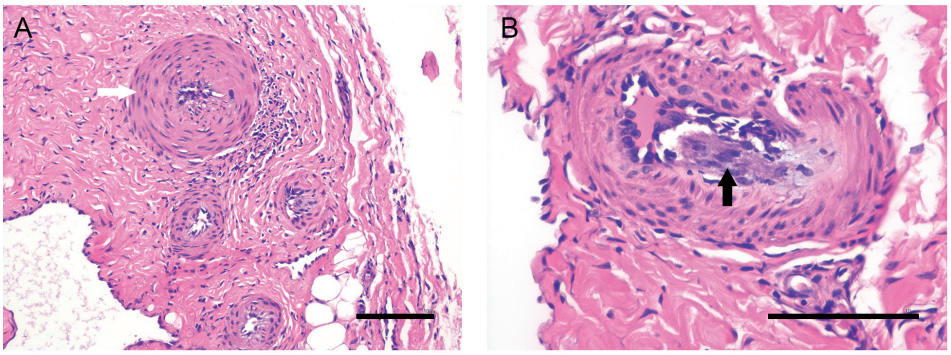
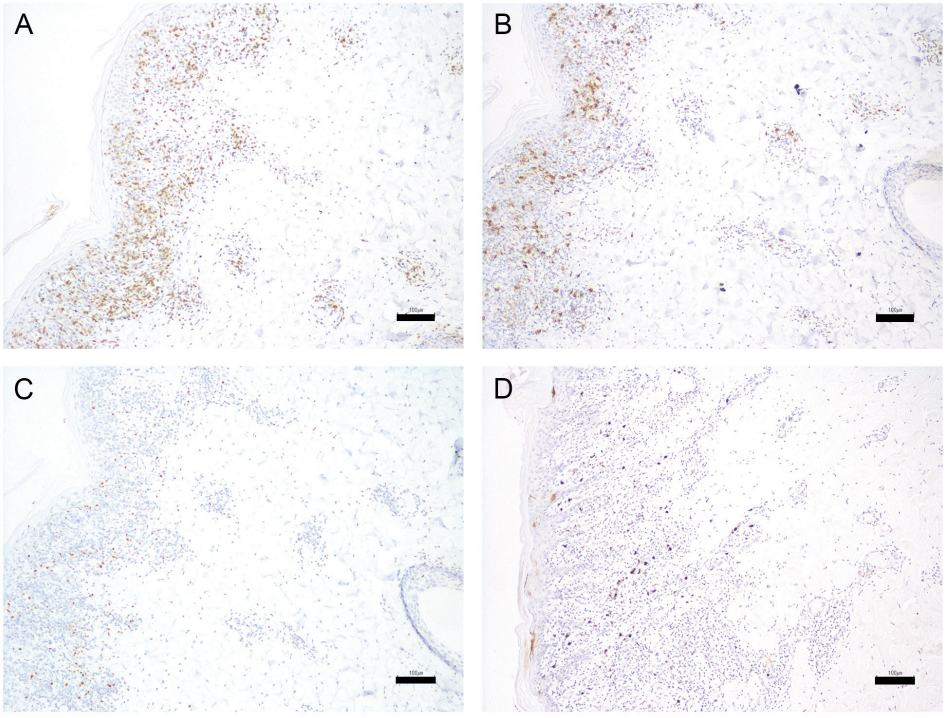
Supplemental figures



Supplemental Figure 1: Example of arteriopathy in VCA rejection. Arteriopathy consists of small and medium sized arteriolar medial hypertrophy and hyperplasia (A, white arrow; 200X) and intimal hyperplasia (B, black arrow; 400X). Scale bars are 100mm.



Supplemental Figure 2: Immunohistochemical staining for leukocyte cell phenotype in representative swine skin samples: A) T cells staining positive for CD3; B) B cells staining positive for CD20; C) Foxp3+ regulatory T cells; D) macrophages, staining positive for S100A9.

Part II

Improving transplant candidate access: desensitization strategies in VCA

Chapter 5

Desensitization and Prevention of Antibody-Mediated Rejection in Vascularized Composite Allotransplantation by Syngeneic Hematopoietic Stem Cell Transplantation

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Howard D. Wang, MD,[1] Samuel A.J. Fidder, MD,[1,2] Devin T. Miller, MD,[1] Georg J. Furtmüller, MD,[1] Ali R. Ahmadi, BSc,[3] Felix Nägele, BSc,[1] Joseph Lopez, MD, MBA,[1] Amy Quan, MD, MPH,[1] Joshua Budihardjo, BSc,[1] Denver M. Lough, MD, PhD,[1] Burcu Akpinarli, BSc,[1] Joanna W. Etra, MD,[1] Dalibor Vasilic, MD, PhD,[2] Giorgio Raimondi, PhD,[1] W.P. Andrew Lee, MD,[1] Robert A. Montgomery, MD, PhD,[4] Zhaoli Sun, MD, PhD,[3] and Gerald Brandacher, MD[1]

1. Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory, Johns Hopkins University School of Medicine, Baltimore, MD.
2. Department of Plastic and Reconstructive Surgery, Erasmus Medical Center, Rotterdam, The Netherlands.
3. Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD.
4. Department of Surgery, New York University (NYU) Langone Medical Center, NYU Langone Transplant Institute, New York, NY.

H.D.W. and S.A.J.F. contributed equally to this work.

Abstract

Background: Candidates for vascularized composite allotransplantation (VCA) are frequently sensitized, putting them at risk for antibody-mediated rejection. Current desensitization strategies are imperfect and require a living-donor setting. Here we investigated the impact of sensitization on and the efficacy of a desensitization protocol utilizing syngeneic hematopoietic stem cell transplantation (HSCT) to prevent antibody-mediated rejection in VCA.

Methods: Skin transplants from Dark Agouti to Lewis rats were performed for sensitization. Orthotopic hind limb transplants from Dark Agouti donors were performed to sensitized and nonsensitized recipients, and the animals were treated with either daily tacrolimus or no immunosuppression. A desensitization protocol consisting of total body irradiation, fludarabine, and syngeneic HSCT was applied to sensitized animals. Graft rejection was monitored by clinical assessment and histological analysis. Serum levels of donor-specific antibodies (DSA IgG) were measured using flow cytometry.

Results: Sensitized recipients exhibited accelerated rejection by 5.5 ± 1.2 days without immunosuppression and 10.2 ± 3.6 days with daily tacrolimus compared with 8.7 ± 1.2 days and longer than 30 days in nonsensitized recipients, respectively. Serum levels of DSA IgG were markedly elevated (37.3 ± 3.34-fold from baseline) in sensitized recipients after VCA and correlated with histologic evidence of rejection and C4d deposition. Desensitization significantly reduced DSA compared with sensitized controls (2.6 ± 0.5-fold vs 6.0 ± 1.2-fold, $P < 0.01$) and along with daily tacrolimus led to improved VCA survival longer than 30 days without evidence of C4d deposition ($n = 6$).

Conclusions: In summary, sensitization leads to accelerated rejection of VCA, and syngeneic HSCT combined with conventional immunosuppression effectively reduces DSA and improves allograft survival in sensitized rats**.**

Introduction

For patients with severe injuries from burn, trauma, or tumor resection, vascularized composite allotransplantation (VCA) offers a promising alternative to restore form and function when conventional reconstruction proves to be inadequate.[1] However, the initial clinical management of this patient population frequently requires multiple blood transfusions or skin allografts, leading to formation of donor-specific antibodies (DSA) and a high degree of sensitization.[2,3] In solid organ transplantation, sensitization is known to be a major risk factor for allograft rejection and long-term graft loss.[4,5] In fact, patients with DSA and positive crossmatch are frequently excluded as candidates for transplantation, leading to prolonged waiting times and decreased chance of receiving a transplant.[6] Furthermore, DSA play an essential role in the development of antibody-mediated rejection (AMR), which has emerged as the major clinical challenge in transplantation and is the most frequent cause of renal allograft failure.[7]

Despite the prevalence of sensitized recipients, the role of DSA and AMR in VCA remains largely unexplored, with only a few clinical reports of AMR in upper extremity and face transplantation in the literature and only, to our knowledge, a single experimental study that attempted to define the role of DSA in a rat model of VCA.[8-11]

Currently available desensitization protocols, which all entail several courses of pretransplant plasmapheresis, IVIG or antibody treatment, are only feasible in a living donor scenario and therefore are not applicable to VCA where deceased donors are the only option.[12,13] Recently, a strategy to ablate and repopulate the bone marrow of sensitized recipients using chemotherapy and total body irradiation (TBI) induction followed by syngeneic hematopoietic stem cell transplant (HSCT) was successful in improving kidney allograft survival in sensitized rats.[14] However, there remains an unmet need to develop a better understanding of the impact of sensitization on VCA and to develop strategies to improve access to and outcomes after VCA in sensitized patients. In this study, we therefore examined the effect of sensitization on VCA using a small animal model of orthotopic hind limb transplantation and investigated the effectiveness of a clinically relevant desensitization protocol using TBI and fludarabine preconditioning followed by syngeneic-HSCT that would be applicable in a deceased donor setting. Our results indicate that sensitization leads to accelerated rejection of the hind limb allograft and that our proposed HSCT-based protocol reverts the immune reactivity of sensitized hosts and restores the ability of conventional immunosuppression to preserve VCA.

Table 1: Experimental groups. Flu, Fludarabine; HSCT, hematopoietic stem cell transplant; DA, Dark Agouti; VCA, vascularized composite allograft.

Group #	Group Name	Donor	Recipient	Sensitization
1	Non-sensitized	DA	Lewis	No
2	Non-sensitized + FK	DA	Lewis	No
3	Sensitized	DA	Lewis	DA Skin
4	Sensitized + FK	DA	Lewis	DA Skin
5	Flu + HSCT	DA	Lewis	DA Skin
6	Flu + HSCT + FK	DA	Lewis	DA Skin
7	Naïve Serum + FK	DA	Lewis	Naïve serum
8	Sensitized Serum + FK	DA	Lewis	Sensitized serum

Flu, Fludarabine; HSCT, hematopoietic stem cell transplant; DA, Dark Agouti; VCA, vascularized composite allograft.

Materials and Methods

Rat Strains and Care

Six- to 8-week-old male Lewis (LEW; RT11) and Dark Agouti (DA; RT1Aa) rats were purchased from Harlan Laboratories (Indianapolis, IN) and were used as the recipients and donors, respectively, for skin and hind limb transplantation. Animals were divided into 8 experimental groups (Table 1): (1) nonsensitized recipients; (2) nonsensitized recipients treated with tacrolimus; (3) sensitized recipients; (4) sensitized recipients treated with tacrolimus; (5) sensitized recipients that received Fludarabine and underwent syngeneic HSCT; (6) sensitized recipients that received Fludarabine, underwent syngeneic HSCT and were treated with tacrolimus; (7) nonsensitized recipients infused every other day with naive serum and treated with tacrolimus; (8) nonsensitized recipients infused every other day with sensitized serum and treated with tacrolimus. This study was approved by the Johns Hopkins University Animal Care and Use Committee (RA13M310), and all animals were cared for according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Skin Transplant Sensitization

Lewis recipients were sensitized by skin transplants from DA donors. A 2 cm by 2 cm full thickness skin graft was harvested from the dorsum of donor animals and transplanted to the dorsum of recipients. A bolster dressing was applied and maintained for at least 5 days before dressing removal.

Flu	HSCT	Transplant	Tacrolimus	Survival (Days)
No	No	VCA	No	8, 8, 8, 8, 9, 11
No	No	VCA	0.5 mg/kg/day	>30, >30, >30, >30, >30, >30
No	No	VCA	No	5, 5, 5, 5, 6, 7
No	No	VCA	0.5 mg/kg/day	7, 8, 9, 9, 11, 17
25 mg/kg/day (7 days)	Lewis HSC	VCA	No	13, 14, 15, 16
25 mg/kg/day (7 days)	Lewis HSC	VCA	0.5 mg/kg/day	>30, >30, >30, >30, >30, >30
No	No	VCA	0.5 mg/kg/day	>30, >30, >30, >30, >30
No	No	VCA	0.5 mg/kg/day	14, 14, 28

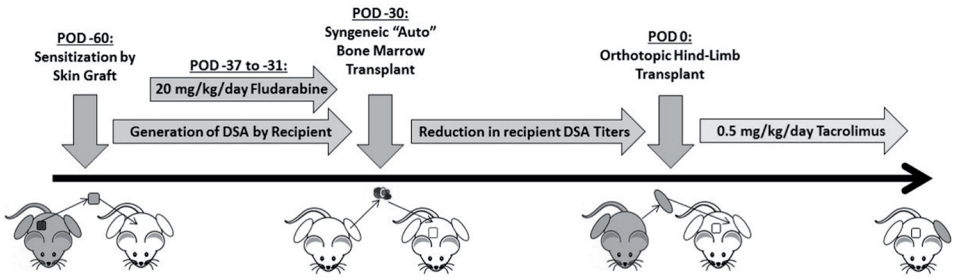

Figure 1: Schematic of desensitization protocol using fludarabine and syngeneic HSCT. Skin transplant from DA donors were performed to Lewis recipients 60 days prior to hind-limb transplantation to allow for generation of DSA. Preconditioning was performed with a 7-day course of Fludarabine (20 mg/kg/day) followed by a single dose of TBI at 12 Gy. Syngeneic HSCT was performed 30 days after the skin transplantation. A 30-day recovery period followed, which allowed for reduction of DSA titers and immune reconstitution in the recipient animals. Orthotopic hind-limb transplantation was then performed from DA donors to the desensitized recipients, and recipients were maintained with 0.5 mg/kg/day of Tacrolimus.

Desensitization Protocol With Fludarabine and Syngeneic HSCT

Desensitization was performed using a previously described protocol for solid organ transplantation.14 At 30 days after skin transplantation, sensitized recipients undergoing desensitization were treated with a 7-day course of fludarabine (25 mg/kg per day) administered intraperitoneally, followed by myeloablative TBI with a single dose at 12 Gy using Gammacell 40 (Nordion, Ottawa, ON, Canada). Bone marrow cells (3-4 × 108) were isolated from wild-type Lewis rats and were injected intravenously into sensitized Lewis recipients 24 hours after irradiation (Figure 1).

Orthotopic Hind Limb Transplantation

Rat orthotopic hind limb transplantation was performed as previously described.[15] For the donor operation, a circumferential inguinal skin incision was made to access the femoral vessels, which were then dissected and divided proximally. The thigh muscles were then transected, and a femoral osteotomy was performed. The graft was stored in cold storage (4°C) while the recipient operation took place. The recipient procedure was performed in a similar fashion, except that the division of the femoral vessels occurred more distally to ensure maximal pedicle length for vascular anastomosis. Osteosynthesis was performed using an 18-gauge needle as an intramedullary rod. Muscle approximation was performed with 4-0 vicryl sutures, and the microvascular anastomosis was performed with interrupted 10-0 nylon sutures. After transplantations, animals assigned to the immunosuppression groups received daily tacrolimus (0.5 mg/kg intraperitoneally).

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Adoptive Serum Transfer

Donors were sensitized by skin transplant as described. Sensitized serum was obtained on postoperative day (POD) 10, and control serum was obtained from naïve animals without skin transplant. Serum levels of DSA were measured as described above to confirm sensitization. All animals received 1.5-mL serum at post-VCA day -1, and 1 mL of serum was injected every other day starting at post-VCA day 1.

Graft Monitoring

Hind limb allografts were inspected daily for clinical signs of rejection and graded as 0, no rejection; 1, edema; 2, erythema; 3, epidermolysis or desquamation; 4, necrosis; and 5, mummification.[16] Study endpoint was defined as either POD 30 or grade 3 rejection.

Detection of Serum DSA Levels

Antidonor IgG and IgM levels were determined using flow cytometry with donor (DA) thymocytes as target cells. Cells were incubated with 50 µL of diluted heat-inactivated sera (1/125). Fluorescein isothiocyanate-conjugated goat antirat IgG at 1:125 (Jackson ImmunoResearch Laboratories, West Grove, PA), PE-conjugated goat antirat IgM at 1:125 (Jackson ImmunoResearch Laboratories), and Alexa Fluor 647 conjugated antirat CD3 antibody at 1:400 (Biolegend, San Diego, CA) were then added. Data acquisition was performed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). DSA levels in the serum drawn from naïve Lewis recipients before any transplants served as baseline presensitization levels. Data was analyzed by gating for viable and CD3 positive cells using FlowJo V10 software (Tree Star Inc., Ashland, OR) and expressed as fold increase in mean fluorescence intensity compared with baseline levels.

Histological Analysis

Skin biopsies were performed at 7 days after transplantation and again at 30 days if the graft has not reached grade 3 clinical rejection before that point. Formalin-fixed and paraffin-embedded tissue was cut into 5-μm sections for C4d and hematoxylin-eosin (H&E) staining. Immunohistochemistry (IHC) for C4d was performed on unstained sections, which were deparaffinized in HistoClear (National Diagnostics, GA) and then hydrated in graded alcohol. Endogenous peroxide was blocked by incubation in a solution of 3% H2O2 in 70% ethanol. Antigen retrieval was performed using a heat-induced method in Dako target-retrieval solution (Dako, Carpenteria, CA). After blocking with Dako serum-free protein block (Dako), the slides were then incubated overnight at 4 °C with Rabbit antirat C4d primary antibody at 1:25 (Hycult, Polymouth Meeting, PA). Sections were then washed and incubated with goat antirabbit biotinylated secondary antibodies for 30 minutes at room temperature. VECTASTAIN ABC method (Vector Laboratories, Burlingame, CA) was then applied, and the reaction was developed with 3, 3'-diaminobenzidine (Sigma-Aldrich Corp., St. Louis, MO) under direct microscopic visualization. Mayer's hematoxylin (Sigma-Aldrich) was used for counterstaining. All stained slides were imaged using a Zeiss Axio Observer microscope (Carl Zeiss Microscopy, Jena, Germany).

Immune Phenotyping After HSCT

To determine the kinetics of immune reconstitution, 7 Lewis animals were sensitized with DA skin transplants and then desensitized with the Fludarabine and HSCT protocol. Complete cell counts (CBC) were obtained from peripheral blood samples at baseline, post-HSCT day 1, and day 30. Similarly, T and B cell percentages of lymphocytes were determined using flow cytometry at the same time points. Four animals then received a hind limb transplant at post-HSCT day 30, and CBC was obtained at post-VCA day 15 to continue to follow the trend of immune cells.

Statistical Analysis

All analyses were performed using Prism Graphpad (GraphPad Software, Inc., La Jolla, CA). Graft survival analysis was performed using the Kaplan-Meier method. Comparison of serum DSA levels between groups was performed using unpaired Student t tests. All data were expressed as mean ± SD, and a P value less than 0.05 was deemed significant.

RESULTS

Elevated Levels of Serum DSA After Sensitization With Skin Transplants

Lewis recipients receiving DA skin transplants without immunosuppression rejected their allografts by an average of 15.8 ± 2.2 days after transplantation. Serum levels of

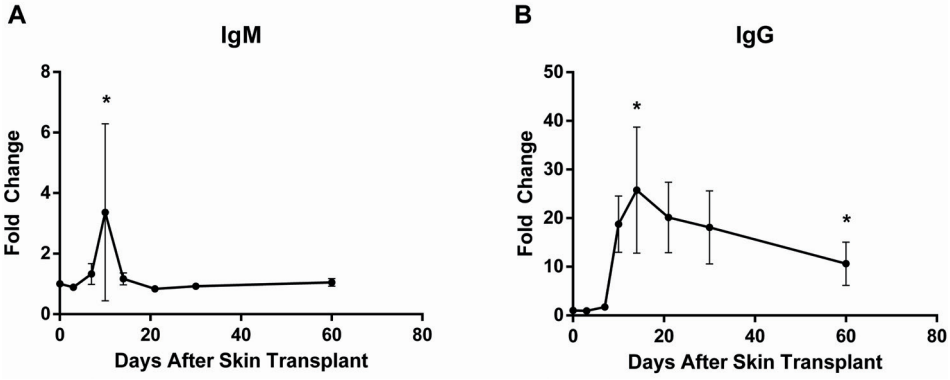

Figure 2: Serum donor-specific antibodies after skin transplantation. Skin allografts from DA rats were transplanted to Lewis recipients. Serum levels of DSA IgM (A) and IgG (B), following skin transplantation, were determined by flow cytometry using donor (DA) splenocytes as target cells. Data was expressed as fold increase in mean fluorescence intensity from presensitization levels. Serum samples from three animals at each time point were analyzed. *p<0.01 compared to presensitization levels.

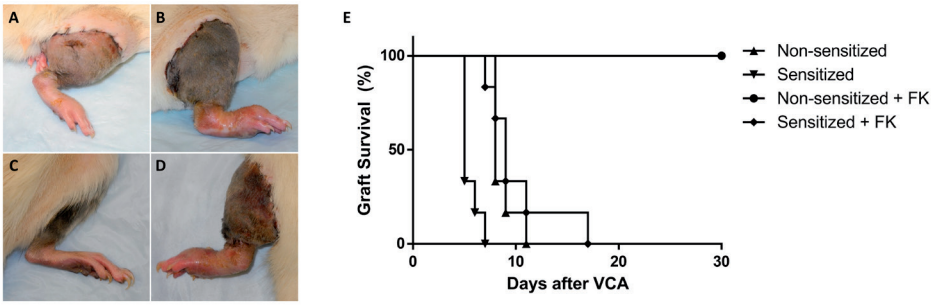

Figure 3: Accelerated rejection in sensitized recipients. Orthotopic hind-limb transplants were performed from DA donors to Lewis recipients. Treated groups received 0.5 mg/kg/day of Tacrolimus (FK). Graft rejection was determined by clinical observation and defined as grade 3 rejection with evidence of epidermolysis or desquamation. (A) Representative image of the hind-limb allograft of a non-sensitized animal on POD7 with evidence of erythema and edema (grade 2). (B) Image of the hind-limb allograft of a sensitized animal on POD7 showing significant edema, erythema and desquamation (grade 3). (C) Non-sensitized animal with daily FK treatment on POD30 without any evidence of rejection (grade 0). (D) Sensitized animal hind-limb allograft with daily FK treatment on POD7 showing significant erythema, edema and desquamation (grade 3). (E) Without immunosuppression, non-sensitized recipients rejected their allografts by POD 8.7±1.2, and the sensitized recipients rejected their allografts on POD 5.5±0.8. When treated with daily Tacrolimus, all non-sensitized animals maintained graft survival beyond 30 days. In contrast, the sensitized animals rejected their grafts by POD 10.2±3.6 on average despite immunosuppression. Six animals were included in each group.

DSA-IgM peaked on day 10 (3.4 ± 2.6-fold) and returned to baseline levels soon afterward (Figure 2). In contrast, the DSA-IgG levels peaked on day 14 (25.8 ± 7.5-fold) and remained elevated at 60 days (10.6 ± 2.6-fold) after skin transplantation.

Accelerated Allograft Rejection in Sensitized VCA Recipients

Hind limb transplantation from DA donors to sensitized and nonsensitized Lewis recipients was performed to investigate the impact of sensitization on VCA survival. The animals were further separated into groups that received daily therapeutic tacrolimus immunosuppression and those that were left untreated (Table 1). Nonsensitized animals without immunosuppression (n = 6) rejected their hind limb grafts by 8.7 ± 1.2 days after transplantation (Figure 3). In comparison, sensitized animals without immunosuppression (n = 6) rejected their grafts by 5.5 ± 0.8 days after transplantation ($P < 0.01$). All nonsensitized animals treated with daily tacrolimus (n = 6) maintained graft survival without evidence of rejection for the entire duration of drug administration (30 days). In contrast, sensitized animals rejected their hind limb allografts on an average of 10.2 ± 1.5 days ($P < 0.01$) after transplantation despite daily tacrolimus treatment. This indicated the inability of conventional immunosuppression to prevent graft rejection in our sensitization model.

Accelerated Allograft Rejection in Sensitized Serum Transfer Recipients

Animals in the adoptive serum transfer groups received sensitized serum (n = 3) or naïve serum (n = 5). All animals received daily tacrolimus injections of 0.5 mg/kg intraperitoneally. Animals in the naïve serum group showed no signs of rejection at the study endpoint of POD 30, whereas recipients of sensitized serum reached graft rejection at POD 14, 14, and 28 (Figure 7, and Figure S1).

Reduced DSA Titers After Fludarabine and Syngeneic HSCT

To investigate the impact of the desensitization protocol, sensitized recipients underwent treatment with fludarabine, TBI and syngeneic HSCT. Serum levels of DSA-IgG were measured in the animals before and 30 days after undergoing the protocol HSCT. By 30 days after HSCT, the serum levels of DSA-Ig in the HSCT and fludarabine group was significantly lower compared to sensitized animals that did not receive the desensitization protocol (2.6 ± 0.5-fold vs 6.0 ± 1.2-fold, $P < 0.01$) (Figure 4A).

Fludarabine and Syngeneic HSCT Improved Hind Limb Allograft Survival in Sensitized Rats With Tacrolimus Treatment

Orthotopic hind limb transplantation from DA donors was performed 30 days after HSCT to investigate whether this desensitization protocol can improve VCA survival. The desensitized recipients received daily tacrolimus injection after hind limb transplantation. All animals in the Fludarabine and HSCT group maintained graft survival without evidence of rejection to at least 30 days after hind limb transplant compared

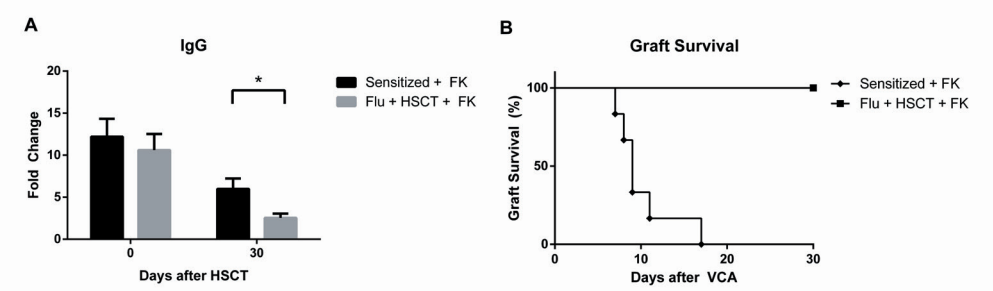

Figure 4: Decreased DSA titers and improved graft survival with Syngeneic HSCT. (A) DSA levels were measured at 30 days after skin transplant and just prior to hind-limb allograft transplantation. Both the sensitized group and the Fludarabine and HSCT group had a decrease in the serum levels of DSA, but the Fludarabine and HSCT group had a significantly lower level compared to sensitized controls by 30 days after the desensitization protocol (2.6±0.5-fold vs 6.0±1.2-fold, respectively). *p<0.05. (B) Orthotopic hind-limb transplants were then performed in both groups, and all animals were treated with daily Tacrolimus injections (0.5 mg/kg). All animals in the Fludarabine and HSCT group maintained graft survival without evidence of rejection to 30 days or more after hind-limb transplant. In contrast, the sensitized group rejected their allografts by 10.2 days on average. Six animals were included in each group.

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with an average rejection time of 10.2 ± 1.5 days ($P < 0.05$) in the sensitized group that received tacrolimus (Figure 4B, and Figure S2, SDC,http://links.lww.com/TP/B522).

Functional Immune Reconstitution at 30 Days Post-HSCT

Both total white blood cells and total lymphocytes showed reconstitution by post-HSCT day 30 (Figure S3). Total lymphocyte counts have returned to 69% of baseline by 30 days after HSCT. Hind limb transplant was performed at 30 days after HSCT, and total lymphocyte levels were measured again at 15 days after VCA. At which time, lymphocyte counts were 87% of baseline. Flow cytometry was performed to analyze the percent of B and T cells. B cells constituted 25.7 ± 5.3% of lymphocytes at 30 days post-HSCT. T cells constitute 15.2 ± 2.3% of lymphocytes at 30 days post-HSCT (Figure S4). All animals treated with fludarabine and HSCT without daily tacrolimus after hind limb transplant rejected their grafts at an average time of 14.5 ± 1.3 days ($n = 4$).

DSA Titer Elevation in Sensitized Recipients But Not in Desensitized Recipients After VCA

To investigate the humoral response of the recipients after a repeat exposure to DA alloantigens in the form of a hind limb allograft, serum levels of DSA-IgG was measured at various time points after the hind limb transplantation. In the sensitized group, there was a rapid increase in the levels of DSA-IgG despite daily tacrolimus treatment to 37.3 ± 3.34-fold by POD 14 (Figure 5). In the sensitized animals that received fludarabine and HSCT, the serum levels of DSA-IgG not only did not increase but instead decreased to near presensitization levels by POD 14 (1.8 ± 1.3-fold, $P < 0.01$).

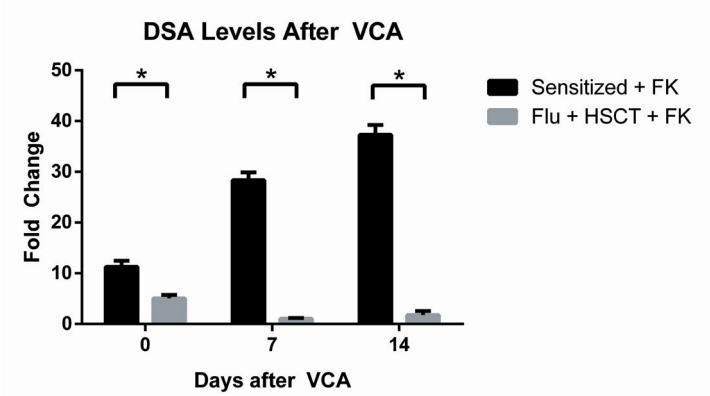

Figure 5: DSA titer elevation in sensitized recipients. After orthotopic hind-limb transplants were performed in sensitized controls and animals who received fludarabine and HSCT, serial measurements of serum DSA titers were performed using flow cytometry. In the sensitized animals, the serum levels of DSA had a marked elevation after hind-limb transplantation to 37.3±3.34-fold by POD14. No elevation in DSA titers were seen in the fludarabine and HSCT group in the immediate period after hind-limb transplant. In fact, the DSA titers in the fludarabine and HSCT group decreased to presensitization levels by POD 14 (1.8±1.3-fold). Samples from three animals were analyzed in each group. *P<0.05.

Reduction of C4d Deposition in Animals Treated With Fludarabine and Syngeneic HSCT

To analyze the impact of altered levels of circulating DSA on tissue rejection and complement deposition, graft skin biopsy specimens were obtained from sensitized and nonsensitized animals that were treated with daily tacrolimus. On histology of the sensitized animal specimens, diffuse infiltration of mononuclear cells with loss of dermal-epidermal junction architecture were seen on the H&E stains at 7 days after transplantation (Figure 6A). This correlated with visible signs of rejection of the skin component on clinical examination. In contrast, no evidence of rejection was present on H&E stains of skin biopsies from nonsensitized animals (Figure 6B). Similarly, there was no evidence of rejection at 7 or 30 days after transplantation in the fludarabine and syngeneic HSCT group (Figures 6C and D).

Evidence of antibody interaction with the vasculature, such as complement deposition, is used as a marker of AMR in solid organ transplantation. IHC for C4d was performed to look for evidence of complement activation, and C4d deposition was consistently detected in the dermal vascular endothelium of the sensitized animals at 7 days after transplantation (Figure 6E). In the nonsensitized animals treated with tacrolimus, no evidence of C4d deposition was detected on IHC (Figure 6F). Similarly, in the desensitized animals, there were minimal C4d staining at both 7 and 30 days after transplantation (Figures 6G, H).

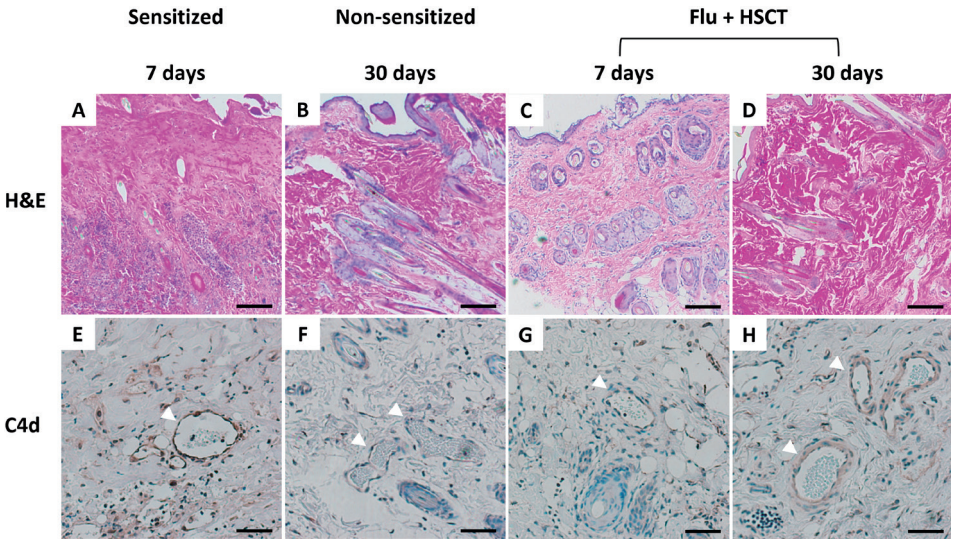

Figure 6: C4d Deposition in sensitized recipients. Graft skin biopsies were performed at various time points. Tissue was fixed in formalin and embedded in paraffin. 5µm thick sections were used for staining with hematoxylin and eosin and immunohistochemistry for C4d. A-D.) Representative H&E image. Note the Banff grade 3 rejection in the skin of sensitized animals as evidenced by diffuse cellular infiltration by 7 days after transplantation. In contrast, there is no evidence of rejection seen in the non-sensitized and desensitized animals at 7 days or 30 days after transplantation. White arrows indicate dermal vasculatures. Scale bar = 200µm. E-H.) Representative C4d staining images. Strongly positive C4d staining (panel E) was detected in the dermal vascular endothelium of sensitized animals but not in the non-sensitized (F) or desensitized (G and H) animals. Scale bar = 50µm.

Discussion

Potential VCA candidates are frequently sensitized, which can significantly increase their wait time and limit access to reconstructive transplantation that could enhance their quality of life. In solid organ transplantation, the response to sensitization and the presence of preformed DSA varies depending on the type of organ transplanted.[7] VCA is unique in its composition and can include skin, muscle, and vascularized bone marrow components. In this study, we initially investigated the impact of sensitization on VCA survival in sensitized rats. Our results indicated that VCA in sensitized recipients experience accelerated rejection compared with nonsensitized animals and was not controlled by tacrolimus immunosuppression. This finding is consistent with previous work by Wu et al,10 where the authors observed accelerated rejection of myocutaneous VCA in a rat model, but not hyperacute rejection that typically characterizes acute AMR in renal transplantation. Taken together, these results highlight the risk of performing VCA in highly sensitized recipients but also suggest that sensitized VCA recipients would not experience hyperacute rejection as seen in renal transplantation.

This could potentially provide a window for prompt intervention to prevent graft loss. The Banff 2013 statement described 3 features that must be present for the diagnosis of AMR in renal allografts: histologic evidence of acute tissue injury; evidence of antibody interaction with vascular endothelium, such as C4d staining in the peritubular capillaries; and serologic evidence of DSA.[17] The diagnosis of AMR in skin containing composite tissue allografts remains poorly defined, in part because of the limited and inconclusive clinical reports on AMR, DSA, and C4d with regard to VCA in the literature.[18-21]

In the only reported case of acute AMR after face transplantation performed in a highly sensitized recipient with positive donor crossmatch, the acute rejection episode was associated with both elevated DSA and positive C4d staining.[8] This is consistent with the results from our study. Sensitized rats that experienced accelerated rejection of hind limb transplant had a dramatic increase in the serum DSA titers and consistently showed positive C4d staining in the dermal vasculatures. Combined with gross and histologic evidence of rejection, these findings suggest that AMR is likely contributing to the accelerated rejection of VCA seen in our sensitization model.

The H&E stains of the skin samples of the accelerated rejection animals were marked by extensive mononuclear cell infiltrates, suggesting that cellular-mediated rejection may be playing a role as well. Furthermore, the addition of tacrolimus, a calcineurin inhibitor that targets T-cell response, did prolong average graft survival time in sensitized recipients from 5.5 ± 1.2 days to 10.2 ± 3.6 days, which would be in line with cellular-mediated rejection contributing to the accelerated rejection. It seems likely that the accelerated rejection in sensitized recipients may occur through a combination of cellular-antibody-mediated rejection and AMR mechanisms. Memory T cells, which are more resistant to conventional immunosuppression and have been implicated as a potential contributor to skin rejection in sensitized recipients, may play a role in the observed accelerated rejection.[22]

Clinical management of highly sensitized patients and AMR is significantly different from T cell–mediated rejection. Currently available desensitization protocols all entail several courses of pretransplant plasmapheresis, IVIG, or antibody treatment immediately before transplantation, which require a living-related donor situation where pretransplant planning can be performed.[12] This is not feasible in VCA, where deceased donation is the only option. Autologous HSCT for immune reconstitution has shown clinical efficacy and safety for the treatment of severe autoimmune diseases and hematologic malignancies.[23] Furthermore, it is applicable in a deceased donor setting. In patients with severe, refractory autoimmune disease, autologous HSCT appears to act by eliminating autoreactive T cells, antigen-presenting cells and even plasma cells by irradiation and chemotherapy followed by immune reconstitution by autologous HSCT.[24] Fu et al demonstrated success of using TBI, fludarabine and syngeneic HSCT to reduce DSA and improve survival of renal allografts in sensitized rats.[14]

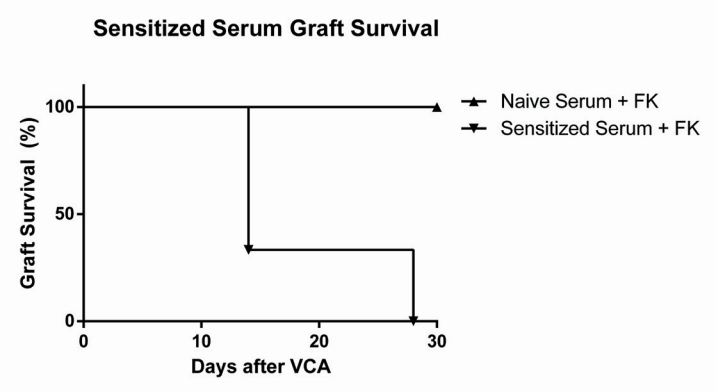

Figure 7: Accelerated graft rejection in sensitized serum recipients. Sensitized serum recipients treated with Tacrolimus rejected their grafts on POD 14, 14, and 28 (N=3). All naïve serum recipients treated with Tacrolimus maintained graft survival beyond 30 days (N=5).

We applied this strategy to sensitized rats in the setting of VCA. Similar to the study by Fu et al, we found a reduction in the level of serum DSA after syngeneic HSCT compared to sensitized controls.[14] More importantly, there was a dramatic improvement in the graft survival in the syngeneic HSCT group with all animals having graft survival beyond 30 days. Additionally, there was no evidence of AMR in the syngeneic HSCT group with respect to elevated serum levels of DSA after hind limb transplantation or C4d deposition on tissue biopsies. These results suggest that the desensitization protocol effectively reduced preformed DSA and eliminated the antibody producing alloreactive immune cells that likely contributed to the accelerated rejection in the sensitized group.

At the time of transplant, the serum levels of DSA in the syngeneic HSCT group was significantly lower than that of sensitized controls but was still elevated compared to presensitization levels. However, this did not appear to have a noticeable adverse impact on graft survival when recipients were treated with tacrolimus. Additionally, the serum levels of DSA in the group that received syngeneic HSCT and daily tacrolimus quickly decreased to presensitization levels after hind limb transplantation, likely due to adsorption of preformed DSA by the hind limb graft but with no appreciable negative impact.

To further delineate the effect of serum DSA on the accelerated rejection seen in sensitized recipients, an adoptive serum transfer experiment was performed that showed that sensitized serum transferred to naive recipients of hind limb transplant was sufficient to cause accelerated rejection of VCA (Figure 7), albeit at a slower pace than in the sensitized recipients. This may be due to a lower level of serum antibody present in the animals that received sensitized serum transfer. Although only a small number of animals were included in the sensitized serum transfer group, they demonstrated a significantly different outcome compared to the naïve serum transfer group. Notably, these animals began to demonstrate early signs of rejection (erythema and edema, grades 1-2) by the second week after transplantation, and all 3 progressed to fully rejecting their allografts (grades 3-4). In contrast, the animals that received naïve

serum did not exhibit any evidence of rejection (grade 0) up to the study endpoint of POD 30. This preliminary data suggests that sensitized serum containing DSA can induce accelerated rejection of VCA.

Based on the results of this study, syngeneic HSCT after preconditioning with fludarabine and TBI is effective in improving VCA survival in sensitized rats. Desensitization of potential VCA candidates presents unique challenges because deceased donors are the only option. The desensitization protocol described in this study holds promise because it targets the cellular source of DSA in contrast to existing strategies such as plasmapheresis or IVIG. In the clinical setting, this desensitization protocol may be performed on highly sensitized patients before enrollment on waitlists for VCA. This therapeutic approach has the potential to improve access to reconstructive transplantation.

A limitation of this study is that it does not address the impact of de novo DSA formation and chronic rejection, which may play a critical role in long-term VCA graft survival as illustrated by a case from the Innsbruck group of AMR 9 years after bilateral upper extremity transplantation.9 Another limitation of this study is the potentially significant toxicity associated with the combination of TBI, fludarabine and autologous HSCT, which is an important concern in the setting of nonlife-saving reconstructive transplantation. Although the current regimen may be too risky for clinical translation, future work will aim to identify the effect of each component of the protocol, with the goal of improving understanding and developing modifications that may minimize toxicity while preserving efficacy. Additionally, future studies will focus on elucidating the relative contribution of and the impact of the desensitization protocol on cellular and antibody-mediated responses in sensitized recipients.

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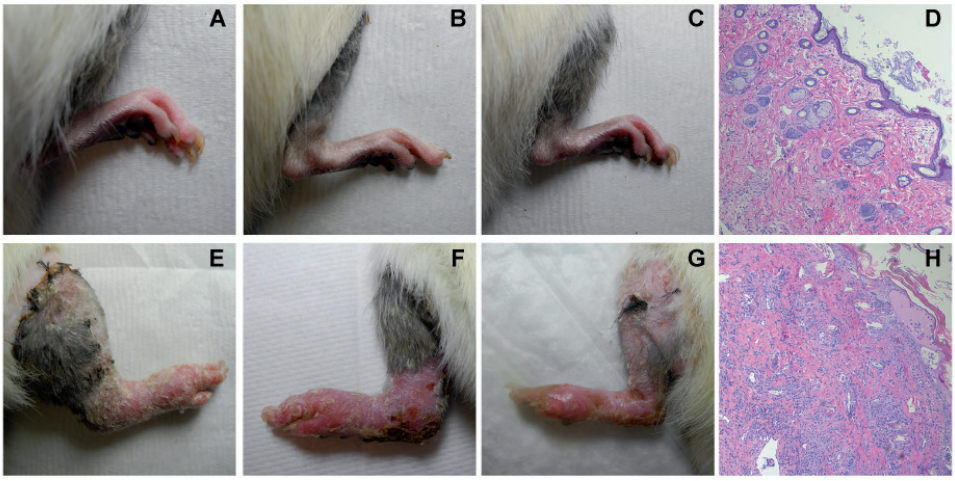
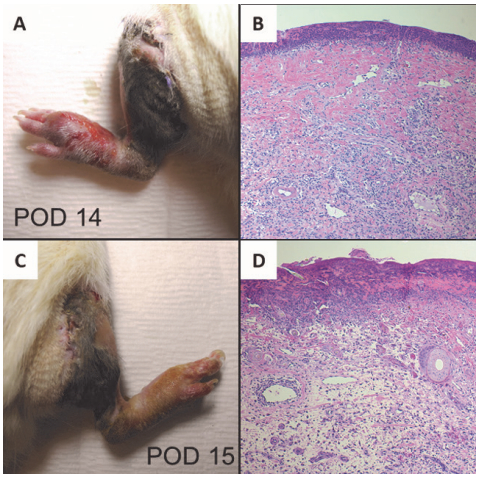
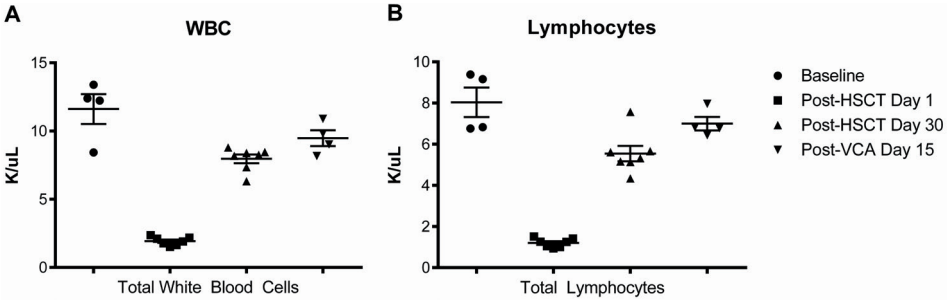
Supplemental figures



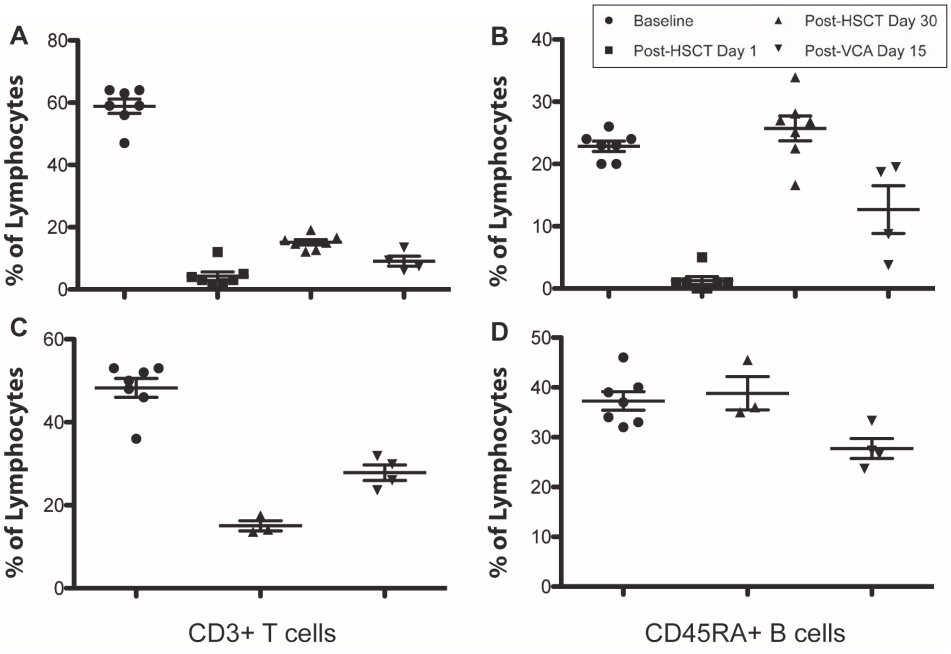
Supplemental Figure 1: Clinical and Histological outcomes of sensitized and naïve serum recipients. Animals received 1.5 ml of sensitized or naïve control serum intravenously at VCA POD-1. After transplant, animals received serum 1ml/every other day. Both groups received 0.5 mg/kg/day of Tacrolimus (FK). (A-C) Representative images of hind-limb allografts of control serum recipients on POD30 without any evidence of rejection (grade 0). (D) Representative corresponding H&E stained histology at POD14 shows no signs of rejection. (E-G) Representative images of hind-limb allografts of sensitized serum recipients on POD14 (E,F) and 28 (G), showing significant erythema, edema and desquamation (Banff Grade 3). (H) Representative corresponding H&E stained histology at POD14 shows cellular infiltration and skin breakdown.



Supplemental Figure 2: Clinical and Histological outcomes of sensitized animals treated with Fludarabine and HSCT, without Tacrolimus. Flu+HSCT recipients without Tacrolimus maintenance treatment showed clinical grade 3 rejection by POD 13-16. (A,B) Representative image of hind-limb allograft with endpoint rejection at POD 14 and severe cellular infiltrate on corresponding histology. (C,D) Representative image of hind-limb allograft with endpoint rejection at POD 15 and severe cellular infiltrate on corresponding histology.



Supplemental Figure 3: White blood cells and Lymphocyte Repopulation after Fludarabine and HSCT. Flu+HSCT recipients without Tacrolimus maintenance treatment had complete blood cell counts (CBC) taken at Post Hematopoetic stem cell transplantation (HSCT) day 1 and 30. Four animals received a hind-limb transplant at Post-HSCT day 30 and had additional CBC performed at 15 days post VCA. (A) White Blood cell counts are 68% and 82% of baseline at post-HSCT day 30 and post-VCA day 15 respectively. (B) Lymphocyte counts are 69% and 87% of baseline at PRD 30 and post-VCA day 15 respectively.



Supplemental Figure 4: B- and T-cell reconstitution in splenocytes and peripheral lymphocytes after Fludarabine and HSCT. Flow cytometry was performed to analyze the percent of B and T-cells. (A) T-cells constitute 15.2 ± 2.3% and 9.1± 3.2% of peripheral lymphocytes at 30 days post HSCT and 15 days post-VCA respectively (B) B-cells constituted 25.7 ± 5.3% and 12.7± 7.7% of peripheral lymphocytes at 30 days post HSCT and 15 days post-VCA respectively. (C) T-cells constituted 15.7 ± 2.1% and 27.85± 3.7% of splenocytes at 30 days post HSCT and 15 days post-VCA respectively. (D) B-cells constituted 38.8 ± 5.8% and 27.7± 4.0% of splenocytes at 30 days post HSCT and 15 days post-VCA respectively.

Part III

Expanding graft availability: high subzero ice-free graft storage

Chapter 6

Successful long-term survival of vascularized composite allografts after extended preservation at subzero temperatures using bioinspired next-generation cryoprotectants

Submitted

Samuel A.J. Fidder, MD[1,2], Andrea Callegari, PhD[3], Andres Matoso, MD[4,5,6], Byoung Chol Oh, DVM, PhD[1], Kara Lombardo, MSc[6], Adam Childs, MSc[3], Joanna W. Etra, MD[1], Franka Messner MD, PhD[1], Dalibor Vasilic, MD[2], Richard J. Redett III, MD[1], Xiaoxi Wei, PhD[3], Mark Kline, PhD[3*], Gerald Brandacher, MD[1*]

1. Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
2. Department of Plastic and Reconstructive Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands.
3. X-Therma Inc., Berkeley, California, USA
4. Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
5. Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
6. Department of Urology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

* These authors contributed equally to the manuscript.

Abbreviations: DMSO, Dimethylsulfoxide; **H**, Hour; **HSP**, High Subzero Preservation; **HTK**, Histidine Tryptophan Ketoglutarate; **LEW**, Lewis; **LSP**, Low Subzero Preservation; **POD**, Postoperative day; **PS**, Biomimetic Preservation Solution Containing Peptoids; **UW**, University of Wisconsin; **VCA**, Vascularized Composite Allotransplantation.

Abstract

Transplantation is constrained by limited time for organ preservation. Extending ischemia tolerance allows for better immunological organ matching, recipient conditioning, and broader organ sharing. We here investigate a novel biomimetic solution for extended organ preservation at subzero temperatures containing novel antifreeze peptoids. Syngeneic Lewis rat penile grafts were either promptly transplanted, perfused with HTK (stored at 4°C), or peptoid solution (PS, stored at -5°C). HTK and PS grafts were transplanted after 24, 48, or 72h (hours). Grafts were clinically monitored daily until study endpoints of post-operative day (POD)3 and POD30 and assessed by H&E and Caspase-3. Complete graft necrosis was observed in 33.3%, 44.4% and 83.3% of HTK treated animals at 24h, 48h, and 72h of static cold storage, respectively; in contrast, no complete graft necrosis was observed in any PS perfused grafts. PS grafts showed significantly less distal necrosis than HTK grafts with none occuring in the 24h and 48h PS groups. Irrespective of the timepoint, post-transplant H&E and Caspase-3 analysis showed that HTK preserved distal tissue samples displayed more inflammation than grafts with minimal ischemic injury or PS preserved grafts. We report the first successful long-term survival of vascularized composite allografts after 72h of preservation at subzero temperatures.

Introduction

Organ transplantation has transformed medicine by dramatically extending the lives of patients with end stage organ failure.[1,2] In addition, over the past two decades, reconstructive transplants have been introduced to restore devastating tissue defects of hand, face, and reproductive organs.[3–6] Though transplant medicine has achieved excellent patient and graft survival rates, challenges remain. These include side effects and toxicities of maintenance immunosuppression, chronic rejection, and constraints due to limited ex-vivo tissue storage times.[7]

Organ preservation is an integral part of transplantation as it bridges the time between graft procurement in the donor and reperfusion in the recipient. Static cold storage (SCS) remains the gold standard for organ and tissue preservation.[8,9] SCS can preserve organs and tissues from 4h to up to 24h depending on graft type.[9–11] Cold ischemic time, however, results in progressive organ damage eventually leading to early allograft dysfunction or primary non-function. Extending tolerated ischemic time to days instead of hours could revolutionize the current practice of transplantation. A transplant would become a plannable/elective procedure enabling organ sharing and exchange across longer distances (in turn enabling better immunological matching between donor and recipient) or pre-conditioning of recipients in the setting of deceased organ donation.[9]

In order to achieve prolonged ex-vivo preservation, multiple approaches are currently under (pre)clinical investigation. Currently studied methods are normothermic machine perfusion,[12,13] low subzero preservation (LSP; <-100°C, most commonly through vitrification),[14] and ice-free high subzero preservation (HSP; ~ -20 to 0°C).[11] The main challenge for HSP lies in the prevention of crystal (ice) formation, which is critical for graft survival.[12] Ice crystals can induce widespread tissue damage, resulting in graft failure. Multiple molecules are known to prevent crystal formation in water (i.e., dimethylsulfoxide), but they are also toxic to most tissues.[15,16] Natural examples of proteins that prevent ice formation in high subzero temperatures exist in many arctic species of fish and insects; mammals such as the arctic ground squirrel have the ability to survive repeated cycles of subzero core temperatures.[17–21] Recently, progress has been made in the synthetic development of such antifreeze proteins, with promising results in the preservation of cells, but few studies have reported on the feasibility of this approach in vivo.

We here describe a study demonstrating the feasibility of a subzero temperature approach to preserve vascularized composite allografts employing a novel biomimetic preservation solution containing antifreeze peptoids (PS), a fully synthetic non-protein chemomimetic of antifreeze proteins, in a syngeneic rat penile transplant model.[22,23]

Table 1: Study groups. Group 1: control with minimal ischemia. Group 2-4: control with ischemic times of 24-72 hours, preserved with HTK, stored on ice at 4°C. Groups 5-7: intervention groups with ischemic times of 24-72 hours, preserved with biomimetic preservation solution containing peptoids (PS) and stored at -5°C.

Group	Preservative	Purpose	Ischemic Time	Preservation Temperature	Total group size n=	Endpoint POD3 n=	Endpoint POD30 n=	Preservation only n=
1	Saline	Minimal ischemia control	1h	4°C	8	3	3	2
2	HTK	24h Control	24h	4°C	7	3	3	1
3	HTK	48h Control	48h	4°C	7	3	3	1
4	HTK	72h Control	72h	4°C	7	3	3	1
5	PS	24h Intervention	24h	-5°C	10	4	4	2
6	PS	48h Intervention	48h	-5°C	10	4	4	2
7	PS	72h Intervention	72h	-5°C	10	2	6	2

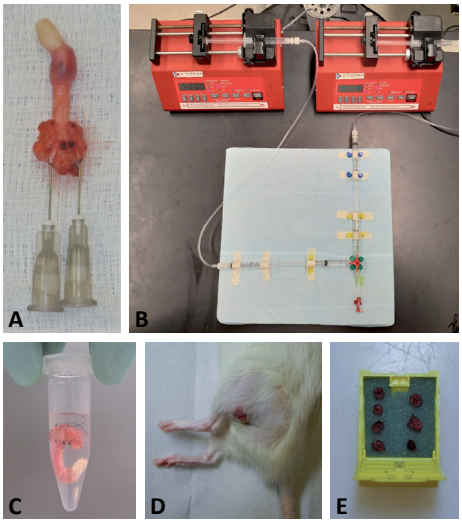


Figure 1: Study procedures. Grafts were obtained from their donors and both internal pudendal arteries were cannulated (panel A). Grafts were perfused with heparinized saline and a linearly increasing fraction of either HTK or PS through both arteries (panel B). Grafts were stored in 5mLs of their study solution (panel C). To prevent local tissue freezing, peptoid solution perfused samples were kept from touching the container wall by surrounding them with perfusate saturated foam while in the solution. After preservation at their respective temperatures, grafts were implanted and photographed at predetermined postoperative days (panel D). Histological samples were collected at respective endpoints (panel E).

Materials and Methods

Animals

8 week-old male inbred Lewis rats (Envigo Inc.) were used as penile transplant donors and recipients in a syngeneic model.[23] The study was approved by the JHMI IACUC under protocol number RA18M258.

Study groups

Seven study groups were used **(Table 1)**. Seven animals were allocated to each control group and 10 to every intervention group. Post-operative day (POD)3, POD30 or complete graft necrosis were used as endpoints for each group. Vascular complications or necrosis occurring on or before POD1 was considered surgical failure and led to exclusion of the transplant from the study. For the HTK control groups and PS groups, 3 different graft ischemia times were tested: 24, 48, and 72h. For each group and time point, 1-2 penile grafts were analyzed after undergoing their preservation procedure without being transplanted to a recipient as preservation controls.

Transplant surgery and animal care

All grafts were transplanted using a heterotopic surgical penile transplant method previously developed by our group.[23] The grafts were implanted heterotopically in the groin of the recipient animal. Donor dorsal penile vein and internal pudendal arteries were anastomosed to the recipient superficial femoral artery and the superficial epigastric artery and vein using a cuff-technique. The glans and prepuce were subcutaneously tunneled to the dorsal thigh for standardized macroscopic follow-up. All animals received enrofloxacin 10 mg/kg/day subcutaneously for 1 week for antibiotic prophylaxis and received buprenorphine 0.02mg/kg subcutaneously as pain medication twice daily for 1 week.

Graft treatment and *ex vivo* preservation protocols

Upon recovery, all grafts were promptly flushed with 5 mL of cold (4°C) heparinized (30 IU/mL) saline. Minimal ischemia control group grafts were immediately transplanted. HTK control group grafts were flushed with 6mL of Custodiol HTK solution (3mLs through each dorsal penile artery) and statically stored in 5mL of HTK solution at 4°C for 24, 48 or 72h. PS intervention groups were flushed with 6ml of peptoid preservative solution (3mls through each dorsal penile artery) and stored in 5mL of peptoid preservative solution at -5°C for 24, 48 or 72h **(Figure 1)**.

HTK and PS graft perfusion was performed using two syringe pumps (NE-1000, New Era Pump Systems, Farmingdale, NY, USA) to ensure standardized speed and volume of graft perfusion in all groups **(Figure 1, supplement 1)**. Perfusion of the first artery was performed at a rate of 500 uL/min, starting with 100% saline and 0% PS. Concentrations were programmed to linearly adjust over the course of the perfusion to end with 0% saline and 100% PS (total volume 6 mL). The second artery was then perfused with 100% PS at 500 uL/min (total volume 3 mL).

For the control group, a laboratory cold room with constant temperature monitoring was used. A freezer with constant temperature monitoring (CoolFreezer CFX, Dometic, Sweden) was utilized in the intervention group. Cooling and rewarming of grafts occurred by placing the graft in the fridge or freezer and by thawing at room temperature, respectively.

Biomimetic preservation solution containing peptoids (PS; XT-ViVo™)

XT-ViVo™ is a chemically-defined DMSO-, serum-, and protein-free biomimetic cryopreservation product developed by X-Therma Inc. (Richmond, CA, USA) that utilizes a peptoid oligomer as a novel cryoprotectant (US patents US10694739B2, US20200170241A1). Peptoids are not proteins or peptides, but are closely related versatile and synthetically accessible biomimetic building blocks that bridge the material gap between proteins and bulk polymers. Peptoids have a body of literature to support their use as functional mimics of naturally occurring peptides[24] while offering a wide range of benefits.[25] In addition to biomimetic peptoid-based cryoprotectants, the solution contains saccharides, salts, membrane stabilizers, antioxidants, and molecules to maintain proper osmotic balance.

Clinical follow up and macroscopic assessment

Animals and grafts were followed up daily for overall health and graft viability in the first week post-transplant and then at POD14, POD21, and POD30. All grafts were photographed at these time points using a NIKON P7700 camera with a fixed aperture. Graft injury was categorized as 1) minor distal necrosis (any necrosis of less than 50% of the glans), 2) major distal necrosis (necrosis of 50% of the glans or more), and 3) complete graft necrosis.

Histological sample collection and analysis

Transverse sections of standardized thickness were made of every penile graft. The distal glans (G) and the proximal shaft (S) were divided in 3 (1G, 2G, and 3G) and 4 (1S, 2S, 3S, and 4S) tissue slices, respectively. Final assessment was done by comparing distal (1-3G) and proximal sections (1-4S) **(Figure 1, supplement 2)**. At endpoint, all samples were fixed in formalin for 24h and then stored in 100% ethanol.

Non-transplanted samples

Non-transplanted samples were stained by an external histopathology group (Ensigna, Inc., San Leandro, CA, USA) with H&E to visualize histopathological changes and TUNEL (ApopTag S7101 kit; Merck; Darmstadt, Germany) to quantify apoptosis. Apoptosis was assessed as the number of positive nuclei divided by the total nuclei in the complete tissue slide (1G-3G and 1S-4S).

Transplanted samples

All samples were embedded in paraffin, sectioned, placed on glass slides, and stained with hematoxylin and eosin (H&E) and activated Caspase-3 (Asp175; dilution 1:200; Cell Signaling Technology, Danvers, USA). Caspase-3 served as a stain to identify apoptotic cells and was quantified as the number of positively stained cells per 40x high powered field (HPF). H&E slides were assessed for inflammation using a penile inflammation grading system developed by our group and can be found in the **figure 6** legend.

All samples were graded by an expert genitourinary pathologist (AM) in a blinded fashion.

Statistical analysis

Counts were reported as percentages and continuous variables as median with range. According to the non-normal distribution of the presented data, Kruskal Wallis test and Dunn's correction for multiple comparison were used for intergroup comparison. For survival analysis, Kaplan Maier graphs were utilized and the log rank test was applied. A two-sided p-value < 0.05 was considered statistically significant. All analyses were performed using Prism 7 software (GraphPad Software, La Jolla, USA).

Results

Macroscopic graft assessment

In all groups, animal survival was 100% and no adverse effects were found on overall animal health. For all groups, a pattern was observed where any significant tissue damage was first seen at the distal end of the graft, starting around POD2. This damage either led to complete graft thrombosis and necrosis on POD3-4 or demarcation of the affected tissue, after which the remaining proximal part of the graft clinically recovered. Macroscopic signs of generalized inflammation peaked at POD7, after which (residual) graft recovery was observed in all groups for the grafts that did not develop complete necrosis (**Figure 2**). The clinical progression of each individual transplanted graft included in this study can be found in **supplements 3&4**.

Graft survival

Graft survival for all groups is summarized in **Figure 3** and **supplements 3, 4 and 5**. Grafts transplanted with minimal ischemia showed no signs of tissue necrosis and all parts of each graft, including the distal glans and preputial skin, stayed homogenously perfused and viable at all timepoints post-transplantation.

Nearly all HTK preserved grafts demonstrated minor distal necrosis (24h storage group: 77.8%; 48h storage group: 100%; 72h storage group: 100%) (**Figure 3**). Median time to occurrence of minor distal necrosis was 5, 4, and 3 days after transplantation in the 24h, 48h, and 72h HTK groups, respectively. Major distal necrosis occurred in 77.8% (median POD5), 83.3% (median POD5), and 100% (median POD3) and complete graft necrosis was observed in 33.3%, 44.4% and 83.3% of HTK preserved grafts at 24h, 48h, and 72h of storage, respectively (**Figure 3**). In nearly all animals, complete graft necrosis manifested around POD3. Only one animal in the 48h HTK group experienced complete graft necrosis on POD11 and one in the 72h HTK group at POD4.

In contrast to HTK treated groups, PS preserved grafts at subzero temperature showed significantly less (log rank $P<0.0001$) minor distal necrosis, with none occur-

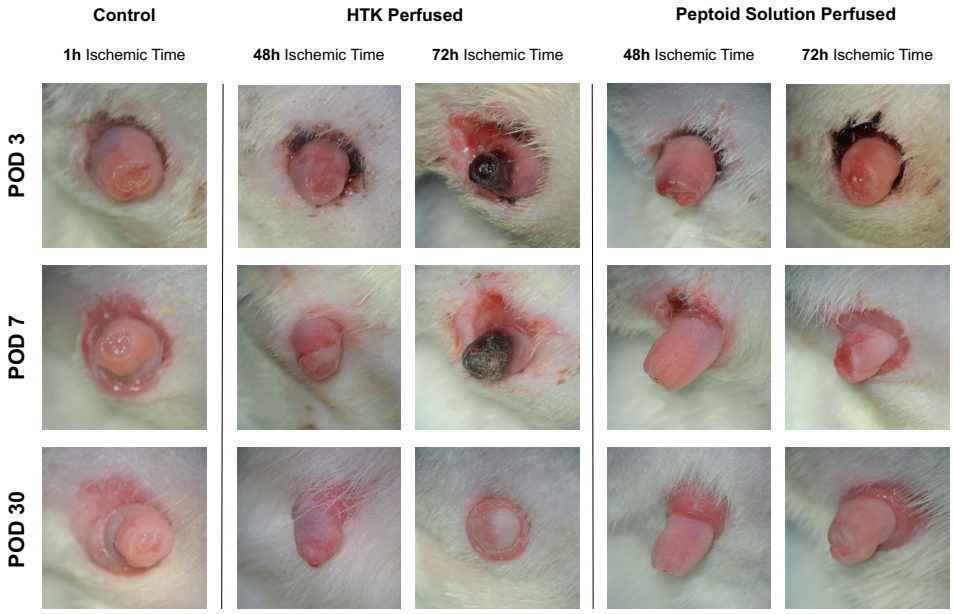


Figure 2: Clinical graft survival. Grafts are shown at POD3, 7 and 30. Promptly transplanted control grafts showed no signs of necrosis or significant tissue damage at any time point. When transplanted after 48h all HTK perfused grafts had distal necrosis at POD3. In 67% of grafts this extended to major graft loss at POD30. PS perfused grafts showed no signs of significant tissue damage at any time point when transplanted after 48h. In grafts transplanted after 72h, HTK perfused grafts underwent major graft loss in all grafts before POD30; PS perfused grafts showed no major graft loss. Tissue damage and associated inflammation follows a distal-to-proximal pattern. More inflammation was observed in distal tissue samples across all groups. Possibly this is related to tissue perfusion being a particular challenge in the distal end of grafts after prolonged ischemia. Multiple grafts showed unilateral necrosis of distal tissue. This pattern may indicate that vascular damage and/or clotting plays a significant role in adverse outcomes.

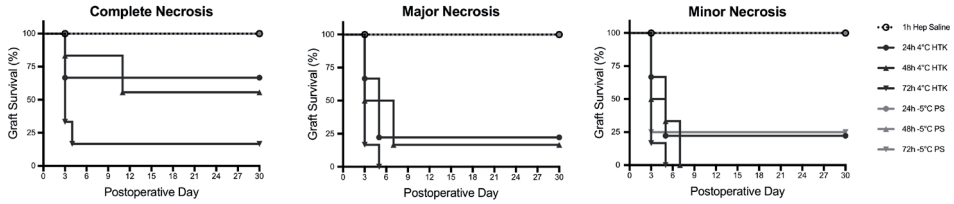


Figure 3: Clinical graft survival. Kaplan-Meier graphs showing occurrence of complete necrosis, major necrosis and minor necrosis. (1) Complete necrosis did not occur in any PS perfused graft. Complete graft necrosis was observed in 33.3%, 44.4% and 83.3% of HTK treated animals at 24h, 48h, and 72h of storage, respectively. For most HTK grafts complete graft necrosis manifested on POD 3. (2) Major distal necrosis did not occur in any PS perfused graft. Major distal necrosis occurred in 77.8% (median POD 5), 83.3% (median POD 5) and 100% (median POD 3) of HTK treated animals at 24h, 48h, and 72h of storage, respectively. (3) For HTK perfused groups, minor distal necrosis occurred in 77.8% (24h storage, median time to occurance 5 days), 100% (48h storage, median time to occurance 4 days), and 100% (72h storage, median time to occurance 3 days). Minor necrosis did not occur in 24 and 48h PS groups but did occur in 75% of 72h PS perfused grafts (median time to occurance 3 days).

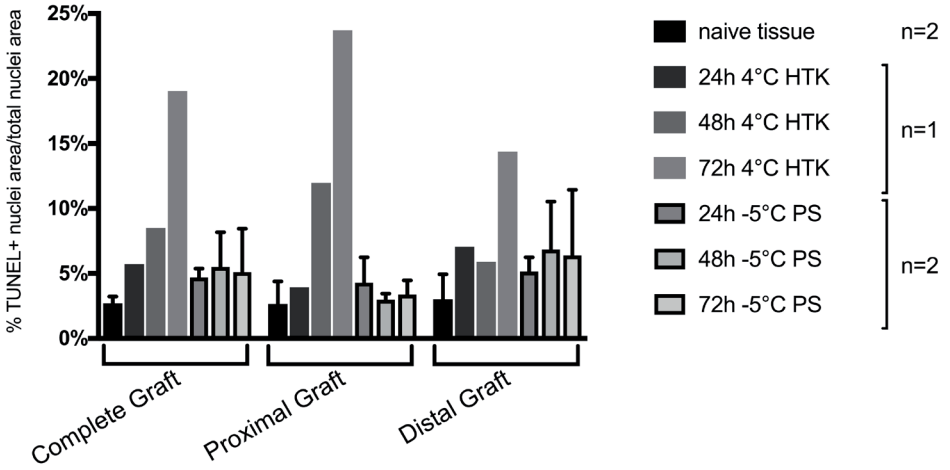


Figure 4: Tissue viability of samples through TUNEL staining. TUNEL staining was performed to analyze the preservation of whole rat penises perfused with saline (fresh), HTK at 4°C, or PS at -5°C and preserved for up to 72h of ischemia. Rat penis samples were stained with TUNEL to visualize apoptotic nuclei. A low percentage of TUNEL to total nuclei was reflective of a well preserved tissue with low cell death/loss. As was observed clinically, differences between intervention and control groups were found at 48h and 72h of ischemic time.

ring in the 24h and 48h groups (**Figure 3**). 75% of PS grafts after 72h of storage experienced minor distal necrosis with a median time to occurrence of 3 days. Grafts stored in PS solution for 24h, 48h and 72h did not display major distal or complete graft necrosis. Compared to HTK solution, a significant reduction of major (log rank $P<0.0001$) and complete graft loss (log rank $P<0.0005$) was achieved and both were comparable to the results of 1h control grafts (log rank $P>0.9$) (**Figure 3**).

Histologic assessment and graft viability

Post-preservation H&E

Post preservation H&E showed no significant structural changes in both the control and the intervention groups.

Post-preservation viability

TUNEL staining demonstrated consistent levels of apoptotic nuclei of around 5% (24h PS: 4.7%; 48h PS: 5.6%; 72h PS: 5.1%) when using PS at -5°C. In contrast, in HTK stored samples, an increase of apototic cells was seen with prolonged storage time. While HTK samples stored at 4°C for 24h contained 5.7% $TUNEL^+$ cells, the 48h and 72h HTK samples had 8.5% and 19% $TUNEL^+$ cells (**Figure 4**). Due to the limited number of samples, no intergroup comparison was performed.

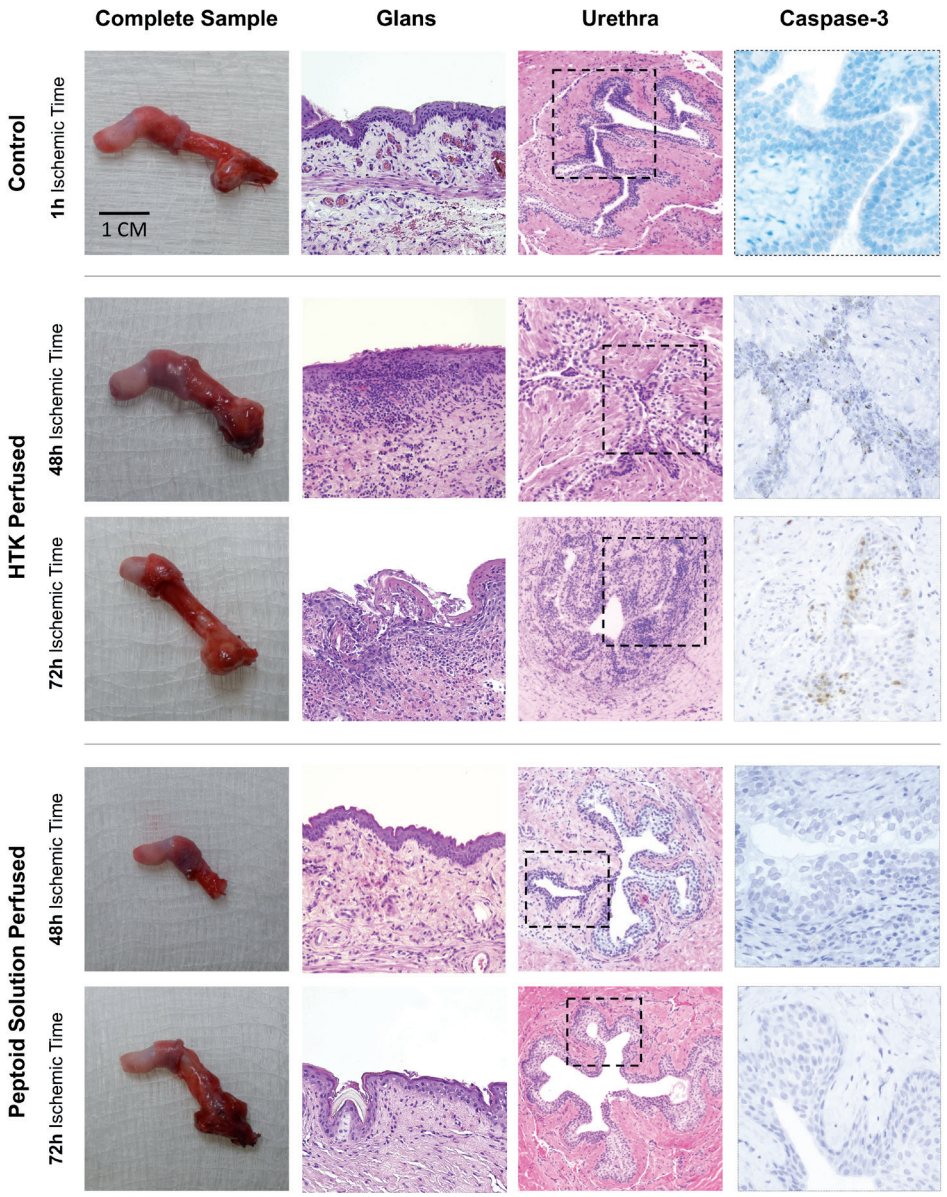


Figure 5: H&E histology and Caspase 3 immunohistochemistry of distal tissue samples at POD30. Differences between intervention groups were most evident after 48 and 72h of ischemia. There was significantly increased inflammation in the glans skin and distal urethra in samples from HTK groups but not PS groups. Immunostaining for caspase-3 in distal urethra shows more necrotic (Caspase-3+) cells in HTK group at 48h and 72h than in the samples from the PS group at 48 and 72h. The differences found for histological distal inflammation and cell death at POD30 are an underrepresentation of the clinical reality. At POD30 fully necrotic tissue in both HTK and PS groups had been lost and grafts had healed to the point where the remaining tissue had clinically recovered. Distal samples in the control groups at POD30 are in most cases from an area far proximal from intervention group samples where all tissue had survived.

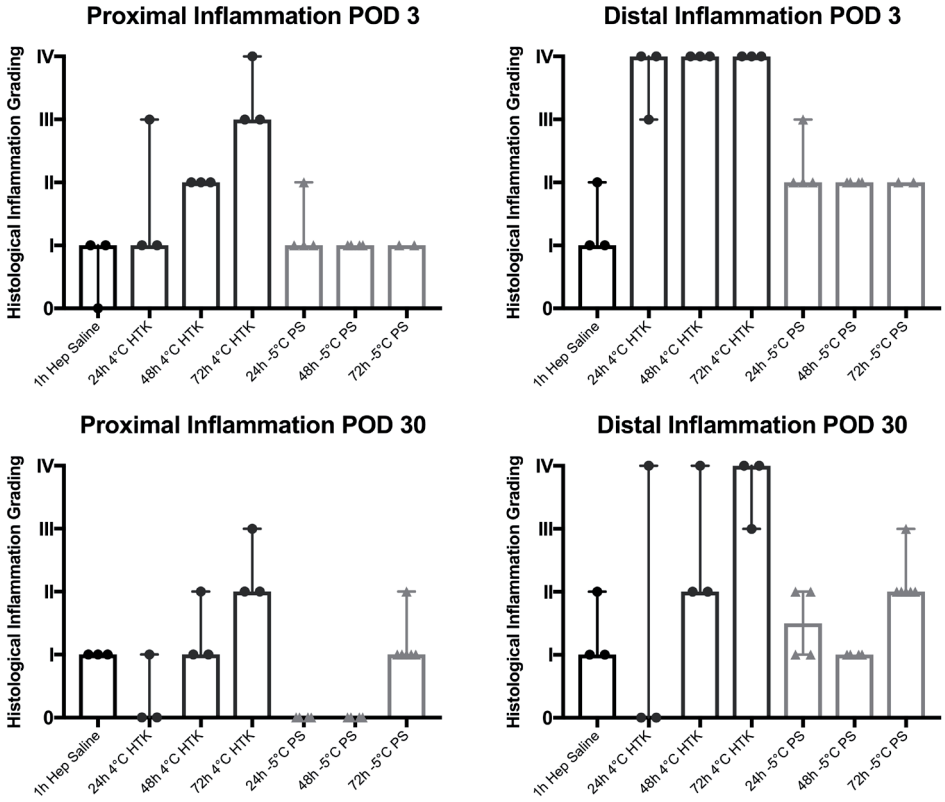


Figure 6: Histological inflammation. In all grafts inflammation was more pronounced at POD3 compared to POD30. A trend was found where PS perfused samples had lower inflammation grades compared to HTK perfused samples at all timepoints. *Histological Inflammation Grading Scale* **Grade 0:** No or rare inflammatory infiltrates. Minimal to mild urethritis may be present. **Grade I:** Mild perivascular inflammation with no involvement of epidermis. Minimal to mild urethritis may be present. No to minimal infiltration of tunica albuginea or corpora cavernosa. **Grade II:** Moderate-to-severe perivascular inflammation with mild epidermal involvement and mild to moderate urethritis. Mild to moderate Infiltration of tunica albuginea without involvement of corpora cavernosa. **Grade III:** Dense inflammation and epidermal involvement with apoptosis, dyskeratosis, or keratinolysis. Moderate-to-severe urethritis with focal urothelial ulceration. Moderate-to-severe inflammation of tunica albuginea and corpora cavernosa. **Grade IV:** Severe inflammation with necrosis of epidermis, urothelium, tunica, and/or corpora.

Post-transplant H&E

Minimal ischemia control grafts displayed mild, mostly perivascular situated inflammatory infiltrate at POD3 and POD30. Samples taken from a more proximal part of the graft tended to display lower inflammation grades (median I°, range 0-I°) than those taken from a more distal part (median I°, range I-II°) (**Figure 5&6**).

Grafts that were preserved with HTK at 4°C displayed, especially in distal samples irrespective of the timepoint, higher levels of inflammation than minimally ischemically injured samples or grafts stored in PS at -5°C. The median inflammation grade in

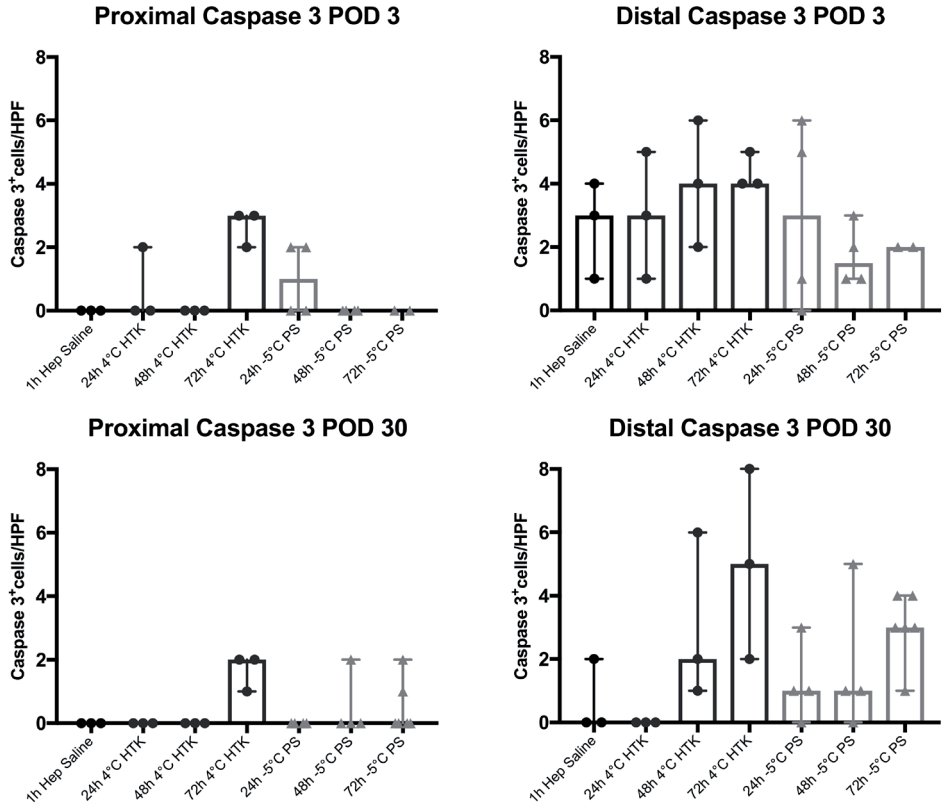


Figure 7: Post transplant viability after staining with activated Caspase-3. Distal tissue samples displayed higher levels of Caspase-3+ cells than proximal ones for all groups. Caspase-3 expression was significantly increased in proximal HTK samples stored for 72h compared to the other samples at POD3 (P=0.027) and POD30 (P=0.036). At POD3 distal Caspase-3 levels were comparable between all groups (P=0.50). At POD30, Caspase-3 expression was significantly higher in samples stored with HTK and subjected to prolonged (≥ 48h) preservation time (P=0.035). Caspase-3 Scoring: Nuclear staining was considered positive and positive cells were counted in 40xHPF in area of staining.

distal tissue samples at POD3 for HTK at 24h, 48h and 72h was IV° compared to II° in PS preserved grafts at the same timepoints (**Figure 6**).

Inflammation levels decreased in most groups until POD30, with shorter storage times experiencing a more pronounced reduction in inflammatory infiltration. A complete overview of histological grading and imaging for each group can be found in **supplements 5&6.**

Post-transplant viability

Overall, immunohistochemistry for Caspase-3 showed that distal tissue samples displayed higher levels of Caspase-3^{+} cells than proximal ones. In proximal samples, compared to the other samples taken at similar timepoints, Caspase-3 expression

was significantly increased in HTK samples stored for 72h at POD3 (P=0.027) and POD30 (P=0.036). Early after transplantation, distal Caspase-3 levels were comparable between all groups (P=0.50) and only late after transplantation, Caspase-3 expression was significantly higher in samples stored with HTK and subjected to prolonged (≥ 48h) preservation time (P=0.035) (**Figure 7**).

Discussion

In this work, we report on the efficacy of a subzero preservation technique utilizing a novel antifreeze peptoid containing solution to improve graft survival after exposure to prolonged periods of ischemia. PS was superior in preserving vascularized composite allografts (rat penis) for extended periods of time before transplantation compared to a standard static cold storage with HTK at all time points, including after 72h of ischemia. All grafts transplanted after 24h and 48h of subzero ischemia using PS at -5°C had favorable clinical outcomes, with no proximal or distal necrosis, while clear clinical signs of tissue damage were observed when using HTK at 4°C, even at the shortest time point of 24h of ischemic time.

Tissue preservation by supercooling has been studied for decades[26] and has been shown to be effective in the ex vivo preservation of human livers in combination with machine perfusion.[27] No clinical application of supercooling in complex tissues has however been reported and many challenges remain for the field.[28] The use of fully synthetic non-protein chemomimetics of antifreeze proteins (peptoids) found in freeze-tolerant animal species is a novel approach to supercooling and has the expected advantage of limited toxicity to cells in vivo. This study's use of peptoids with simple one-time perfusion and static storage yielded graft survival results comparable to a study applying supercooling in combination with machine perfusion in rat livers using University of Wisconsin (UW) and 3-O-methyl-D-glucose solution[29] and a study preserving mouse hearts using UW solution with added PEG, glucose, trehalose, and lidocaine.[30] Though these studies use different organ models and HSP approaches, their outcomes confirm our study's findings regarding the viability of HSP for short-term tissue preservation.

For clinical application, the minimal metabolic activity achieved through LSP (below -100°C) makes it the most suitable approach for long-term tissue storage (weeks-months). High subzero preservation as used in this study is most suited for the short term (days), as metabolic activity of tissues preserved at high subzero temperatures remains at levels that cannot enable long term preservation.[9,28] Of all preservation strategies, HSP however has the lowest barrier for clinical implementation as it does not necessarily require highly trained personnel and high-end equipment such as ultra-low temperature freezers or normothermic perfusion devices. Translation of HSP to the clinic could thus result in rapid application of this relatively simple approach.[9]

Study limitations and avenues for improvement

Our current study does not provide insight into the response of microvasculature to the perfusion with cryoprotectant. As such, we do not know with certainty if every part of the graft was perfused by sufficient amounts of perfusate with the current approach or that longer perfusion and larger volumes of perfusate are needed for a higher success rate at ischemic times of 72h and beyond. Similarly, it is not known if the amount of perfusate the grafts are stored in has significant impact on the outcomes. This study's other limitations are the limited sample size and heterotopic transplant model that does not allow for urinary function. Lastly, the study only tested a single preservation temperature and cooling/rewarming approach and did not measure in-graft temperatures during the preservation process. Additional research could elicit the role varying temperatures and cooling/rewarming rates play in preservation success.

Conclusions and future perspectives

In this study we showed reliable complete survival of syngeneic rat vascularized composite allografts after ischemia time of 48h using subzero preservation at -5°C and a novel cryoprotectant solution. Improvements to the preservation solution and protocols will be needed for reliable complete graft survival after 72h of ischemic time. Further studies are needed to assess tissue-to-volume perfusion ratios of the cryoprotectant, complete cryoprotectant tissue perfusion, and translateability to human-sized grafts. This work lays the foundation for extending tolerable ischemia time using subzero temperature in conjunction with novel preservation solutions, which would ultimatly allow global transport of transplantable tissues and organs.

Acknowledgements

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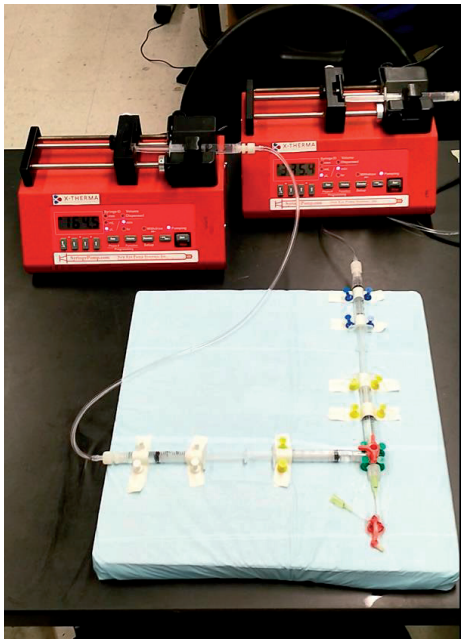
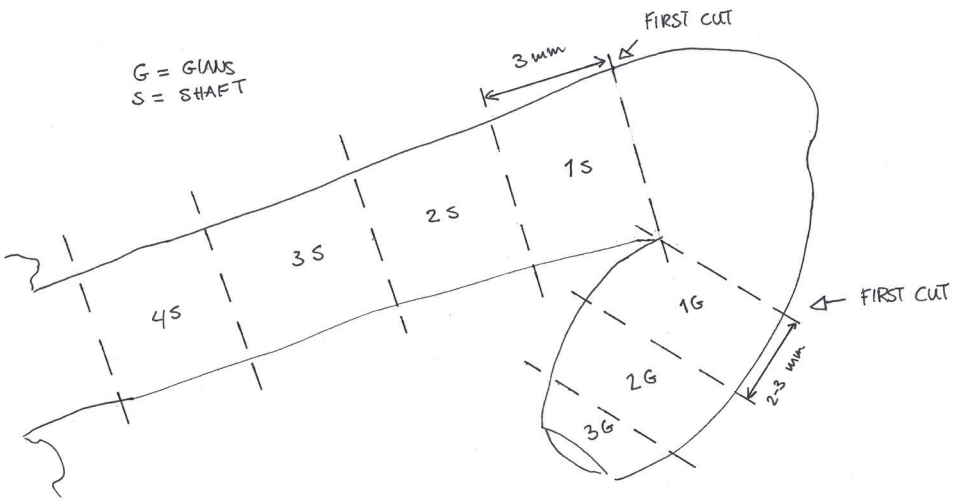
Supplemental information



Supplement 1: Video showing the linearly increasing gradient perfusion of a cannulated penile graft using preprogrammed linked pumps.



Supplement 2: Figure showing standardized sectioning locations of penile grafts.

Group	Animal ID	Description	Tissue Sampling	Photos	Date of transplant	Epidermolysis POD	Minor Necrosis (<50% of glans) POD	Major necrosis (>50% of glans) POD	Complete necrosis (complete graft) POD
Baseline	A1	No ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	Feb 20th 2019	none	none	none	none
Saline	A2	No ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	March 1st 2019	none	none	none	none
	A3	No ischemia	POD3	Postop, POD1, POD3	March 7th 2019	none	none	none	none
	A4	No ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	Apr 15th 2019	none	none	none	none
	A5	No ischemia	POD3	Postop, POD1, POD3	May 21st 2019	none	none	none	none
	A6	No ischemia	POD3	Postop, POD1, POD3	May 21st 2019	none	none	none	none
Control	B1	24 hr cold ischemia	POD3	Postop, POD1, POD3	Jan 29th 2019	POD3	POD3	POD3	POD3
24 H	B2	24 hr cold ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	Jan 31st 2019	POD3	POD5	POD5	none
HTK	B3	24 hr cold ischemia	POD3	Postop, POD1, POD3	Feb 16th 2019	POD3	none	none	none
	B4	24 hr cold ischemia	POD3	Postop, POD1, POD3	Feb 19th 2019	POD3	POD3	POD3	POD3
	B5	24 hr cold ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	March 27th 2019	POD3	POD5	POD5	none
	B6	24 hr cold ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	April 3rd 2019	none	none	none	none
Control	C1	48 hr cold ischemia	POD11	Postop, POD 1,3,5,7,14,21,30	Jan 25th 2019	POD3	POD5	POD7	POD11
48H	C2	48 hr cold ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	Apr 12th 2019	POD3	POD7	POD7	none
HTK	C3	48 hr cold ischemia	POD30	Postop, POD 1,3,5,7,14,21,30	Apr 26th 2019	POD5	POD7	none	none
	C4	48 hr cold ischemia	POD3	Postop, POD1, POD3	Jun 5th 2019	POD3	POD3	POD3	none
	C5	48 hr cold ischemia	POD3	Postop, POD1, POD3	Jun 12th 2019	POD3	POD3	POD3	POD3
	C6	48 hr cold ischemia	POD3	Postop, POD1, POD3	Jun 12th 2019	POD3	POD3	POD3	none
Control	D1	72hr, 4C with HTK	Endpoint/POD30	Postop, POD 1,3,4	September 16 2019	POD3	POD3	POD3	POD4
72 H	D2	72hr, 4C with HTK	Endpoint/POD30	Postop, POD 1,3,4	September 16 2019	POD3	POD3	POD3	POD3
HTK	D3	72hr, 4C with HTK	Endpoint/POD30	Postop, POD 1,3,5	September 16 2019	POD3	POD5	POD5	none
	D4	72hr, 4C with HTK	POD3	Postop, POD 1,3	September 23 2019	POD3	POD3	POD3	POD3
	D5	72hr, 4C with HTK	POD3	Postop, POD 1,3	September 23 2019	POD3	POD3	POD3	POD3
	D6	72hr, 4C with HTK	POD3	Postop, POD 1,3	September 23 2019	POD3	POD3	POD3	POD3
XT-Vivo	X1	24hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	Apr 12th 2019	none	none	none	none
24 H	X2	24hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	Apr 23rd 2019	none	none	none	none
	X3	24hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	Apr 30th 2019	none	none	none	none
	X4	24hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	May 21st 2019	none	none	none	none
	X5	24hr, -5C with XT-vivo	POD3	Postop, POD1, POD3	June 10th 2019	none	none	none	none
	X6	24hr, -5C with XT-vivo	POD3	Postop, POD1, POD3	Aug 28 2019	none	none	none	none
	X7	24hr, -5C with XT-vivo	POD3	Postop, POD1, POD3	Aug 13 2019	none	none	none	none
	X8	24hr, -5C with XT-vivo	POD3	Postop, POD1, POD3	September 4 2019	none	none	none	none
Y1	Y1	48hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	June 6 2019	none	none	none	none
48 H	Y2	48hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	June 6 2019	none	none	none	none
	Y3	48hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	June 6 2019	none	none	none	none
	Y4	48hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	June 11 2019	none	none	none	none
	Y5	48hr, -5C with XT-vivo	POD3	Postop, POD 1, POD3	June 21 2019	none	none	none	none
	Y6	48hr, -5C with XT-vivo	POD3	Postop, POD 1, POD3	June 26 2019	none	none	none	none
	Y7	48hr, -5C with XT-vivo	POD3	Postop, POD 1, POD3	June 26 2019	none	none	none	none
	Y8	48hr, -5C with XT-vivo	POD3	Postop, POD 1, POD3	Aug 28 2019	none	none	none	none
XT-Vivo	Z1	72hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	August 26 2019	POD3	POD3	none	none
72 H	Z2	72hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	August 26 2019	POD1	POD3	none	none
	Z3	72hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	August 26 2019	POD3	POD3	none	none
	Z4	72hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	September 9 2019	POD5	none	none	none
	Z5	72hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	September 9 2019	POD5	none	none	none
	Z6	72hr, -5C with XT-vivo	POD30	Postop, POD 1,3,5,7,14,21,30	September 9 2019	POD3	POD3	none	none
	Z7	72hr, -5C with XT-vivo	POD3	Postop, POD 1,3	September 30 2019	POD3	POD3	none	none
	Z8	72hr, -5C with XT-vivo	POD3	Postop, POD 1,3	September 30 2019	POD3	POD3	none	none

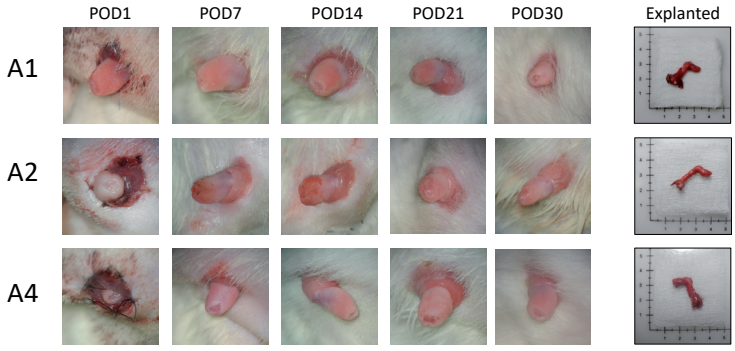
Supplement 3: Complete overview of all samples included in the study with surgery date and occurrence of necrosis.

▼ **Supplement 4:** Complete macroscopic overview of all samples included in the study for each post-operative day (POD) 3,5,7, 14, 21 and 30.

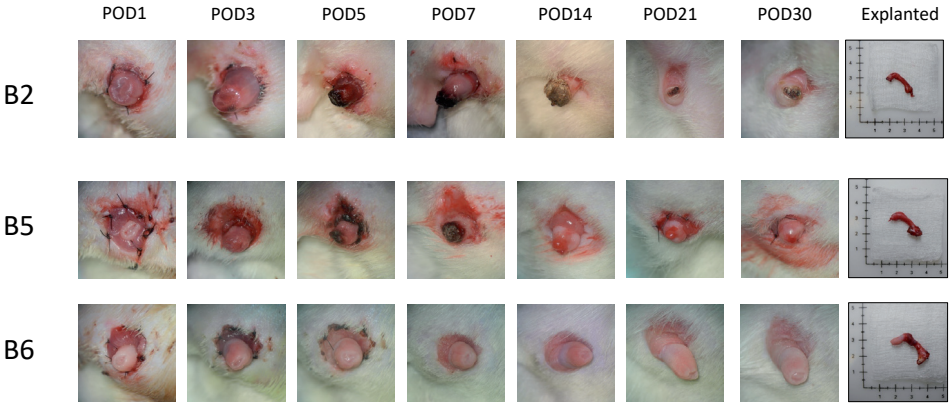
Study groups

Group	Preservation solution	Preservation temperature	Preservation time	Number in Group
A	Saline	4 °C	1 Hour	6
B	HTK	4 °C	24 Hours	6
C	HTK	4 °C	48 Hours	6
D	HTK	4 °C	72 Hours	6
X	XT-vivo	-5 °C	24 Hours	8
Y	XT-vivo	-5 °C	48 Hours	8
Z	XT-vivo	-5 °C	72 Hours	8

Minimal Ischemia (1H Hep Saline Flushed)



24 Hour 4°C Ischemia (HTK perfused)



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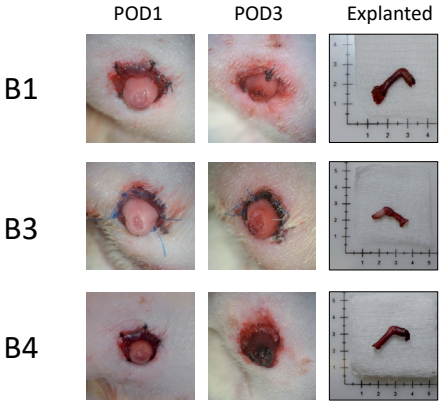
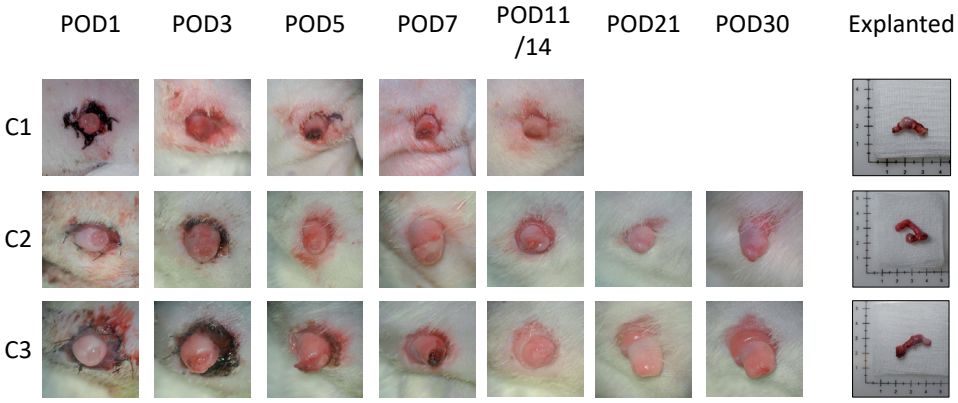


48 Hour 4°C Ischemia (HTK Perfused)



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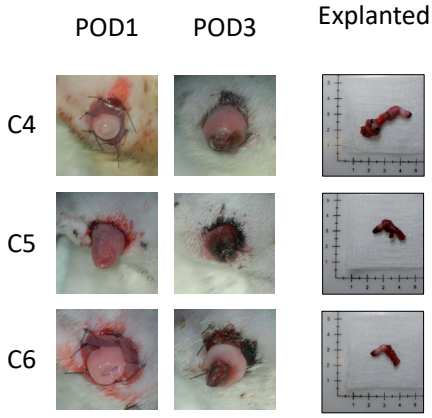
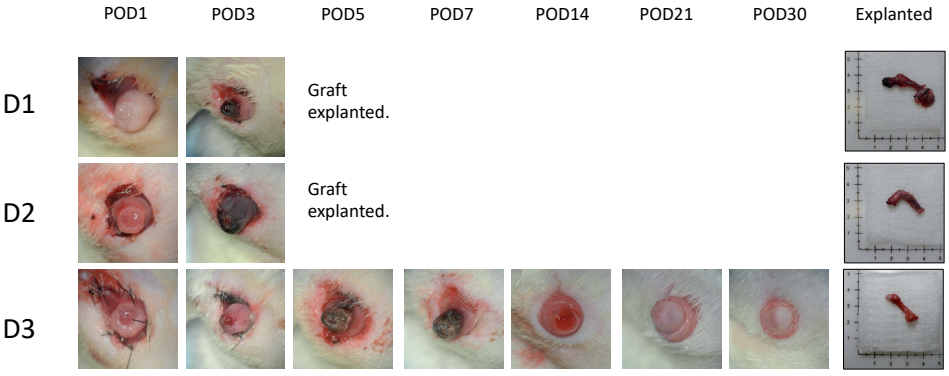
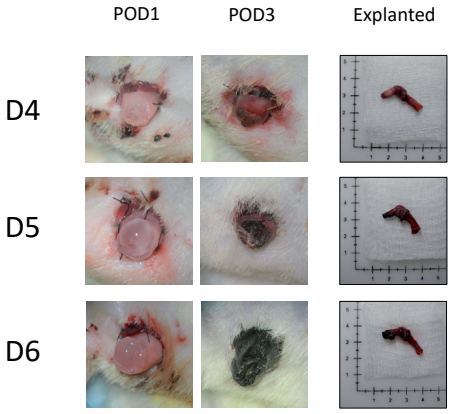


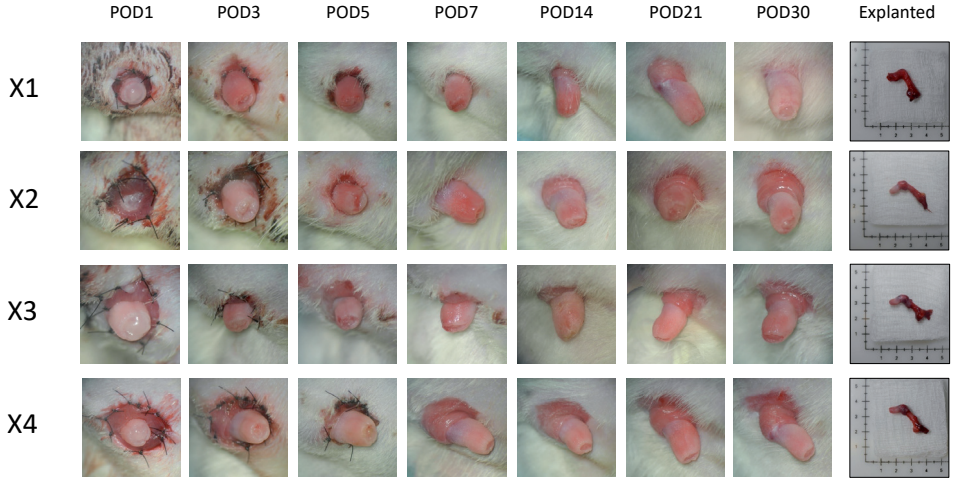
72 Hour 4°C Ischemia (HTK perfused)



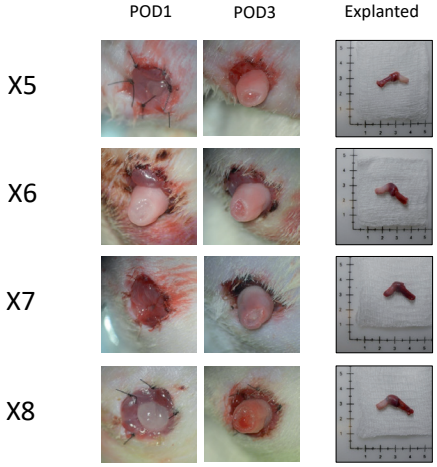
72 Hour 4°C Ischemia (HTK perfused)



24 Hour -5°C XT-vivo perfused)



24 Hour -5°C Supercooled (XT-vivo perfused)



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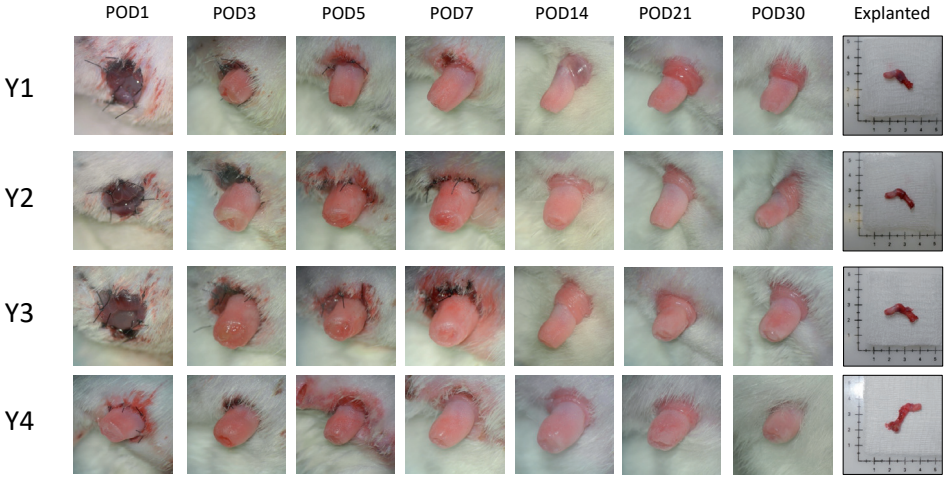
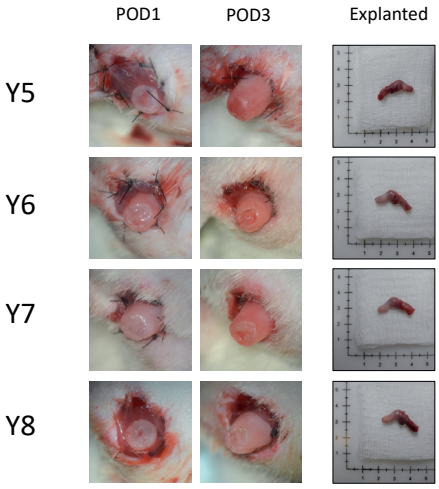
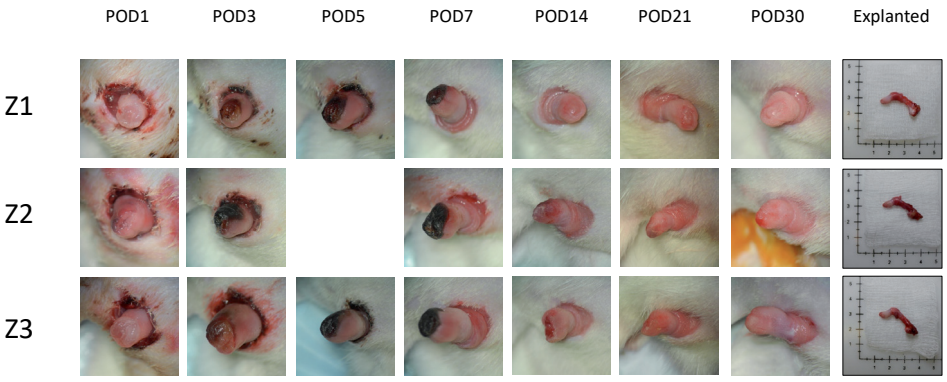


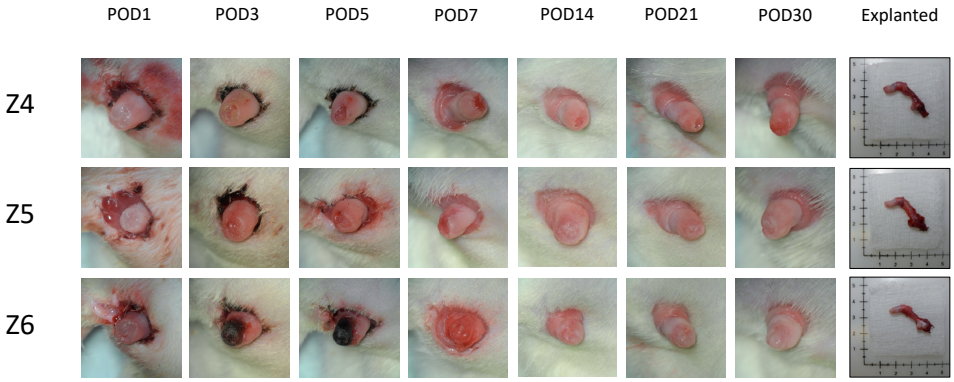
48 Hour -5°C Supercooled (XT-vivo perfused)



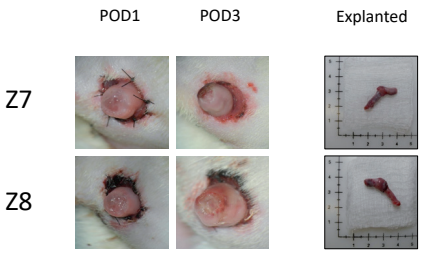
72 Hour -5°C Supercooled (XT-vivo perfused)



72 Hour -5°C Supercooled (XT-vivo perfused)



72 Hour -5°C Supercooled (XT-vivo perfused)



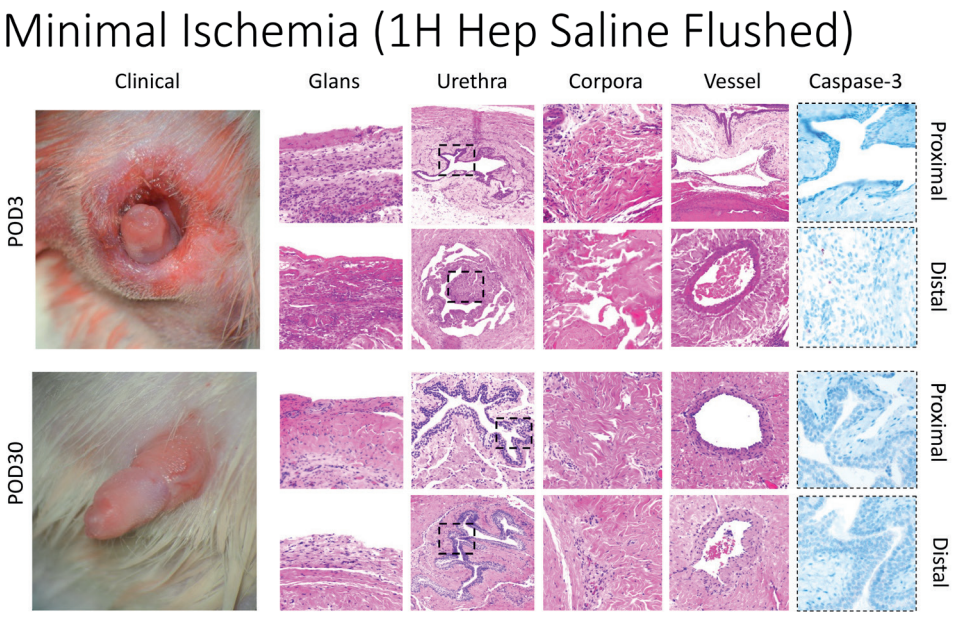
Supplement 5: Table 1: Graft survival for each study group. For each group the percentage of grafts suffering minor distal necrosis (<50% of glans), major distal necrosis (>50% of glans) or complete graft necrosis at POD3 and POD30 is given, with raw numbers in brackets. Table 2: Histological grading of transplants. This table shows the histological grading in the different groups. Proximal and distal inflammation is graded on a 0-4 scale detailed in the legend of **figure 6**. Grades are given for each sample. Caspase 3 positive cells were counted in 40xHPF in area of staining and are given for each sample with the average in brackets.

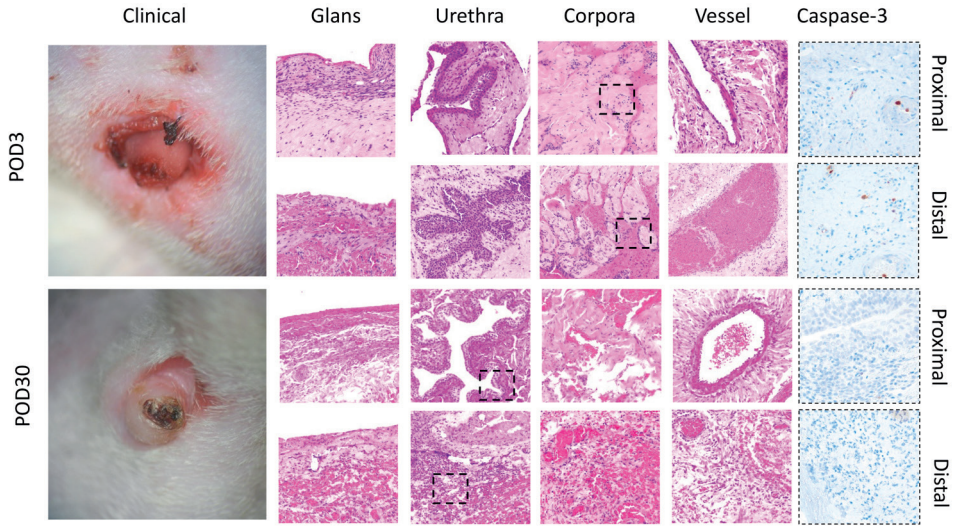
Group	Preservative	Purpose	Transplant group size N=	Minor distal necrosis at POD3	Minor distal necrosis at POD30	Major distal necrosis at POD3	Major distal necrosis at POD30	Complete graft necrosis at POD3	Complete graft necrosis at POD30
1	Saline	1h Control	6	0% (0/6)	0% (0/3)	0% (0/6)	0% (0/3)	0% (0/6)	0% (0/3)
2	HTK	24h Control	6	33% (2/6)	67% (2/3)	33% (2/6)	67% (2/3)	33% (2/6)	0% (0/3)
3	HTK	48h Control	6	50% (3/6)	100% (3/3)	50% (3/6)	67% (2/3)	17% (1/6)	33% (1/3)
4	HTK	72h Control	6	83% (5/6)	100% (3/3)	83% (5/6)	100% (3/3)	66% (4/6)	66% (2/3)
5	Peptoid Solution	24h Intervention	8	0% (0/8)	0% (0/4)	0% (0/8)	0% (0/4)	0% (0/8)	0% (0/4)
6	Peptoid Solution	48h Intervention	8	0% (0/8)	0% (0/4)	0% (0/8)	0% (0/4)	0% (0/8)	0% (0/4)
7	Peptoid Solution	72h Intervention	8	75% (6/8)	67% (4/6)	0% (0/8)	0% (0/6)	0% (0/8)	0% (0/6)

Supplemental Table 2: Graft inflammation grades and caspase-3 stain results.

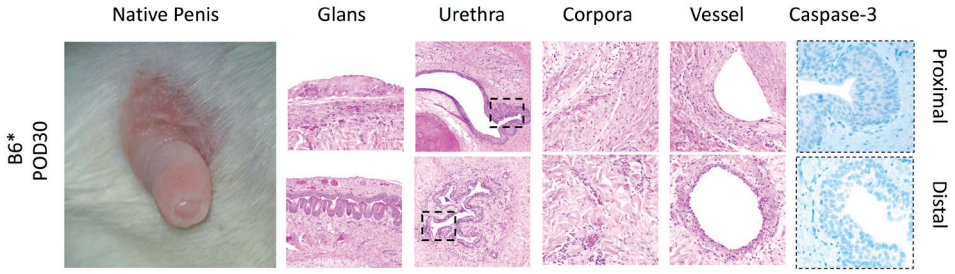
Group	Proximal Inflammation Grade*	Distal Inflammation Grade*	Prox Casp3 (positive cells/ HPF)	Dist Casp3 (positive cells/ HPF)
1H Hep Saline				
POD30 (N=3)	1,1,1	1,1,2	0,0,0	0,0,2
POD3 (N=3)	1,1,0	1,1,2	0,0,0	1,3,4
24h 4C HTK				
POD30 (N=3)	0,0,1	0,0,4	0,0,0	0,0,0
POD3 (N=3)	1,1,3	3,4,4	0,0,2	1,3,5
48h 4C HTK				
POD11/30 (N=3)	1,1,2	2,2,4	0,0,0	1,2,6
POD3(N=3)	2,2,2	4,4,4	0,0,0	2,4,6
72h 4C HTK				
POD 30 (N=3)	2,2,3	3,4,4	1,2,2	2,5,8
POD3 (N=3)	3,3,4	4,4,4	2,3,3	4,4,5
24h -5C PS				
POD30 (N=4)	0,0,0,0	1,1,2,2	0,0,0,0	0,1,1,3
POD3 (N=4)	1,1,1,2	2,2,2,3	0,0,2,2	0,1,5,6
48h -5C PS				
POD30 (N=4)	0,0,0,0	1,1,1,1	0,0,0,2	0,1,1,5
POD3 (N=4)	1,1,1,1	2,2,2,2	0,0,0,0	1,1,2,3
72h -5C PS				
POD30 (N=6)	1,1,1,1,1,2	2,2,2,2,2,3	0,0,0,0,1,2	1,3,3,3,4,4
POD3 (N=2)	1,1	2,2	0,0	2,2

▼ **Supplement 6:** Complete overview of representative histological images of proximal and distal H&E (glans, corpus, urethra, vessel) and Caspase 3 (Urethra) for each group at POD3 and POD30.



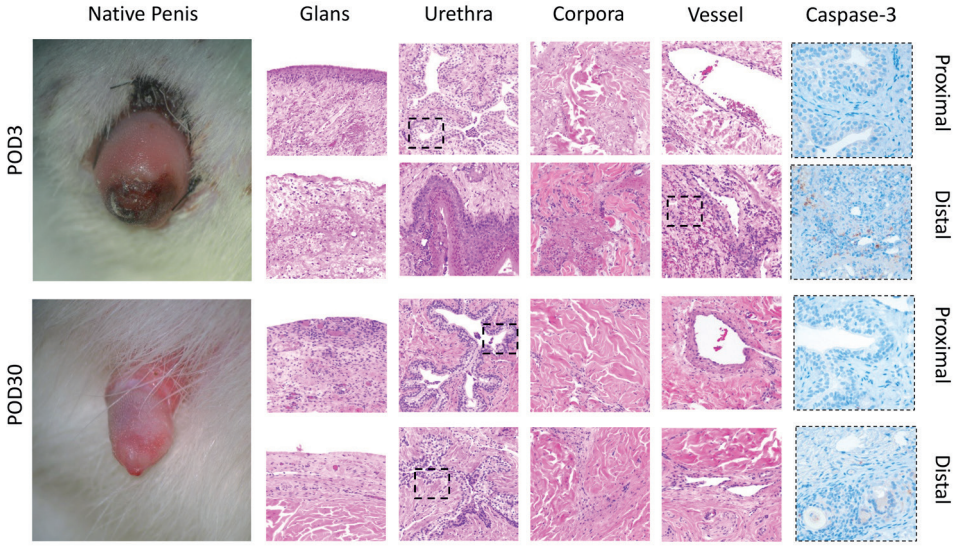


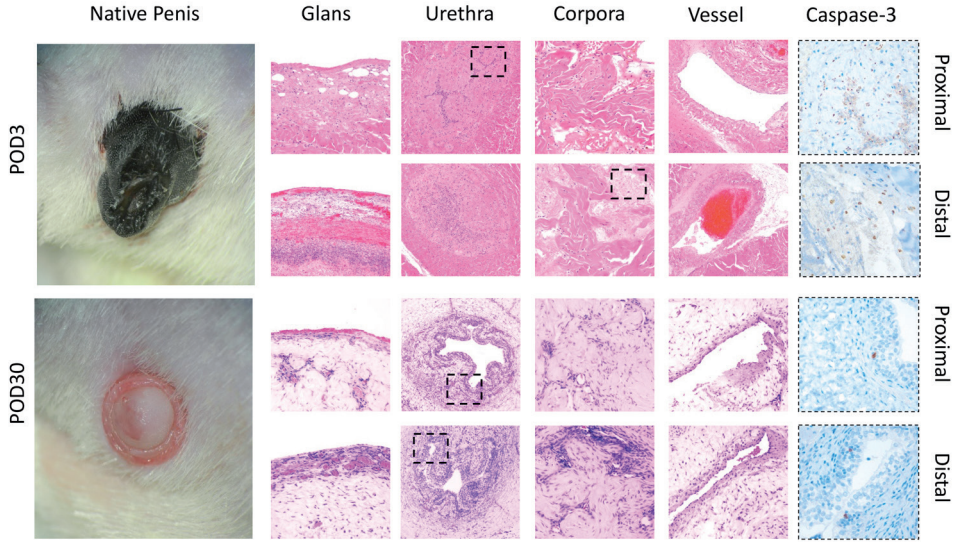
24 Hour 4°C Ischemia (HTK perfused)

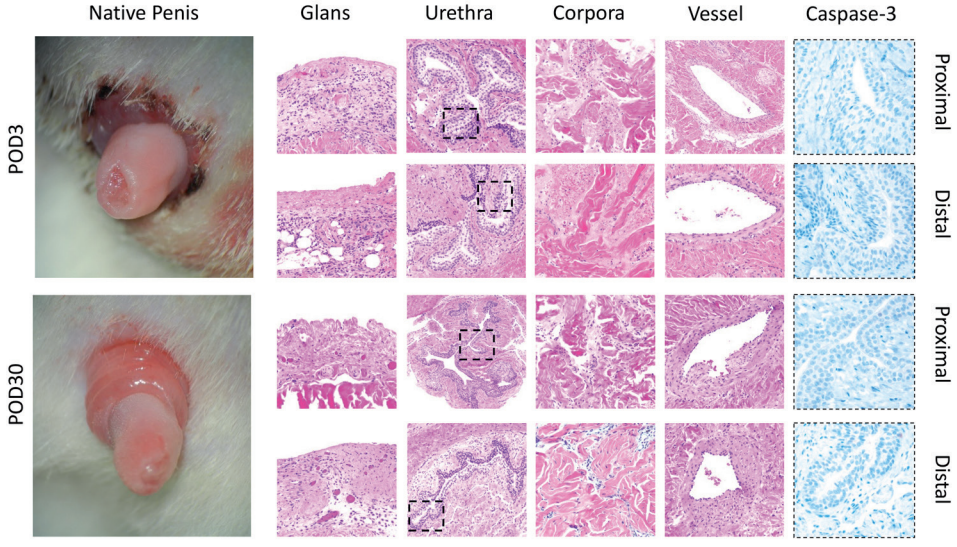


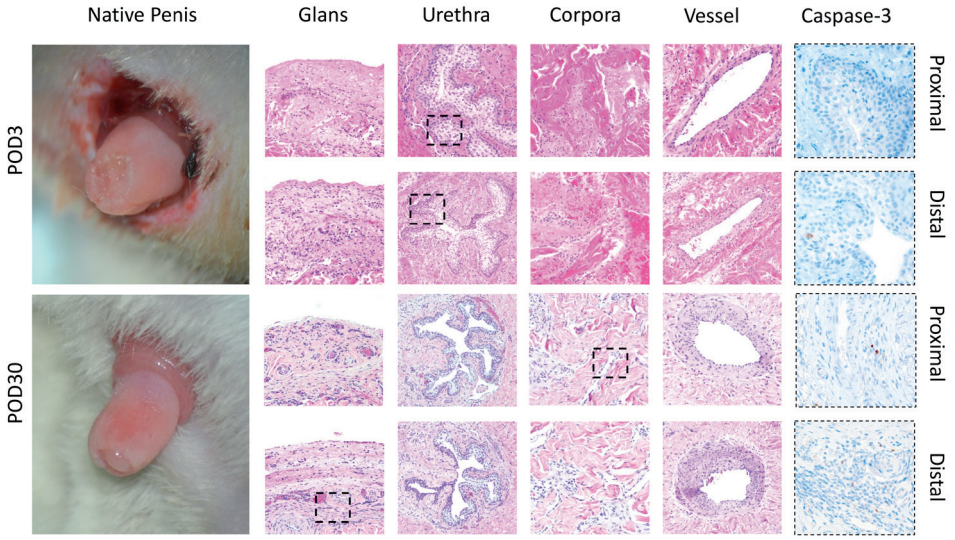
* One graft clinically fully survived despite 24h of ischemia

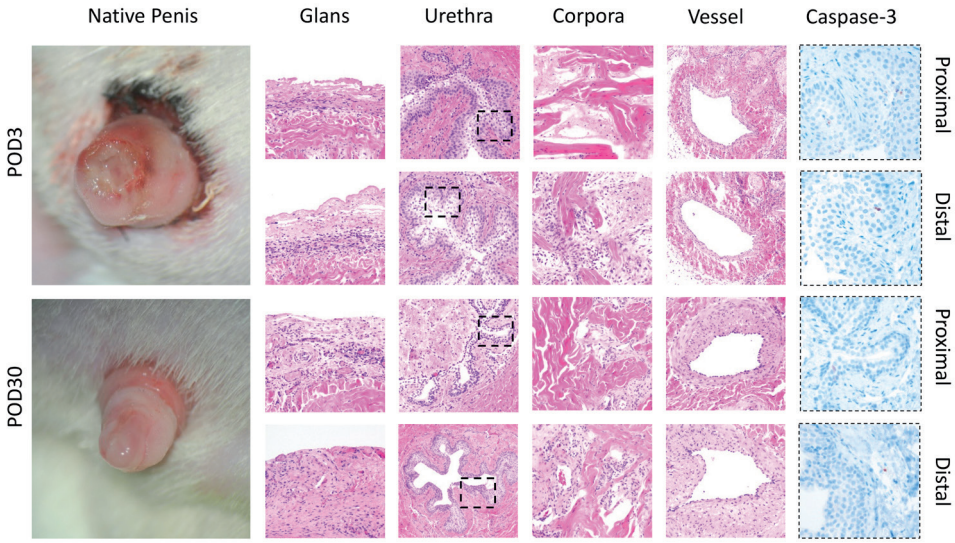
48 Hour 4°C Ischemia (HTK Perfused)




72 Hour 4°C Ischemia (HTK perfused)
Native Penis
Glans
Urethra
Corpora
Vessel
Caspase-3
POD3
POD30
Proximal
Distal
Proximal
Distal

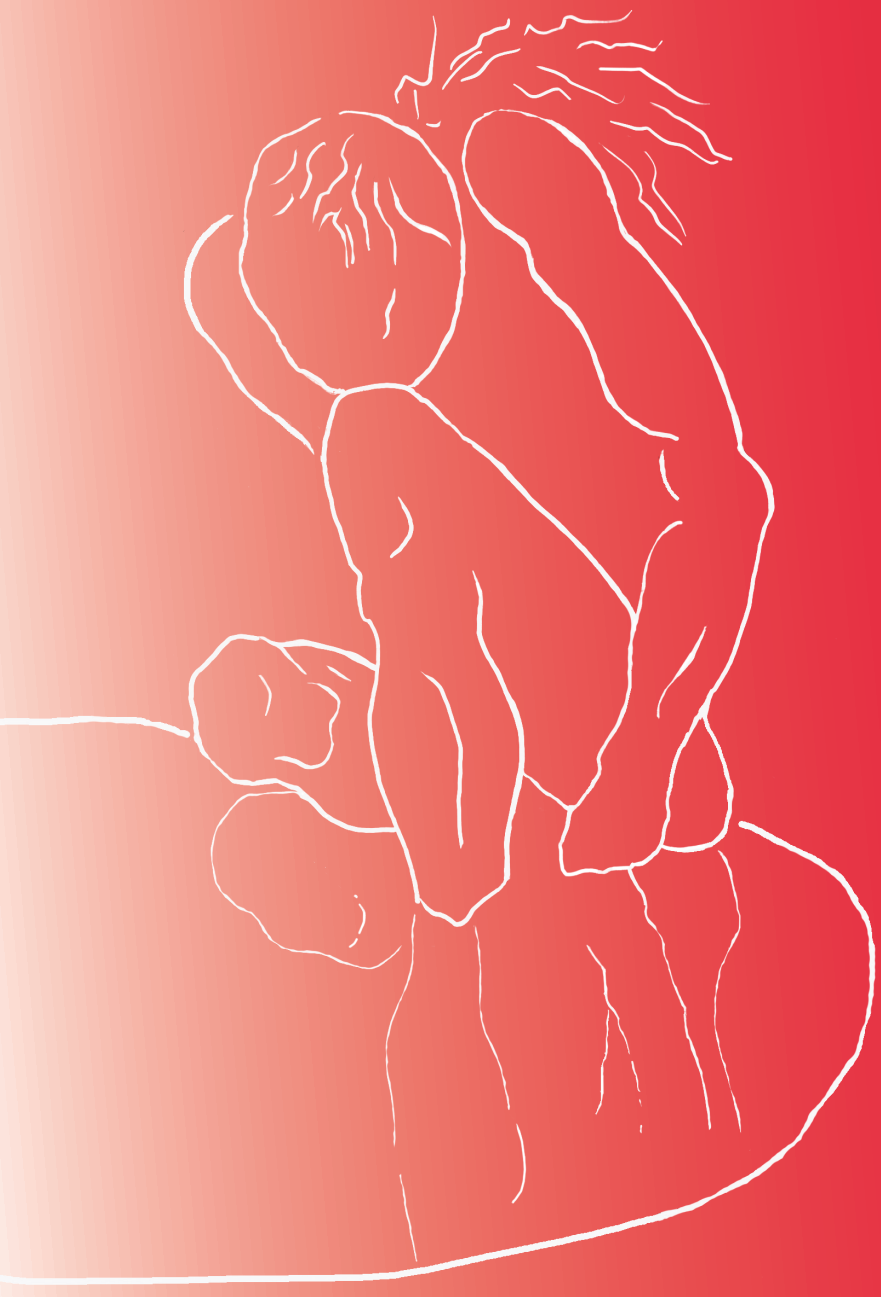

24 Hour -5°C Supercooled (XT-vivo perfused)
Native Penis
Glans
Urethra
Corpora
Vessel
Caspase-3
POD3
POD30
Proximal
Distal
Proximal
Distal


48 Hour -5°C Supercooled (XT-vivo perfused)
Native Penis
Glans
Urethra
Corpora
Vessel
Caspase-3
POD3
POD30
Proximal
Distal
Proximal
Distal


72 Hour -5°C Supercooled (XT-vivo perfused)
Native Penis
Glans
Urethra
Corpora
Vessel
Caspase-3
POD3
POD30
Proximal
Distal
Proximal
Distal

Part IV

Ethical clinical application of penile transplantation



Chapter 7

Experience Establishing and Maintaining a Penile Allotransplantation Program: Ethical Considerations and Transplant Program Obligations

Submitted

Samuel A.J. Fidder, MD[1,2*]; Carisa M. Cooney, MPH[1*]; Jordan L. Coleman, MDiv[1]; Gerald Brandacher, MD[1]; Jaimie T. Shores, MD[1]; Joanna W. Etra, MD[1]; Dalibor Vasilic, MD[2]; W.P. Andrew Lee, MD[3]; Frederick S. Berlin, MD, PhD[4]; Jeremy Sugarman, MD, MPH, MA[5]; Jeffrey Kahn, PhD[5]; Richard J. Redett, MD[1†]; Damon S. Cooney, MD, PhD[1†]

1. Department of Plastic and Reconstructive Surgery; Johns Hopkins University School of Medicine, Baltimore, MD, USA
2. Department of Plastic and Reconstructive Surgery; Erasmus Medical Center, Rotterdam, The Netherlands
3. UT Southwestern Medical School, Dallas, TX, USA
4. Department of Psychiatry and Behavioral Sciences; Johns Hopkins University School of Medicine, Baltimore, MD, USA
5. Johns Hopkins Berman Institute of Bioethics; Johns Hopkins University School of Medicine, Baltimore, MD, USA

*Co-first authors (These authors contributed equally)
†Co-last authors (These authors contributed equally)

Abstract

Background: Vascularized composite allotransplantation is currently used as an experimental treatment option for profound physical defects. To date, four successful penile transplants have been performed worldwide. Penile transplantation raises ethical questions that must be properly addressed when it is offered and used.

Methods: A description of the guiding principles of the Johns Hopkins Human Penile Allotransplantation Program, which outlines the clinical and ethical obligations that accompanyoffering penile transplantation.

Results: In select patients, penile transplantation promises significant advantages over conventional reconstruction in terms of urethral reconstruction and voiding, donor site morbidity, return of erogenous sensation and the ability to have sexual intercourse. In providing penile transplants special consideration must be given to informed consent, patient selection, public opinion, privacy, and post-transplant psychosocial support.

Conclusions: It is paramount that all team members involved in penile transplantation proceed ethically to enable the availability of this procedure to appropriate patients who stand to benefit from it and respect these patients' right to choose care that best aligns with their goals.

Introduction

Vascularized composite allotransplantation (VCA) is a viable treatment option for devastating physical defects. To date, over 213 VCA procedures have been performed globally, with upper extremity transplantation being the most common.[1–6] VCA centers in the United States include programs for lower extremity, abdominal wall, uterus, and penile transplantation.[7] Given the limited treatment options provided by conventional reconstruction, a team at the Johns Hopkins University School of Medicine combined its expertise in VCA[1,6,8–12], conventional penile reconstruction[13–15], and transplant immunology[16,17] to offer experimental penile transplantation as a part of genitourinary reconstructive care (IRB #NA_00089306).

To date, five penile transplants have been reported worldwide. The first attempt, performed in China in 2006, was removed 14 days post-transplantation, due to psychological rejection by the patient.[18,19] The second transplant, performed at the Tygerberg Hospital in South Africa in 2014, was succesfully integrated both physiologically and psychologically by the recipient enabling the successfull impregnation of his partner.[20–22] Later transplants were performed at the Massachusetts General Hospital[23], again at Tygerberg Hospital, and at the Johns Hopkins Hospital.[24] With the exception of the initial Chinese case, all patients reportedly have had good initial outcomes with limited rejection episodes, proper voiding function, and varying degrees of erectile function.

However, penile transplants involve particular ethical issues that must be addressed by programs considering performing them.[25] In a previous articel (Ledibabari et al, 2019) we addressed ethical concerns surrounding penile transplantation and program guidelines.[26] In the current article, we discuss the establishment and maintainance of an active human penile transplantation program consistent with these guidelines and outline the clinical and ethical obligations that accompany offering penile transplantation.

Why Penile Transplantation? Demand for New Treatment Options in Genitourinary (GU) Injuries

Since the release of a 2011 report by the Surgeon General of the Army's Dismounted Complex Blast Injury Task Force,[27] the subject of mutilating GU injuries sustained by active-duty service members has gained an increasing amount of attention. Per this report, the frequency of GU trauma rose sharply in the latter years of Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF).[27,28] Increased use of improvised explosive devices and the tactical need for ground troops contributed to the significant spike in GU injuries uncommon in previous wartime conflicts.[27–29]

While injury rates from OEF and OIF raised awareness regarding penile injury, penile loss secondary to trauma, cancer, or congenital absence or deformity of the penis also affects civilians.[30] Despite a paucity of literature, GU injuries have profound

effects on sexual, psychological, and social health.[31] Soldiers who have experienced GU trauma have higher prevalence of both post-traumatic stress disorder (PTSD) and depression.[27] Among cancer patients with penile loss, psychological effects include issues with intimacy, which often lead to complete avoidance of sexual encounters and considerable strain on relationships with their partners.[31–33] For single men, the prospect of discussing penile loss can be unbearable and prohibit forming romantic relationships. One patient shared: "I felt like [the injury] banished me from a relationship, […] I struggled with even viewing myself as a man for a long time."[34] Anecdotes of soldiers asking not to be saved in the case of severe GU injury are not uncommon and speak to the strong psychological ties between a man's penis and his sense of identity.[28]

Penile reconstruction using a radial forearm flap, commonly considered the best reconstructive option for a neophallus, may be precluded for recipients who might be concerned that the distinctive scar could raise unwanted questions regarding their medical histories. Additionally, individuals who sustain devastating penile injuries often have concomitant injuries affecting the forearms, excluding this donor site. For individuals with total penile loss, penile transplantation may provide a more acceptable option and may be the only viable option for those who have insufficient tissue/donor sites for autologous reconstruction as seen with war-related blast injuries. Penile transplantation replaces like with like, using tissue that has the same function and aesthetics as the part it replaces. Successful reconstructive transplants restore the appearance, anatomy, and function of the recipient in a manner expected to be superior to autologous reconstruction. This expectation is based on our combined knowledge of the patient population, previous experience with traditional phalloplasty and reconstructive transplantation, and cadaver studies.[8–10,14,24,35]

Comparing Potential Benefits and Harms of Penile Transplantation and Reconstruction

Penile reconstruction and transplantation require consideration of several functional and physical aspects to assess the risk/benefit ratio associated with any proposed approach. Several of these considerations are presented here.

Urinary function. Urinary function and standing voiding are primary goals of penile reconstruction. However, the most prevalent complications associated with a neophallus are urethral strictures and fistulas,[36] which pose significant reconstructive challenges and may require multiple surgical revisions. Given that a penile transplant involves anastomosing normal urethra to normal urethra, the expectation (and limited clinical experience) is that these complications occur significantly less frequently compared to a phalloplasty which has a neourethra partially constructed out of tubed skin.

Erectile function. Currently, there is no perfect option for sexually functional penile reconstruction, leaving demand for innovation.[37] Autologous free flaps used to create a neophallus[38] lack erectile tissue, making insertion of an implant necessary for

successful intercourse. Patients who undergo phalloplasty have a high prosthesis extrusion and infection rate, due to the flap's inability to provide long term support of the implant.[14,39] This can cause significant scarring and necessitate implant removal. With penile transplantation, spontaneous erections are possible when the recipient is feeling sexually aroused,[24] potentially obviating the need for an erectile prosthesis. If spontaneous erection does not occur following transplant, an implant can be placed inside the existing corporal structure, which is associated with much lower extrusion rates compared to implants placed outside corpora when studied in conventional prosthesis reconstructions in native penises.[40]

Erogenous function. The speed of nerve regeneration, which is approximately 1mm/day, poses limitations for full functional recovery of VCA.[41] Nerve regeneration in autologous penile reconstructions occurs within 4-12 months of nerve coaptation and return of erogenous sensation is common.[13] Based on these data, one would expect return of sensation in a transplanted penis with proper coaptation of the dorsal sensory nerves.[24] However, as reported for conventional reconstructions, there is a risk of diminished sensation or function when nerve coaptation is performed, and candidates should be made aware through the counseling and consent process that their transplanted penis may feel different from their original penis. Currently, clinical experience and evidence are limited and the potential benefits cannot be guaranteed.

Donor sites. Autologous reconstruction causes considerable donor site scarring. The most commonly used and preferred forearm flap leaves an easily visible scar, potentially threatening patients' privacy, and is not available to some patients due to previous injury and/or tattooing. In patients who have sustained considerable concomitant injuries such as extremity amputations as seen in combat-injured servicemen, few-to-no acceptable donor sites may be available. Transplantation provides an intact penis without creating donor site morbidity and provides a treatment option for patients lacking acceptable donor sites.

Immunosuppression. Lifelong immunosuppression presents significant risk of harm in penile transplantation, as evidenced by the solid organ transplant literature.[35-37] This need necessitates adherence to a strict regimen that could prove especially taxing for patients with comorbidities such as PTSD. Additionally, chronic immunosuppression puts patients at a higher risk of kidney failure, cancer, vascular disease, and infections.[42–44] Younger candidates for this procedure are more likely to experience adverse events related to immunosuppression due to potentially longer lifetime exposure.

Cost. Penile transplantation promises to have a substantial impact on the lives of recipients and their partners. However, the initial surgery and post-operative care is costly and necessitates life-long follow-up care that can be resource-intensive. Conventional reconstruction (phalloplasty) also incurs high operative and post-operative care costs for initial reconstruction, planned revisions, and to address complications; however, it does not incur costs for lifelong immunosuppression and its potential complications. Thus far, cost/benefit analyses comparing penile transplan-

tation and traditional phalloplasty have been difficult due to the limited experience in penile transplantation. As long-term complications and benefits become known, proper analyses should be performed to determine the return-on-investment of penile transplantation.

Practical Measures to Help Ensure Ethically Appropriate Penile Transplantation

Assessing the potential benefits provided by penile transplantation and the manageability of its potential harms, it is clinically and ethically reasonable to consider this option in appropriately selected candidates. Accordingly, we designed our program to weigh the potential benefits against the associated costs and treatment side effects for particular patients, incorporate rigorous patient selection to identify appropriate candidates, cultivate collaborative relationships with organ procurement organizations (OPO) to appropriately approach potential donor families, and address the pre- and post-transplantation obligations associated with the procedure. We have focused our efforts on seven areas to help ensure the ethically appropriateapplication of penile transplantation at our center: (1) patient selection; (2) informed consent; (3) access to transplantation and availability; (4) public opinion and organ donation; (5) privacy for candidate recipients and donors; (6) post-transplant psychosocial support; and (7) ensuring lifelong care.

Patient Selection

Patient selection is critical to successful upper extremity and face transplants.[12,45] Comprehensive screening processes to exclude insurmountable medical and psychosocial challenges help predict post-transplant compliance with immunosuppressive treatment, physical and occupational therapy, regular clinic visits, and vigilant graft monitoring. While optimal functional recovery from penile transplantation does not require occupational or physical therapy, adherence to the immunosuppression regimen, clinic visit schedules, and graft monitoring are crucial.

Given the limited world experience in penile transplantation, we initially offered the procedure to patients aged 18-69 years who had lost 75% or more of their penis due to traumatic injury. In these cases, the anatomy was expected to be predictable, allowing for reasonable re-approximation of vessels, nerves, urethra and corporal bodies. Following the successful transplant in our first patient[24] and that performed by Cetrulo et al,[23] we expanded our eligibility criteria to include penile cancer extirpation patients who are in remission for five or more years and/or genotypic males with congenital absence of the penis or severe penile insufficiency. Employing this treatment for gender affirmation surgery may represent a fourth patient population;[46] however, this application of VCA has its own subset of anatomic, cultural, and ethical considerations that should be addressed before including this indication.

Penile transplantation is one among several reconstructive options. Having multiple options enables patients to make informed decisions regarding their care and choose the treatment that best aligns with their goals. Educating and counseling patients on all feasible treatment options, including forgoing treatment, is imperative to respecting their autonomy. This process, which includes detailed discussions between the patient (and sometimes their partners) and the transplant team, is vital to identifying individuals for whom a penile transplant may be appropriate. As a non-life-saving procedure, the bar for candidacy is higher than that for solid organ transplantation (SOT). Therefore, the patient must be an anatomically and physiologically good candidate for transplantation and for the hours-long transplant surgery. In addition to substantial penile shaft loss, eligibility for penile transplantation includes the absence of many infectious diseases, having low-to-no donor-specific antibodies to minimize risk of rejection post-transplant, absence of significant (micro)vascular disease, and being confirmed cancer-free prior to initiating life-long chronic immunosuppression. Additionally, programs should determine the range of skin-tones the recipient will find acceptable in the transplanted graft.

Careful consideration must also be given to potential candidates' psychological and psychosexual health and social support. Candidate screening should include comprehensive evaluations with a transplant psychologist, a transplant social worker, and a psychologist/psychiatrist experienced in treating patients with sexual dysfunction. The objective is to ensure the candidate's readiness for VCA in general (e.g., identify existing social support, access to resources, ability to take post-transplant medications as prescribed) and for penile transplants in particular (e.g., attitudes regarding intimacy, ability to accept a graft from a deceased donor as his own). Mental health professionals specializing in sexual dysfunction address pre-transplant issues related to sexual function and relationships and explore expectations of post-transplant penile graft function. Establishing baseline psychological and psychosexual status is imperative to identifying any past challenges that could resurface under the physical and emotional stress experienced during and after transplantation. This process also helps develop realistic post-transplantation expectations regarding functional outcomes over time. It should be made clear to candidates whose injury includes the loss of the testes that penile transplantation will not restore reproductive health. Sexual function and activity, considered successful outcomes for penile transplants, may lead to contracting a sexually-transmitted infection (STI), which may result in rejection and partial or full loss of the graft. Therefore, education in STI prevention is an important part of counseling patients how best to care for their transplant.

During the screening process all candidates meet with a transplant social worker to determine their current networks of social, emotional, and financial support. Social workers may assist patients in developing financial plans or connecting patients to resources to improve their ability to comply with care recommendations or to attend follow-up appointments, all of which are expected to improve patient's post-trans-

plant outcomes. As with all VCA, it is important to provide psychological support as needed to transplant candidates and their family members/primary caretakers to set expectations and to prepare them for and support them during life post-transplant. Should the candidate have an intimate partner (i.e., spouse, significant other), the intimate partner should have the opportunity to meet with the psychologists/psychiatrists and transplant social worker to identify any barriers to supporting the candidate.

Informed Consent

Penile transplantation is not an appropriate reconstructive solution for every patient with penile loss and as described earlier is associated with substantial risks. Therefore, obtaining informed consent is essential. The information provided during the informed consent must be complete and balanced, and updated as needed to maximize patient understanding and decision-making. Misrepresentation of the procedure, even if unintentional, must be avoided; to facilitate this, we include a non-conflicted consent designee unaffiliated with the protocol (discussed below). As patients can spend months-to-years on the transplant waiting list, consent is reconfirmed at predetermined intervals (e.g., annually) and in the event of substantial changes effecting the procedure's risk/benefit ratio.

Due to the complexity of the intervention, we employ a stepwise approach to informed consent, separating the process into three discrete steps: consent to a screening interview by phone, consent to a week-long screening process, and consent to transplantation. The complexity of and risks associated with the procedures included in the week-long screening process present sufficient justification for stratifying the consent process. Consent for transplantation is obtained after participants have been found to be physically and psychologically eligible and the multidisciplinary transplant team has formally approved the candidate's eligibility. The team checks in with patients who have provided consent to transplant wait-listing at least quarterly to confirm ongoing eligibility, continued interest in the procedure, and address any questions patients may have. This schedule of regular interactions has the added benefit of complying with United Network for Organ Sharing (UNOS) requirements for active transplant candidates.

The Consent Designee. To help ensure voluntary informed consent, our program employs a consent designee. This individual is not a member of the study team and independently obtains informed consent from candidates after candidates have reviewed all aspects of the transplant process, and its risks, benefits and alternatives with the study team. The consent designee may act as a liaison between the patient and the study team, relaying any questions or concerns the patient may have to the investigators. While the consent designee does not need a medical background, we selected a pediatric urology nurse who has extensive experience working with congenital penile insufficiency patients and their families. Notably, although the consent

designee is not a study team member, our Institutional Review Board requires that they review and approve of the consent designee's qualifications and that they be trained in how to obtain informed consent.

Access to Transplant and Availability

In the United States, penile transplantation falls under the oversight of the Organ Procurement and Transplantation Network Final Rule (42 CFR part 121).[47,48] As such, penile transplant programs may only be run at institutions with specific resources, such as an active, UNOS-registered SOT program. This may mean that certain candidates are geographically remote from penile transplant programs. Study teams need to be sure to connect candidates with transplant social workers and patient financial services to form realistic expectations of short- and long-term costs associated with treatment and any accompanying travel needed to secure necessary treatment, and to identify long-term strategies for fiscally managing being a transplant patient. Finally, creating collaborative care plans to enable transplant candidates/recipients to obtain standard lab work and care that is both close to home and coordinated with the transplant team may be crucial to providing sustainable, uninterrupted care for transplant recipients. Making such arrangements can be challenging in rural areas. Therefore, it is vital to explore resources local to the participant's home pre-transplant and, when located, be aware of and realistic about barriers to attending regular medical appointments (e.g., distance, reliable transportation).

Public Opinion and Organ Donation

While VCAs are regulated by UNOS,[49] they are not implicit organs of donation indicated by the "Organ Donor" designation on state identification/driver's licenses, meaning they cannot be donated without obtaining specific consent from a donor's family. At present, the demand for penile transplants is low, making implicit donation unnecessary. A broader concern is that requests for penis donation, which may be seen by some as distasteful or offensive, should not undermine efforts to obtain life-saving organ donations. We have addressed this in two ways. First, we have worked to educate the public that, as with other VCAs, penis donation requires a specific, additional request and consent process that potential donors' family/legally authorized representatives may decline while still providing consent to the donation of solid organs and other tissues. Second, our team works closely with our local OPO to craft recipient-specific inquiries that, with the recipient's consent, allow OPO family coordinators to share certain pieces of recipient information with the potential donor's loved ones.

Privacy

Candidates and Recipients. Patient privacy and confidentiality is critical. Unlike face or hand transplantation, penile transplantation is readily concealed through normal attire, making the transplant undetectable to the general public. Loss of recipient's

privacy may severely impair future social interactions, romantic or otherwise, and destroy any psychological improvements gained through the transplant.

To help protect participant privacy, penile transplant study participants have the opportunity to receive their care under an alias. All other standard privacy protections are in place to preserve confidentiality. While information regarding availability of penile transplantation programs may be released proactively to help educate the public about this reconstructive option, no individually identifiable patient information is released in these communications without extensive counseling and the express written consent of the patient.

Additionally, care teams need to have HIPAA-compliant protocols in place for handling sensitive patient care images (e.g., penis/genitalia) that are shared electronically. Smart devices and technologies make it easier and more cost-effective for patients to communicate remotely with their care teams, particularly when determining rejection episodes manifesting on the skin. It should be clear that such images are being shared to obtain medical care and plans for secure photo storage and disposal should be specified.

Donors and Donor Families. Following standard donation privacy practices for deceased donor transplantation, no information about the donor is revealed without the express written consent of the donor family. This includes information that may not normally be considered protected health information (i.e., state in which the graft was procured), as the uniqueness of this type of transplant may make donor identification possible. Consent may only be obtained from the donor family by the OPO per standard protocols; the study team and transplant recipient may not contact the donor family without the donor family's express written consent. Nonetheless, part of the consent process for both the donor family and the recipient includes alerting both parties that it ultimately may not be possible to protect their privacy.[26] To this end, efforts are made to educate donor families and recipients about the impact their own disclosures regarding the transplant may have on the other party's privacy.

Finally, efforts are made to make it clear that transplantation of germline tissues (testes/sperm/gametes) from the donor will not be performed. Transplanting this tissue could potentially result in the creation of offspring with the deceased donor's genes, raising complex ethical concerns. Therefore, we work diligently with patients, the OPO, and the press to clearly communicate to potential recipients, their partners, the lay public, and potential donor families that germline tissues will not be transplanted.

Post-Transplant Psychosocial Support

As with all our other transplant programs, our center has a post-penile transplantation support plan. This includes education on rejection episodes and opportunistic infection risk as well as psychological counseling. Patients are educated about symptoms of graft rejection and self-monitoring practices (e.g., redness, rashes, swelling), symptoms of possible treatment side effects (e.g., opportunistic infections) and decreased

kidney function or kidney failure. In addition, education is provided about hand hygiene, dietary considerations, recommended immunizations, frequency of blood draws, and other best practices related to maintaining their health as a transplant recipient. This information is included in a comprehensive genitourinary transplant handbook, given to and reviewed with recipients prior to hospital discharge. In addition to the transplant psychologists and/or social workers who are available to all of our transplant patients, we have included psychologists and/or psychiatrists who specialize in sexual dysfunction in our penile transplant team. These specialists are available to patients to guide them through the first stages of life with a penis transplant with special consideration for interactions with intimate partners. While we are able to extend this counseling to include significant others, patients and significant others must independently choose to engage in counseling sessions.

Ensuring Lifelong Care

Since penile transplantation necessitates lifelong immunosuppression and care, the transplant team considers a transplant program as one that will span decades in which the patient has a reliable team to turn to for advice or when complications arise. This is particularly important since penile graft longevity is currently not known. Plans for re-transplantation or restoration following explantation need to be agreed upon prior to waitlisting a patient for transplantation. The procedure should only be performed when the team is sure that either they themselves or additional healthcare providers local to the patient will provide the necessary care in consultation with the transplant team. Local support is vital to continuity of care and provides recipients with ongoing medical support should the penis transplant program dissolve.

Conclusion

Penile transplantation can be an appropriate option for treatment of penile loss or insufficiency in select cases. Long-term patient outcomes will provide further evidence as to the procedure's risk/benefit ratio. It is paramount that all centers involved in penile transplantation proceed in an ethically sound manner to ensure availability of this procedure to severely injured patients and respect these patients' right to choose care that best aligns with their goals. We look forward to learning from other centers' experiences and revising our practices as needed to continue providing thoughtful and ethical care.

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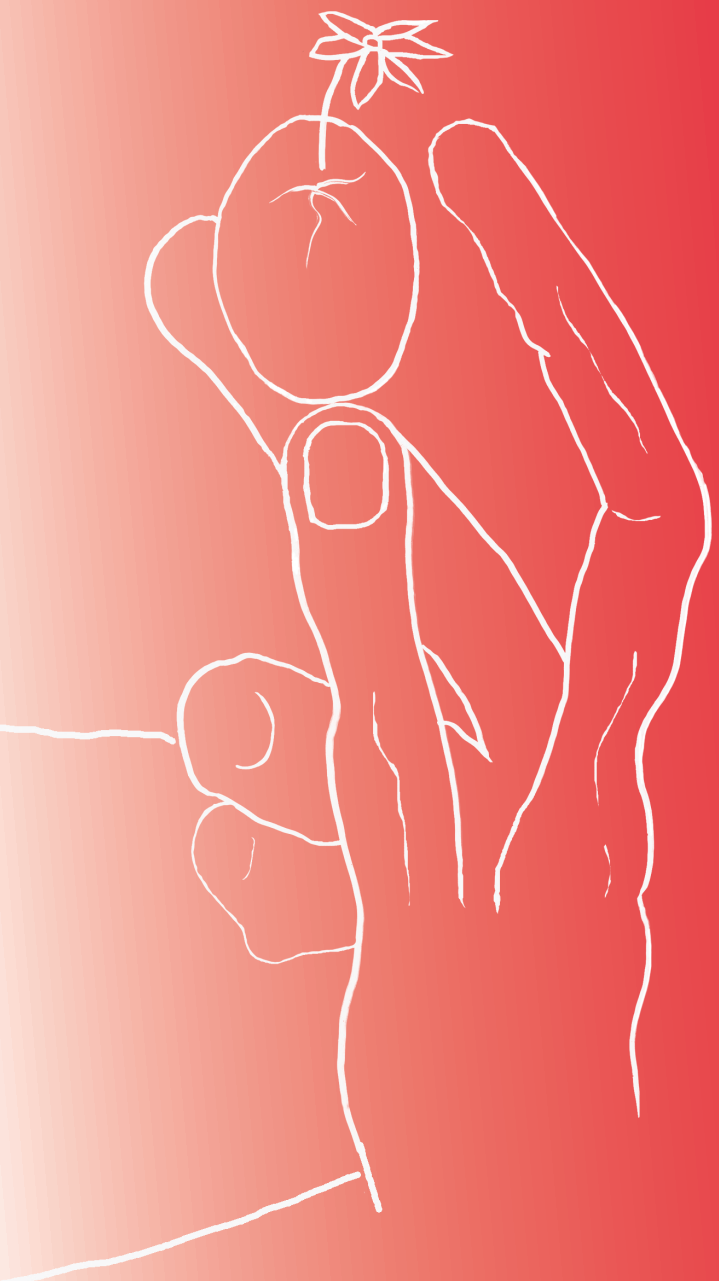

Chapter 8

Total Penis, Scrotum, and Lower Abdominal Wall Transplantation

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Richard J. Redett III MD[1*], Joanna W. Etra MD[1], Gerald Brandacher MD[1], Arthur L. Burnett MD MBA FACS[2*], Sami H. Tuffaha MD[1], Justin M. Sacks MD MBA[1], Jaimie T. Shores MD[1], Trinity J. Bivalacqua MD[2], Stephen Bonawitz MD[3], Carisa M. Cooney MPH[1], Devin M. O'Brien-Coon MD[1], Aliaksei Pustavoitau MD[4], Nicole A. Rizkalla MD[4], Annette M. Jackson PhD[5], Vidhi Javia BS[1], Samuel A.J, Fidder MD[1], Janice Davis-Sproul[6], Daniel C. Brennan MD[7], Inbal B. Sander MD[8], Shmuel Shoham MD[9], Nikolai A. Sopko MD PhD[2], W.P. Andrew Lee MD[1], Damon S. Cooney MD PhD[1*]

1. Department of Plastic and Reconstructive Surgery, Johns Hopkins School of Medicine, Baltimore MD
2. Department of Urology, Johns Hopkins School of Medicine, Baltimore MD
3. Division of Plastic and Reconstructive Surgery, Cooper University Health Care, Camden, NJ
4. Department of Anesthesiology, Johns Hopkins School of Medicine, Baltimore MD
5. Department of Surgery, Duke University School of Medicine, Durham, NC
6. Cell Therapy Laboratory, Johns Hopkins School of Medicine, Baltimore MD
7. Division of Renal Medicine, Johns Hopkins School of Medicine, Baltimore MD
8. Department of Dermatology, Johns Hopkins School of Medicine, Baltimore MD
9. Division of Infectious Disease, Johns Hopkins School of Medicine, Baltimore MD

*co senior authors

Abstract

Male genital tissue loss can have devastating effects on function as well as psychosocial well-being of the injured patient. Conventional reconstructive efforts are often insufficient to restore full function of the male phallus. In 2018, we performed a transplantation of the penis, scrotum, and lower abdominal wall. One year later, the patient urinates without stricture, has near normal sensation of the penis, and has erectile function. The patient is optimistic and satisfied with the outcome with an improved self-image. Penile transplantation, while currently in the early stages of investigation, is a viable and comprehensive solution to male genital tissue loss.

Introduction

In Operations Iraqi Freedom and Enduring Freedom alone, more than 1300 male soldiers had diagnosed injuries to the genitalia, with >40% of those injuries to the penis and/or urethra.[1] Along with functional deficits incurred from penile tissue loss, these injuries carry a significant degree of psychosocial damage for affected patients.[2] Conventional genital reconstruction consists of microvascular phalloplasty, including a variety of procedures employing flap-based reconstructions using tissue from the arm or thigh.[3] A history of significant blast-related trauma poses difficulty in finding a suitable donor site for flap reconstruction and skin grafting, as these injuries are frequently associated with extremity amputation.[1] These different methods of neophallus reconstruction involve complex operative planning and staged surgical procedures for urethral reconstruction and insertion of a penile prosthesis. Urethral fistula formation and strictures are common complications when using fasciocutaneous flaps.[4] Because these penile reconstructive techniques carry significant limitations, including inability to reliably restore major functions of an adult male phallus, alternative methods for male genital restoration must be explored.

Recently, there have been four reported penile transplants: the first in Guangzhou, China in 2006, the next two in South Africa in 2014 and 2016, and the fourth at the Massachusetts General Hospital in 2017.[5–7] The first patient, who had a traumatic injury with loss of all but a small penile stump, had some venous congestion, segmental epidermis necrosis, and reported objections from his spouse; subsequently, the graft was removed on postoperative day (POD) 14. The first South African patient, who had penile loss after infection following ritual circumcision, reportedly gained full function and was able to successfully have intercourse resulting in a pregnancy. The patient transplanted at MGH had partial penectomy for penile cancer and was reported to have regained normal urination, proximal sensation, and partial erectile function. Our patient had loss of bilateral lower extremities, a portion of his lower abdominal wall, both of his testes, scrotum, perineum, and the entire penis after a devastating blast injury while serving in the Armed Forces. We here report on the first transplantation including the entire penis, scrotum, and partial abdominal wall.

Case Methods and Results

Detailed description of methodology of Preoperative Evaluation, Procedural Considerations, Infectious Prophylaxis Management, Immunomodulatory Regimen, Donor Bone Marrow Processing, Chimerism Analysis, and Antibody Screening can be found in the Supplementary Appendix.

Patient Characteristics

The patient presented is a U.S. Military Veteran in his early thirties who sustained traumatic penile loss from an IED explosion. His initial injury also resulted in bilateral lower

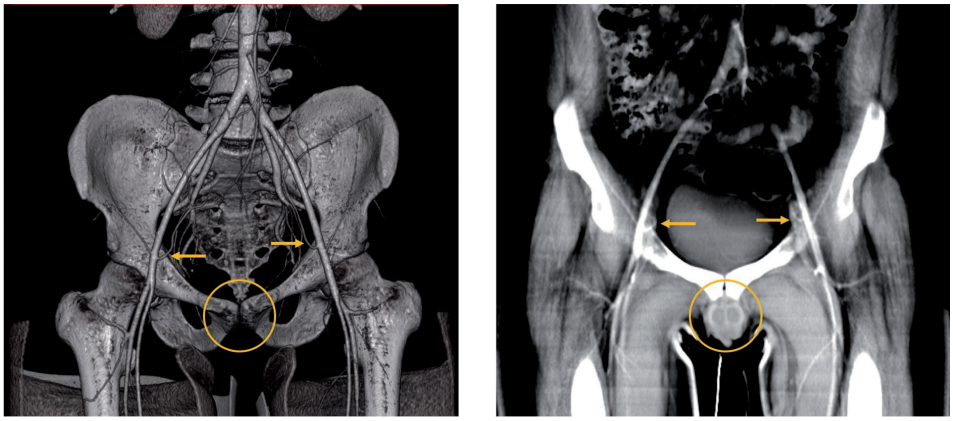

Figure 1: CT angiogram 3D reconstruction. Branches of internal iliac arteries extending toward the rectum and small gluteal vessel branches bilaterally can be seen without visualized dorsal penile or cavernosal arteries (circles). The vessels did not appear to go beyond the upper portion of the pubic symphysis without any significant vessels to the scrotum or penile region. Deep inferior epigastiric arteries were intact bilaterally (arrows).

extremity amputations, significant lower abdominal wall soft tissue injury, and bilateral traumatic orchiectomy and loss of his scrotum. He had no previous reconstructive efforts for his genitalia other than debridement and placement of a skin graft. He also had skin grafting to the abdominal wall, placed on top of rectus muscle. On physical exam, he had about 1.5cm of remnant penile tissue with a urethra at the end of the amputated shaft. Scrotal tissue was essentially absent, and he had no testes. Preoperative imaging revealed normal bilateral inferior epigastric, iliac, and femoral arteries **(Figure 1)** but the dorsal penile and cavernosal arteries were insufficient to support transplantation. MRI showed normal course of the neurovascular pudendal bundles bilaterally with no significant nerve damage noted in the pelvis to confound recovery from transplantation **(Supplemental Figure 1).**

The patient was evaluated and deemed to be an appropriate candidate by experts in sexual psychology, transplantation psychology/social work, and surgery. He was CMV negative, EBV positive, PRA negative, and did not have any other contraindications to transplantation. Due to the extent of the patient's injury and lack of adequate dorsal penile and cavernosal arteries, a surgical technique was developed utilizing the deep inferior epigastric arteries to revascularize the dorsal penile arteries and the graft supplemented by the external pudendal arteries as support to the tissues of the proximal shaft, groin, abdomen, and scrotum.[8]

Donor Characteristics

A donor match was identified by the regional organ procurement organization. Serologic tests of donor serum were positive for EBV IgG and toxoplasma IgG but were otherwise negative. The donor was ABO matched and cytotoxicity and flow cross-

match negative. The patient's serum tested negative for HLA class I and class II specific antibodies. The donor was mismatched for 8 of 12 HLA antigens encoded by HLA-A, -B, -C, DRB1, DQB1, and DPB1 loci.

Penile Transplant Technique

Once the allograft was procured, the recipient team prepared the surgical site and exposed the required vessels and nerves. The transplant began with a primary urethroplasty. The donor and recipient urethra were spatulated, and interrupted 3-0 and 4-0 vicryl sutures were used to perform the anastomosis over a 16-French Foley catheter. The corporal anastomosis was performed next using a running 3-0 monocryl suture repair of the tunica albuginea. The vascular anastomoses included both donor dorsal arteries and veins anastomosed to the recipient's deep inferior epigastric arteries and veins. Next, both recipient dorsal nerves were coapted to the donor graft dorsal nerves. Fluorescence angiography demonstrated perfusion of the glans and most of the shaft but inadequate perfusion of the groin, abdomen, and scrotal tissues. The left donor external pudendal artery was taken with a segment of femoral artery and revascularized by end to side anastomosis to the recipient femoral artery. At the end of the vascular anastomoses, a SPY™ fluorescence imager (NOVADAQ Stryker, Kalamazoo MI) demonstrated complete perfusion of the graft.

Immunomodulatory regimen comprised alemtuzumab and steroid induction, tacrolimus maintenance monotherapy, and a donor bone marrow infusion on POD14, the details of which are included in the Supplementary Appendix.

The patient's postoperative course was notable for a large scrotal hematoma which required a washout five hours after completion of the transplant. At three weeks, no anastomotic leaks were detected on urethrogram.

Immunologic Monitoring

Skin biopsies were performed at weeks 1, 2, 3, 4, 6, 12, 24 (+/- 7 days). Protocol biopsies have shown predominantly no evidence of acute cell-mediated rejection, and few have shown sparse perivascular lymphocytic infiltrate compatible with grade 1 rejection according to the Banff grading system for skin-containing VCA (Figure 3A and B).[9] Multimodal imaging (3-CCD, infrared, and digital) was performed at times of biopsies (**Figure 2**, Supplemental Figure 2).

Postoperatively, the patient has experienced three episodes of slight erythema and rash to the graft. All three of these episodes were treated promptly with topical clobetasol +/- topical tacrolimus with quick and full resolution, at no point requiring increase in systemic tacrolimus or addition of steroid bolus therapy. A biopsy at the time of the third episode showed a moderate perivascular and perineural inflammatory infiltrate within the reticular dermis with admixed eosinophils, compatible with grade II rejection (**Figure 3C**).[9]

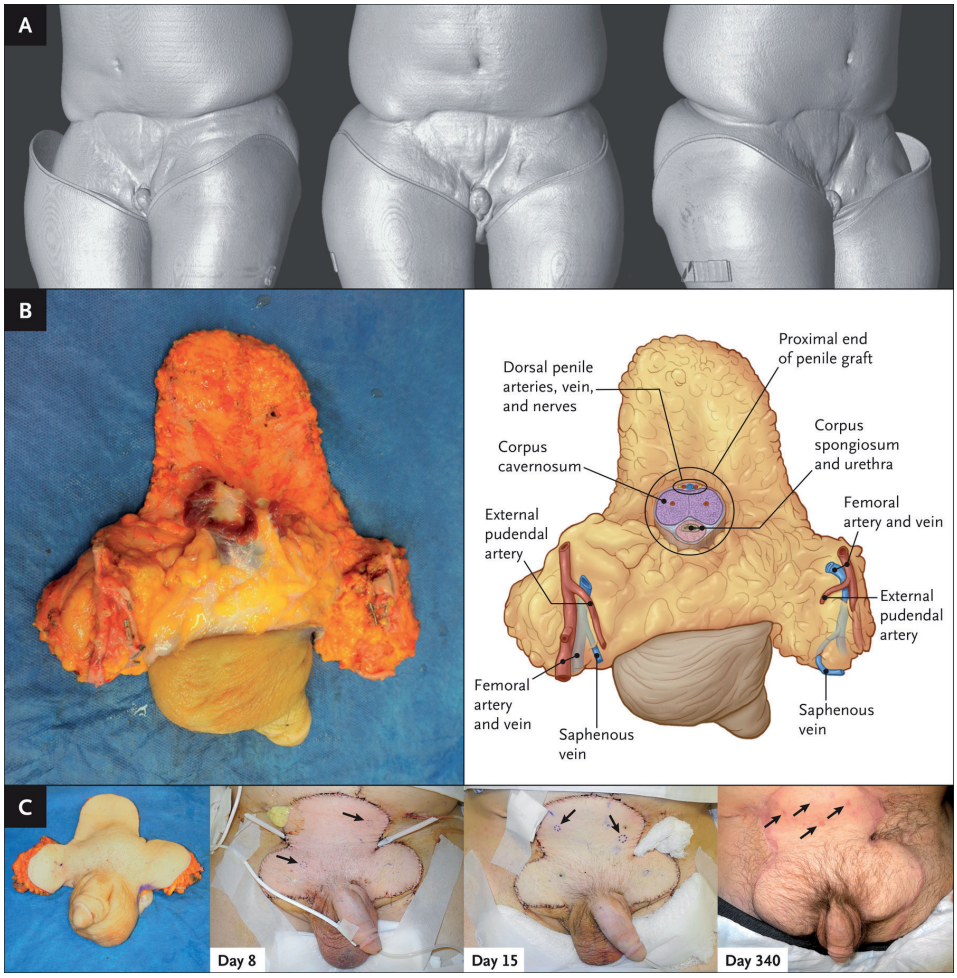

Figure 2: Transplantation of a Penis, Scrotum, and Lower Abdominal Wall.
Panel A shows a preoperative computed tomographic reconstruction of the extent of the injury in the transplant recipient. A small penile stump is visible, with loss of the lower abdominal wall, the entirety of penile shaft, and the scrotum and testes. Panel B shows the graft after explantation from the donor. The graft included the right and left external pudendal artery, a segment of the femoral artery, and the saphenous veins on both sides. Dorsal arteries can be seen on the deep, proximal portion of the penile graft. Panel C shows the graft before the procedure along with clinical images from postoperative day 8, day 15, and day 340. The graft has been incorporated without evidence of rejection. Biopsy sites (arrows) are visible on the skin of the abdomen and groin.

Similar to the previous episodes, this was treated with topical tacrolimus and clobetasol with full resolution within 48 hours and no requirement of systemic steroid treatment or increase in baseline maintenance immunosuppression. The patient has not had any further signs of skin rejection.

Antibody screening for donor specific HLA antibodies (DSA) was performed at scheduled intervals. Beginning 17 days postoperatively, three days after the bone mar-

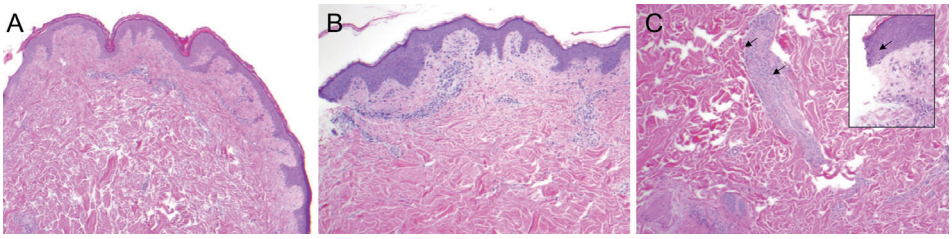

Figure 3: Grade 0 and 1 and 2 rejection, 4x H&E. Punch biopsies of skin showing A) no significant perivascular infiltrate B) sparse to mild perivascular infiltrate without epidermal involvement and C) moderate perivascular inflammation in the reticular dermis with eosinophils (arrows). Inset shows solitary dyskeratotic cell in epidermis (arrow) in otherwise unremarkable epidermis. According to strict interpretation of the Banff criteria, grade II rejection does not have epidermal dyskeratosis, and thus consideration was given to designation as early grade 3 rejection; however, the overall histologic features were notable for a lack of epidermal involvement, favoring grade II rejection. Of note, the significance of the eosinophils in the infiltrate is unclear as the composition of the inflammatory infiltrate is not well understood in the setting of VCA allograft rejection and is not a component of the current staging criteria.

row infusion, the patient was noted to have weak de novo DSA to donor HLA-A24 and -DR15. He did not show any change in his clinical condition, and the level of DSA reactivity remained below that sufficient to yield a positive flow cytometric crossmatch. DSA monitoring was performed weekly for the next 6 weeks and then spaced out to monthly with office visits, and results remained consistent throughout with continued detection of HLA-A24 and -DR15 DSA.

Blood testing for peripheral chimerism was performed on postoperative weeks 1, 2 (immediately prior to bone marrow infusion), 3, 4, 6, and 7 and months 3 and 6. Donor peripheral T cell chimerism was evaluated using peripheral blood CD3+ cells and quantitative multiplex PCR for short tandem repeat markers with an analytic sensitivity of 5%.[10,11]

Chimerism evaluation at weeks 1, 2 and 3 yielded a small percentage of donor DNA identified (<5%), which is below the linear quantification range of the assay. At week 4, CD3+ separated T cell analysis showed 8% mixed chimerism, but none was detected by week 6 with subsequent tests remaining without evidence of donor DNA. CMV quantitative log value as well as plasma EBV log value and viral load were tested by PCR weekly for the first eight weeks and then spaced to monthly and quarterly. All of these tests thus far have been without DNA detection of either virus.

Functional Outcomes

The patient initially had urinary retention with the removal of the Foley catheter in week three. Narcotics were weaned, and the catheter was successfully removed in the fifth postoperative week. Since that time, he has been able to urinate standing with normal continence and flow. There are no concerns for strictures, fistulas, or other urethral complications.

The patient is now one year out from his penile transplant. He had firm nocturnal penile tumescence starting around postoperative month seven and now has near normal erections. He has normal sensation to the shaft and tip of the transplanted penis and can localize touch sensation to those areas. Neurosensory testing with the Pressure-Specified Sensory Device™ (AxoGen, Alachua FL) demonstrates close to normal glans sensibility for one point moving touch and has recovered to lower (better) thresholds than the one-point static touch. The shaft has recovered sensation that has higher thresholds than the glans.

Discussion

As the numbers of VCAs performed increases and patient follow-up lengthens, we are able to expand upon our understanding of the immunology of this multi-tissue transplant type and improve treatment to make these procedures more widely and safely performed. Acute cell-mediated rejection episodes are extremely common in VCA, occurring in 85% of hand transplant patients in the early post-transplant period, with >50% of patients experiencing more than one episode.[12] Our patient has experienced only minor skin symptoms readily abated by topical therapy.

The treatment and maintenance of the penile transplant patients has been largely extrapolated from well-established regimens in both VCA and solid organ transplantation with promising immunologic outcomes. While standard immunosuppression has been validated in solid organ transplantation, the different tissue types involved in VCA have been shown to have different immunogenicity, particularly the skin.[13–15] However, the accessibility of the skin for clinical assessment, photographic monitoring, and biopsy allows for tracking of natural history as well as diagnosis of pathology. In our current protocol, scheduled biopsies allow for monitoring in the absence of visible signs of rejection to evaluate for any sub-clinical manifestations of inflammation. Along with inflammation, architectural changes to the tissue can be tracked over time should there be concern for longer-term alterations.

In penile transplantation, access to the urinary tract might also provide a unique mode of monitoring. Preliminary preclinical data from our institution show that acute rejection in rat penile transplantation appears to affect primarily the skin and urothelium.[16] Rejection in the urothelium of murine penile transplants was accurately reflected in skin manifestations, but evaluation of gene and protein expression in the urine may serve as a viable option for biomarker development as an alternative to invasive tissue biopsies. Studies in kidney transplantation have shown urinary levels of specific mRNA and rRNA products to be diagnostic and prognostic of acute cellular rejection.[17] These findings could indicate alternative monitoring techniques that can be applied to penile transplants, increasing opportunities for non-invasive immune monitoring.

The patient has returned to school full time and continues to live independently using bilateral lower extremity prostheses. He adheres well to his immunosuppressive regimen and has been transitioned to long-acting calcineurin-inhibitor monotherapy (Envarsus XR®,Veloxis Pharmaceuticals, Horsholm, Denmark). Psychologically, he reports and improved self-image and "feeling whole" again and is very satisfied with the transplant and the implications it carries for his future.

Conclusion

Penile transplantation has been demonstrated as technically feasible with reports of promising functional outcomes. We report operative success for a larger tissue defect using multiple tissue-type transplantation and an alternative vascular supply than has been previously described. In carefully selected individuals, the procedure offers full restoration of penile function and represents an exciting new paradigm in reconstructive transplantation.

Acknowledgements

The authors would like to acknowledge the invaluable support of the entire VCA team including Jane Littleton, NP. We also acknowledge and appreciate the contributions of collaborating colleagues including Dr. Ivan Borrello, Dr. M. Victor Lemas, Dr. Elliot Fishman, Dr. Shivani Ahlawat, and Dr. Robin Avery.

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Supplemental information and figures

1. *Preoperative Evaluation.* A protocol for human penile allotransplantation was approved by the Johns Hopkins Institutional Review Board. Potential penile transplantation candidates were identified though review of existing patients in the Johns Hopkins Hospital patient population as well as referrals from the United States military and outside providers. Once identified, potential patients undergo a rigorous screening process including evaluation by a large, multidisciplinary penile transplant team to determine candidacy for transplantation. The team included plastic surgery, urology, transplant surgery, infectious disease, psychology and sexual psychology, and social work. A thorough history and physical exam was performed by plastic surgery and urology, and comprehensive infectious testing was performed. Once a candidate is deemed appropriate for transplantation, preoperative evaluation includes cardiology work up with echocardiogram and electrocardiogram, pulmonary function testing and chest x-ray, HLA typing and panel-reactive antibody (PRA) testing, and imaging including CTA of the abdomen and pelvis, lumbosacral plexus MRI, and MR neurography of the pelvis.
2. *Procedural Considerations.* The recipient's soft tissue defect was extensive and included the skin and fat from the lower abdominal wall, his entire penis and scrotum, and tissue from his medial thighs. The graft included an exact match of these tissues. Recent cadaver studies from our institution have demonstrated the importance of utilizing multiple vascular pedicles to provide optimal graft perfusion.[1,2] The dorsal arteries are the only source of perfusion to the glans and as such are required to prevent distal necrosis. These arteries also provide adequate perfusion to the corpus spongiosum and urethra. Anastomosing the dorsal arteries is important to ensure urethral perfusion and patency since the small size and high variability of the urethral arteries make them ill-suited for consistent use in transplantation. The shaft skin, as well as the surrounding suprapubic, groin, and scrotal skin, is perfused by the external pudendal system arising from the femoral artery.
3. *Infection Prophylaxis Management.* Perioperative antibacterial prophylaxis was with IV piperacillin/tazobactam 3.375 grams prior to and during the operation. Prophylaxis was continued with cefazolin 2 grams IV every 8 hours for an additional 3 days. Donor cultures grew coagulase negative Staphylococcus from urine and Enterococcus from sputum. Neither was considered significant and therefore not treated. Antiviral prophylaxis was with valacyclovir 500 mg twice daily (planned course 12 months). The donor was seropositive for HSV I/II and the recipient was seropositive for varicella zoster virus. Both were addressed by valacyclovir. The recipient and donor were CMV D-/R-, so no direct treatment or prophylaxis was given. Pneumocystis prophylaxis

was with daily trimethoprim-sulfamethoxazole (TMP/SMX) 400 mg/80 mg (planned course 12 months). The donor was seropositive for Toxoplasma. This was addressed by TMP/SMX. Anti-candida prophylaxis was with fluconazole 800 mg loading dose followed by 400 mg daily for 1 month. Infectious management is summarized in Table S1.

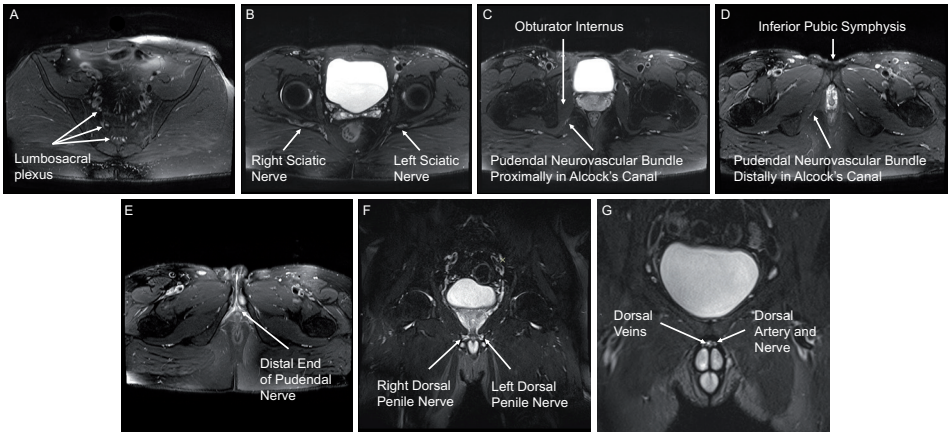
4. *Immunomodulatory Regimen.* The recipient was treated with an immunomodulatory regimen consisting of monoclonal antibody induction, calcineurin inhibitor (tacrolimus) monotherapy maintenance, and a donor bone marrow cell infusion.[3] Specifically, the patient underwent induction therapy with a single dose of alemtuzumab (Campath-1H™, Millennium Pharma, Cambridge MA) 30 mg over 2 hours for lymphocyte depletion administered in the operating room immediately prior to transplantation. 1,000 mg methylprednisolone were given prior to graft reperfusion. The recipient was started on tacrolimus maintenance therapy the day of the transplant with an initial target trough level of 10-15 mg/mL. The dose was briefly lowered and held during the first postoperative week for a mild, transient increase in creatinine (likely a laboratory error) but was quickly restarted with normalization of creatinine within one day (Supplemental Figure 3).
5. *Donor Bone Marrow Processing.* At the time of graft procurement, vertebral bodies were recovered for processing and bone marrow cell isolation per protocols previously published by our group.[3–5] Donor vertebral bodies from T8 to L4 were recovered after removal of the penile graft. The isolation of hematopoietic progenitor cells (HPC) from marrow cells was performed in the Cell Therapy Laboratory at Johns Hopkins Hospital using a process previously used in our upper extremity transplant protocol.[5]

   The vertebral bodies were stored at 2-8C after recovery and before processing; total storage time was 26 hours. The isolation of hematopoietic progenitor cells (HPC) from marrow cells was performed in the Cell Therapy Laboratory at Johns Hopkins Hospital using a modified University of Pittsburgh process.[5] After processing, the product contained 4.2 x 10^{10} nucleated cells of which 1.23% were $CD34^+$ and 10.23% were $CD3^+$ (5.2 x 10^8 total $CD34^+$ cells and 4.3 x 10^9 total $CD3^+$ cells) with 3 mL of RBCs. Sterility results of the initial transport media and the final cells in cryoprotectant were negative; there were no bacteria isolated on the TSA plates. Cell viability was 92%. The product contained hematopoietic progenitors: 3.1 x 10^7 total CFU-GM, 5.1 x 10^7 total BFU-E and 8.0 x 10^6 total CFU-GEMM.

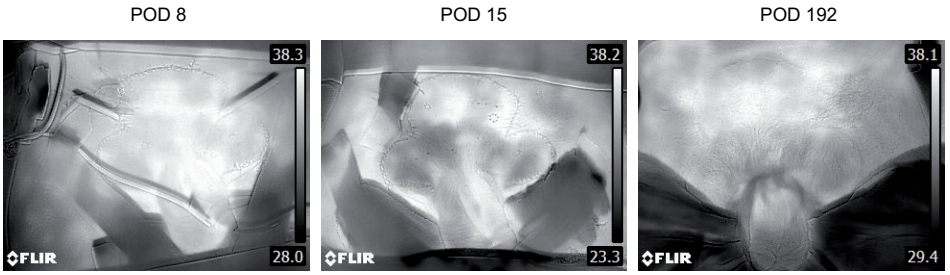
   On postoperative day 14, bone marrow was infused intravenously at a dose of 5.7 x 10^6 $CD34^+$ cells/kg without any concerns or reaction.
6. *Chimerism Analysis.* To evaluate for peripheral T cell chimerism, CD3+ cells are separated from peripheral blood using the RoboSep automated instrument (StemCell Technologies). The assay consists of PCR amplification of fifteen (15)

microsatellite markers and the amelogenin locus using AmpFISTR Identifiler PCR amplification kit (Applied Biosystems). The resulting PCR products are analyzed by capillary electrophoresis and the peak heights of the informative alleles are compared to calculate a percentage engraftment. In general, engraftment is calculated using 2 different microsatellite loci from a single PCR ration. The true limit of detection for an individual reaction is both locus and PCR dependent. The formal limit of detection is 5%. The standard deviation for chimerism values is typically below 5%.[6,7]

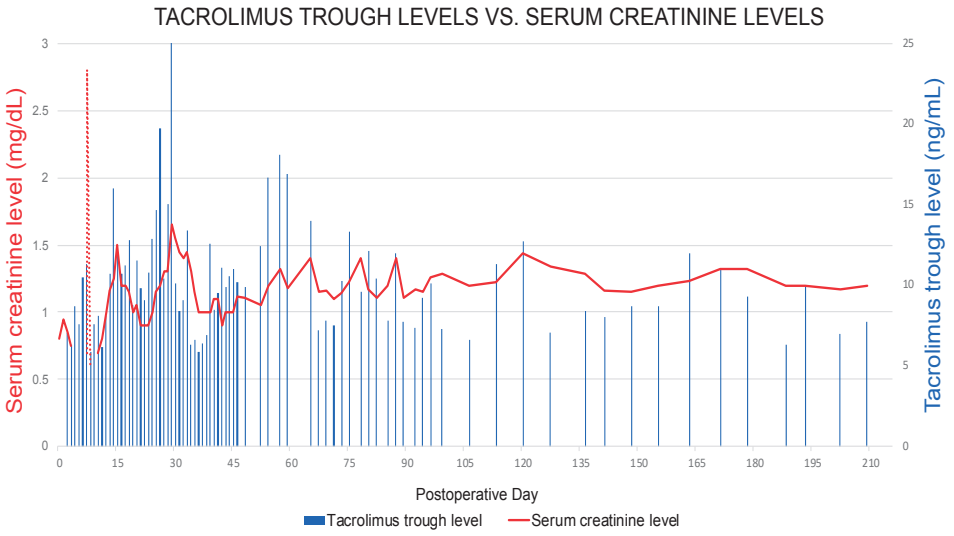
7. *Antibody Screening*. HLA-specific antibodies were evaluated before transplantation and at time of biopsy using multiple solid-phase immunoassays (Lifecodes classes I and II ID panels; Immucor-Lifecodes, Stamford, CT; Single Antigen Beads; One Lambda, Canoga Park, CA) performed on a Luminex platform. Cytotoxicity crossmatch tests with donor cells were performed using standard cytotoxicity B cell and AHG enhanced T cell protocols. DSA levels were correlated and reported relative to crossmatch thresholds; a flow cytometric crossmatch threshold correlates with DSA of moderate strength on a HLA phenotype panel (4000–9000 MFI).



Supplemental Figure 1: Preoperative MRI imaging of patient's penile nerves and vessels. The posterior aspect of the lumbosacral plexus – including the sciatic nerve and posterior femoral cutaneous nerves – demonstrated normal course, size, and signal intensity. There was normal MR neurography appearance of the lateral femoral cutaneous and the obturator nerves. A-E: Transverse cuts showing the path of the patient's sacral nerves from lumbosacral plexus to the distal end of the pudendal nerves. F-G: Coronal images showing the proximal dorsal penile nerves, arteries, and veins, all of which were intact in the patient preoperatively.



Supplemental Figure 2: Representative infrared thermographic imaging of the allograft demonstrating perfusion throughout the postoperative course. POD: Postoperative Day.



Supplemental Figure 3: Tacrolimus trough levels throughout post-transplantation course. The dotted red line on the graph was an isolated spike in creatinine levels with immediate return to normal, deemed a lab error (reported to the IRB). POD: Postoperative Day.

Supplemental Table 1: Overview of JHH Protocol for Infection Prophylaxis Management in VCA. MRSA: methicillin resistant *Staphylococcus aureus*; TMP/SMX: trimethoprim sulfamethoxazole. All doses assume normal renal function.

Time period	Basic	MRSA in donor or recipient	Beta lactam allergy (no MRSA)
Preoperative	Chlorhexidine gluconate bath		
Perioperative	IV cefazolin 2 grams (repeated every 4 hours during the operation).	IV Vancomycin 15 mg/kg (repeated every 12 hours) AND IV ciprofloxacin 400 mg (repeated every 8 hours)	IV ciprofloxacin 400 mg (repeated every 8 hours) AND IV clindamycin 600 mg (repeated every 8 hours)
Post-operative			
Immediate Anti-bacterial	IV cefazolin 1 gram every 8 hours x 72 hours	Above regimen x 72 hours	Above regimen x 72 hours
Anti-staphylococcal	Mupirocin to the nares twice daily x 5 days for patients whose nares are positive for any Staphylococcus aureus strain		
Pneumocystis	Oral TMP/SMX (80 mg/400 mg) daily x 12 months (if unable to tolerate: dapsone 100 mg daily or atovaquone 1500 mg daily)		
Fungal	Oral fluconazole 800 mg x 1 then 400 mg/day x 1 month		
Viral	Recipient seropositive	Donor and recipient CMV seronegative	
	IV ganciclovir 5 mg/kg twice daily x 14 days and then valganciclovir 900 mg daily x 6 months. Valacyclovir 500 mg twice daily from months 6-12	Valacyclovir 500 mg twice daily x 12 months	

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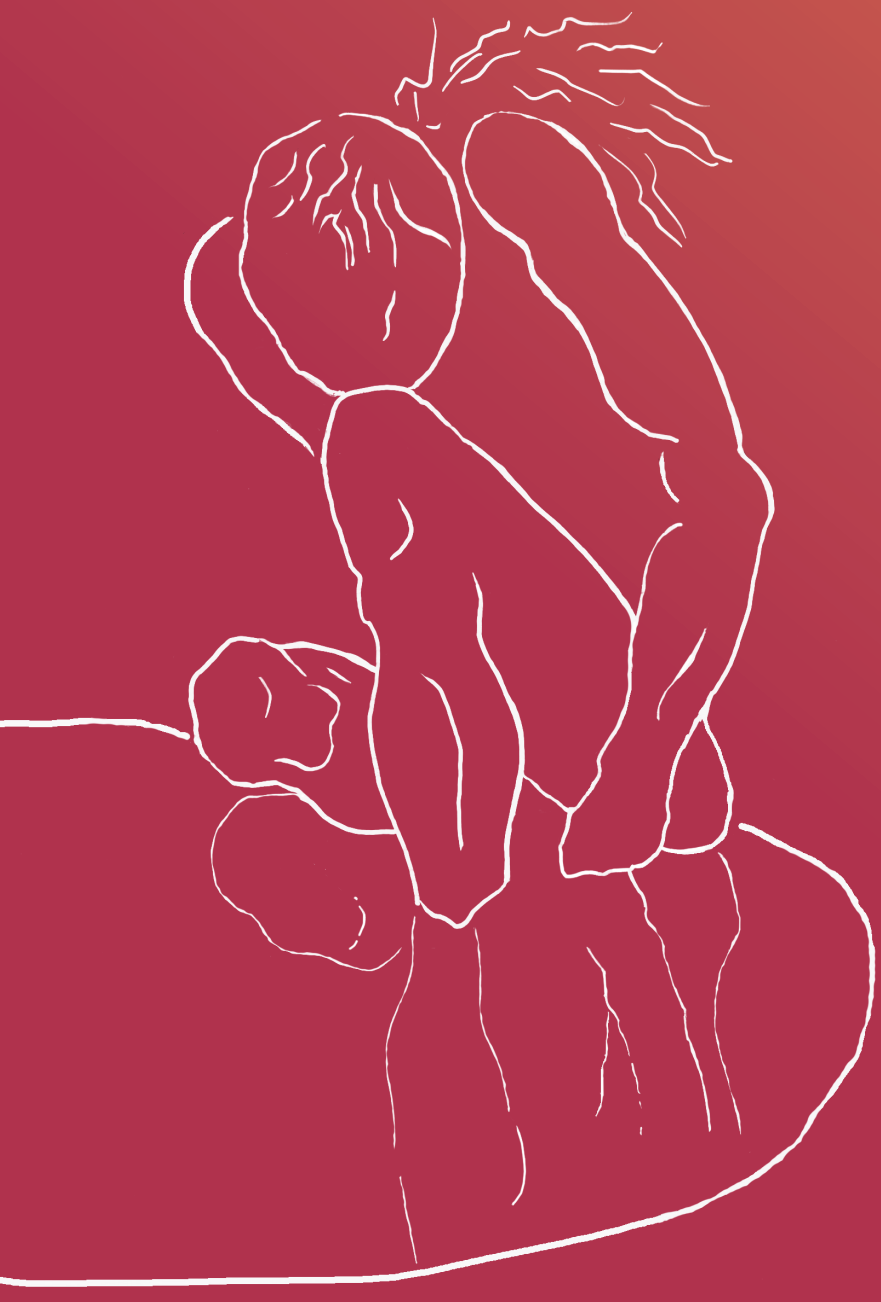

Chapter 9

General discussion and future perspectives

In this thesis, preclinical animal studies and clinical (ethics) studies related to immunology and preservation of vascularized composite allotransplantation (VCA) were presented. This discussion section is divided in four parts that follow the general structure of the thesis. Part 1 discusses the patterns of clinical and histological rejection in VCA grafts in rats (penis) and pigs (hind limb). Part 2 focuses on desensitization strategies in VCA and the possibility of using autologous bone marrow transplants as a means to erase recipients' sensitization to donor tissue. Part 3 describes a novel method applying naturally occurring antifreeze proteins in the preservation of grafts at subzero temperatures. The final part of this thesis explores the ethical framework in which penile transplants can be performed and which steps are necessary for the implementation of a VCA program. These lessons are then applied in a clinical penile transplant in the final chapter.

Objectifying clinical and histological graft rejection in VCA

This part of the thesis aimed to enable understanding of the rejection patterns observed in hand, face and penile transplants and to objectify the role of graft skin as a sentinel for rejection of an entire graft.

In almost all scientific endeavors, models are needed to simplify real world situations and enable the study of interventions. In VCA -the enormous investments required for clinical interventions and the ethical considerations involved- mean that animal studies are essential for scientific progress that enables clinical improvement. Surgical models in animals however remain approximations of clinical reality. For VCA, models generally focus on functional orthotopic designs to study muscle and sensory nervous function[1–3] or on 'non-functional' heterotopic or orthotopic designs to study immunological aspects without nervous function.[4–6] In VCA, the most prevalent swine hind limb model places the graft against the recipient abdominal wall leaving the graft without function and away from its anatomical position in the donor. The limitations coming from an absence of muscle function and lack of nervous coaptation is however accepted in light of the (perceived) impossibility of functional VCA models in mice, rats and pigs. Even when not functional, surgical models do need to be reliable and reproducible to be of use in surgical research and allow for comparison between interventions. The most important factor for surgical animal models in VCA is reliability of perfusion and venous outflow. For penile transplants different models were described which in preliminary experiments for this PhD project were found to be lacking in reliability for transplant studies.[7,8] In this thesis we thus describe a heterotopic penile transplant model that achieves graft inflow through anatomical bilateral perfusion through the pudendal arteries and outflow through the dorsal penile vein. This model showed to be highly reliable (>90% surgical success rate in 80 transplants) and was subsequently used in two other penile transplant studies included in this the-

sis. The third chapter then used this model to elucidate the rejection patterns specific to the components of a penile transplant. The clinical and histological rejection analysis proved that penis transplants have a rejection pattern that is comparable to that of other VCA with the skin being the first graft component to show clinical signs of rejection, together with the urethra. This has implications for clinical practice as it suggests that this pattern could also be true for human penis transplants, meaning that skin monitoring is a good approach for monitoring rejection in the entire graft. Next, the study established a standardized rejection grading system for both clinical and histological rejection that can be used to properly compare between future penile transplant studies regarding tissue rejection.

Analogous to the establishment of a rat penile rejection grading system, the fourth chapter also adapted the human BANFF criteria[9] for VCA rejection to a preclinical model, namely in swine hind limb transplants. Using 214 skin biopsy samples and their time- and graft matched clinical images, a histological rejection scoring system was established and again a clear correlation between clinical skin rejection and histological rejection scoring was found, proving that clinical observation of skin can be used to monitor graft rejection in swine. The BANFF criteria for VCA are still relatively new, and the criteria for acute and chronic rejection are still being refined[10] and debated[11] as longer-term data of VCA transplants is published.[12] The necessity of performing regular skin biopsies is similarly uncertain. Since scarring caused by biopsies is associated with local inflammation and the performance of biopsies is uncomfortable for patients, the insight that clinical observation is reliable in gauging rejection in swine could be used to reduce the amount of clinical biopsies performed.

Several limitations require to be acknowledged concerning the studies in part 1. As mentioned before in this section, the only model that accurately represents real-life clinical care is a real-life clinical situation. Animal transplant models carry inherent limitations related to significant differences in animals' immune responses and general makeup when compared to humans. In transplant research specific animal breeds are in dominant use. For rats the dominant combination is Brown Norway donors into Lewis recipients. For mice BALB/C donors into C57BL/6 recipients. The goal of using these specific strains and specific animal models is to allow for comparison between studies to enable determination of which approaches work best in a major MHC (Major Histocompatibility Complex) mismatched setting. This standardization can even be viewed as essential, since comparison is otherwise impossible and the value of any given study equally impossible to determine. This standardization however presents a major limitation: translating such research to a clinical setting means a change of circumstances from standardized mismatched combinations of inbred animals to matched combinations in a genetically diverse human population. Sadly, budget limitations do not allow for animal sample sizes that are big enough to perform wild type transplants in numbers that make successful translation of findings in animals to a human setting more likely. We are thus limited to the animal studies approach that

we currently have. As such, chapter 3 features Brown Norway grafts transplanted into Lewis recipients as is most common in the field. This combination is known to have a major MHC mismatch and was thus deemed to be a good combination to study graft rejection. However, it cannot be ruled out that findings would have been (mildly) different in a different strain combination or even by transplanting in the other direction (LEW into BN instead of BN into LEW), as this is known to possibly yield other results.[13] More straightforward limitations specific to the penile transplant model described in chapter 2 are related to its heterotopic design and the particulars of rat penises compared to human ones (mainly the presence of a bone marrow containing baculum in the rat penis). The heterotopic model design means that the graft lacks voiding and erectile function. The lack of voiding seemed to correlate with inflammation of the urethra; the fibrosis found in the corpora can possibly be linked to a lack of erectile function.

Another limitation of part 1 is related to animal behavior. In contrast to solid organ transplants, VCA are exposed to the outside world and are impacted by animal behavior. Hind limb transplants in rats can be bitten, licked and otherwise traumatized by the animals and trauma has been shown to impact graft rejection.[14,15] Similarly, pigs scratch themselves regularly and the model used in chapter 4 is insensate, allowing the animals to uninhibitedly scratch and irritate the grafts at varying levels depending on the individual animal. Lastly, our analysis found that there is a relevant difference between findings of simultaneously obtained biopsies from the same graft, suggesting intra-graft variability in inflammation. This highlights the importance of weighing both macroscopic as well as histological factors when assessing the level of graft rejection. Further research will be able to use the findings in these papers for thorough analysis of the effectiveness of immunosuppressive regimens.

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Future perspectives

Scientific progress is impossible without standardized measurements and standardized models. For measurement of rejection the standardized rejection classification scales provided by part 1 can enable rigorous preclinical animal research into VCA rejection. This can be particularly useful in penile transplant research. In clinical face transplants, the oral and sinonasal mucosa are found to regularly show rejection along with skin rejection episodes, proving its role as a prime target of rejection. This has led to an acknowledgement of the importance of monitoring this tissue as well as a graft's skin component.[16,17] The urethral lining tissue present in penile grafts could similarly prove to be a rejection sentinel along with the skin and will need to be studied further. Despite numerous reported clinical transplants few animal studies exist that examine graft rejection and immunosuppressive regimens applied to this specific graft type. The rat penile rejection study included in this thesis shows rejection comparable to other VCA when not receiving any immunosuppressive treatment. It also does not show any rejection under administration of standardized dosages of Tacrolimus

in rats. Relevant novel immunomodulatory regimens have not been tested in penile transplant models and will hopefully be examined for this transplant type in the near future. For the rat penile transplant model itself future researchers may be able to improve upon the model by developing an orthotopic approach with urethral and nervous coaptation to emulate the clinical situation even better.

Improving transplant candidate access: desensitization strategies in VCA

For part 2 this thesis aimed to test if an autologous bone marrow transplant can effectively remove donor specific memory, allowing for successful reconstructive transplantation in sensitized patients.

Sensitization is a major problem for potential transplant recipients and is particularly recognized in kidney transplantation.[18,19] For VCA the challenge of sensitized patients is even more common, as trauma and burn victims that are VCA candidates often become sensitized by the blood transfusions and skin transplants that are necessary for their initial management.[20] Currently, Intravenous immunoglobulins, plasmapheresis, rituximab, toculizumab and eculizumab are all used in the treatment of sensitized patients, with varying degrees of success.[21,22] For VCA, the presence of pre-transplant donor specific alloantibodies as a measure of sensitization is also linked with early rejection and adverse outcomes.[23]

Chapter 5 shows that in a rat hind-limb model, a bone marrow transplant in a sensitized recipient can effectively lower donor-specific antibody levels and allow for complete long-term graft survival. This makes it a promising finding in the definitive treatment of sensitization in transplant candidates.

As highlighted for part 1, a main limitation is the use of a rat model. In chapter 5 Dark Agouti donors are used instead of brown Norway, in part because the immune responses related to sensitization had been clearly defined in this strain combination before by using a kidney transplant model.[24] Though the desensitization approach using an autologous bone marrow transplant and fludarabine induced B-cell depletion is highly successful in rats, translation into pigs and humans can be expected to be challenging. Rats have a high tolerance for severe treatment protocols and are relatively easy to protect from pathogens when in a leukocyte depleted state. Bone marrow transplants are however a significant burden for human patients, even if major steps have been made in mitigating risks of the procedure over the years.[25] The autologous bone marrow transplant design used in the study makes complications such as graft versus host disease unlikely, but the procedure does entail the eradication of the recipient's bone marrow, making them susceptible to infections and bleeding disorders. Recipients would also need to regain all leukocytic disease memory and be vaccinated again for many diseases to achieve this. The risks involved with a bone mar-

row transplant to treat sensitization thus needs to be weighed against the expected disease burden that comes with severe physical defects without the possibility of a transplant due to sensitization.

Future perspectives

In sensitized patients with measurable levels of donor specific antibodies, current clinically used strategies are focused on the acute effects of the circulating antibodies.[21,22] These antibodies are however produced in response to stimulation by memory B-cells. The strategy employed in chapter 5 which removes these memory B-cells is shown to be highly effective in preventing accelerated rejection of hind-limb transplants in rats. Though this approach could be a major improvement over the current clinical practice,[26] it is not known if this approach is clinically viable. With the proven effectiveness of this approach in rats, future work can focus on targeting B-cells in the treatment of antibody related graft rejection with less severe side effects than an autologous bone marrow transplant.[27] As with combined immunosuppressive therapies in current clinical use, a combination of approaches targeting both B-(memory)cells and donor specific antibodies may prove to be successful in large animal models and eventually be applied clinically.

Expanding graft availability: high subzero ICE-free graft storage

Part 3 of this thesis tested the feasibility of expanding permitted penile graft ischemic time through high subzero ice-free graft preservation.

In chapter 6 an approach to high subzero ice free graft preservation is described that uses peptoids that are based upon naturally occurring peptides in arctic animal species.[28–30] Though it has been known for decades that arctic fish, insects and mammals have such antifreeze capabilities, only more recently these insights translated to the production to similar compounds (peptoids) that can be produced in quantities that enables their use in vivo.[31,32] Chapter 6 proves that these peptoids are indeed capable of significantly extending preservation times in rat penile grafts. With this approach itself being novel and applied in a new transplant model, few studies exist that can be compared with the findings of chapter 6.[33] Comparisons thus need to be sought in the other approaches to tissue preservation such as vitrification and normo- and hypothermic machine perfusion in models such as liver and heart transplants.

Vitrification approaches are highly successful in the preservation of cells, with 10% dimethyl sulfoxide solution used as the standard for long-term storage of cells.[34,35] It does however have toxic effects on the stored cells and on the patient receiving those stored cells, for example in the setting of a bone marrow transplant.[36] Limited success has been reported in using this technique for more complex (vascularized) tissues. In contrast, peptoids have not been shown to have toxic side effects on cells and do

not require the resources that are needed for machine perfusion approaches to tissue preservation.

Many avenues related to high subzero preservation still require further study. The use of peptoids has been reported to be highly successful in cell preservation for short durations.[36] With the approach being this novel, many of the procedure's details not pertaining to the chemical components and the interactions on a cellular level need to be taken in consideration when optimizing the procedure for clinical application in more complex tissues. Mainly, this study did not test cooling rates, perfusion rates, perfusate peptoid concentrations, tissue level peptoid concentrations or vascular responses to perfusate.

In kidney transplants, post-transplant perfusion is known to be variable, with the more distal parts of the graft sometimes being relatively malperfused.[37] Anecdotally, histidine-tryptophan-ketoglutarate perfusion of whole kidneys also regularly leads to limited perfusion and thus protection of the kidney cortex. For penile transplants and VCA in general, there is only very limited knowledge on the effectiveness of arterial perfusion in reaching and protecting more 'remote' parts of grafts. The distal-to-proximal and unilateral necrosis patterns found in this chapter further suggests involvement of microvasculature in partial failure of preserved grafts. Further studies will need to determine which strategies can ensure proper saturation of the entire tissue when preserving a VCA.

Cooling and warming rates are known to be of high importance in ultra-low subzero preservation strategies,[38–40] but have mostly been studied in the preservation of gametes.[41] It is not yet established if cooling and warming rates are relevant to high subzero preservation where the main goal is the complete avoidance of ice formation. Future studies have to show if the linear cooling approach used in chapter 6 (through direct graft placement and removal into and out of a freezer) is the optimal one.

Future perspectives

Unlike the bone marrow transplant protocol described in chapter 5, applying the peptoid solution from chapter 6 in large animal models and the clinic faces only reasonable barriers. The peptoids have not shown significant levels of cell and tissue toxicity and no adverse effects on the animal receiving peptoid treated grafts have been observed. This lack of adverse effects on recipient health combined with excellent clinical outcomes in terms of graft survival make the procedure a promising one for translation to larger animals and eventually clinical patients.

Based on the current successes reported for high subzero preservation[33] and the limited side effects, the future of the high subzero preservation approach using peptoids is promising. If these approaches prove to be successful in large animals and clinical application is achieved, they will result in a major step forward in clinical transplantation. The approach does not require extensive training and maintenance of machinery like machine perfusion systems do[42] and would be easily implemented

in most hospital settings. It could greatly improve usage rate of eligible transplantable tissues and allow for transplantation across large distances, further enhancing the eligible donor pool by allowing for matching between larger groups of donors and recipients. Large animal studies are first needed to take the next step towards making this a clinical reality.

Ethical clinical application of penile transplantation

The final section of this thesis aimed to provide an ethical framework for the application of urogenital transplantation in a clinical setting and establish the feasibility of functional urogenital transplantation in a patient suffering from penile loss due to extensive blast trauma.

One of the guiding principles in medicine is primum non nocere; first, do no harm. This is also one of the main considerations related to penis transplantation and VCA in general. Sadly, it has been established that receiving a transplant is highly likely to do harm to a patient. Most patients will to different degrees suffer from the side effects of immunosuppressive treatments such as kidney failure, opportunistic infections, neurological complications and accelerated development of malignancies.[43–46] In solid organ transplants, these risks are acceptable due to the severity of the alternative: short term patient death caused by organ failure. No such risk exists for recipients of non-life-saving reconstructive transplants. Each reconstructive transplant can as such be seen as a tradeoff between quality and quantity of life. Recent history has shown that both patients and physicians have been willing to perform these transplants despite the high likelihood that patients will suffer from the serious side effects in the mid- to long term.[47] For penis transplants, the ethical considerations that come with this tradeoff have to be carefully examined. Chapter 7 considered the different aspects needed to be considered when establishing a program for penile transplantation. It described the clear advantages of penile transplantation over conventional reconstruction in complete penile loss related to voiding, erectile function, erogenous function, donor site morbidity and weighed those against immunosuppression side effects and cost. For practical implementation the utmost importance of patient selection was highlighted along with guidelines for informed consent, privacy considerations and post-transplant psychosocial support and ensuring lifelong care. Finally, chapter 8 describes the first clinical penile transplantation that includes the entire penis, scrotum, and partial abdominal wall. In this chapter the importance of extensive pre-operative evaluation, infection prophylaxis, immunosuppression and rejection monitoring are highlighted. The transplant has a good clinical outcome with excellent voiding through the graft and even erectile function that allows for orgasms.

This 'complete' penis transplant that includes the scrotum and part of the abdominal wall is another step forward when compared to transplants that involved solely the

penile shaft itself.[48,49] It is also very promising that the graft has proven to be functioning as a sexual organ by allowing erections and orgasms. Thus, the aim of recovering voiding and sexual function in cases of total penile loss has been proven to be attainable in the short term.

Future perspectives

Further penile transplants will need to be performed to assess if this latest addition to the field of VCA will be successful in the long term. Penile transplants published in the literature were all reported to be partial shaft transplants,[48,49] with the one described in chapter 8 adding abdominal wall and scrotal tissue. With the proximal shaft deriving from the recipient, existing neurons responsible for delivering and sending the complex signals that enable erections and orgasms are already in place. In these transplants, erectile activity that already existed in the remaining penile stump only needed to incorporate the distal parts of erectile bodies to result in actual erections of the graft penis. More extensive penile transplants that include the entire penile unit will show if the approach is also successful when nervous coaptation is required to enable erections. Longer follow-up will also show if penile tissue can be maintained under immunosuppression comparably to other VCA and solid organ transplants. When penile transplants that include the entire penile unit are proven to give satisfactory functional results and grafts can be maintained long-term, the already existing conversation about the application of this procedure in groups that are not considered biological males will have renewed relevance as it would be feasible to perform if immunosuppression side effects are considered acceptable in these groups. Overall, the positive clinical outcomes shown in these grafts, combined with the great innovations the field has seen since its inception, can make one confident that the field of VCA has a bright future for cases of complete loss of hand, face, and penis.

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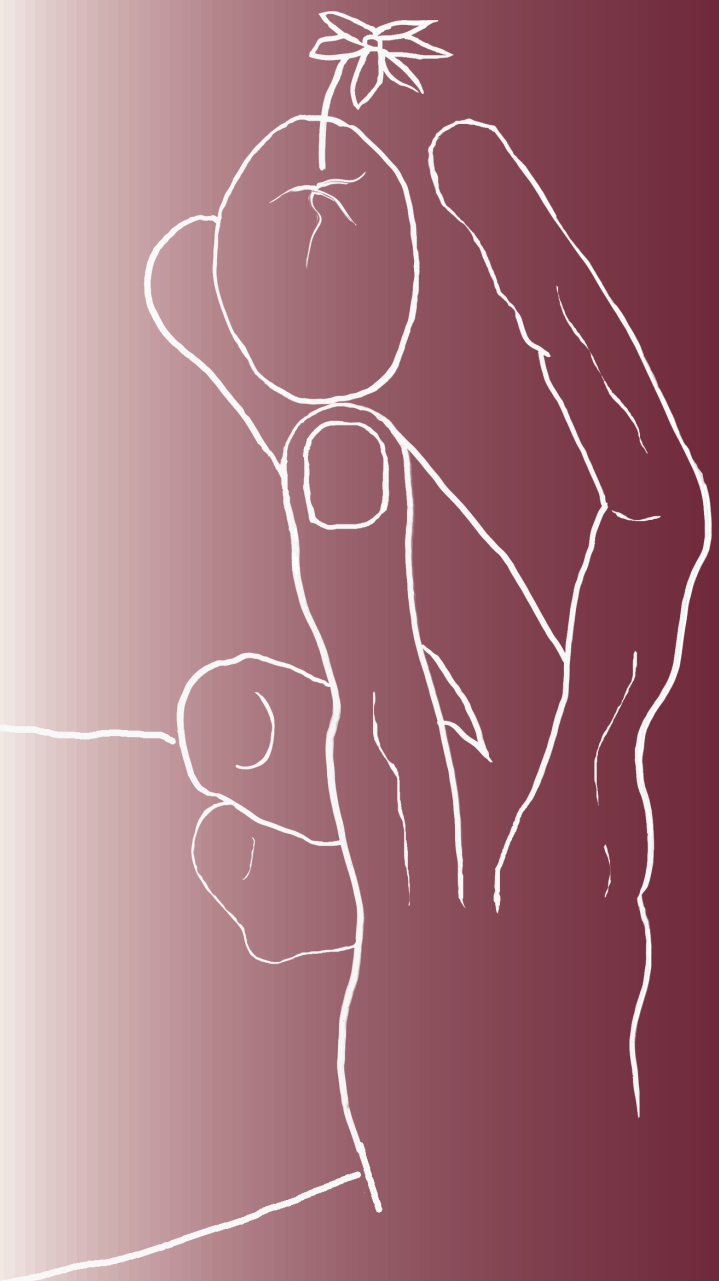

Appendices

Summary
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Summary

This thesis describes studies related to graft rejection, transplant recipient desensitization, graft preservation and transplant ethics related to penile transplantation. In this section the main findings are summarized in accordance with the structure of the overall thesis: 1) Objectifying clinical and histological graft rejection in VCA, 2) Desensitization strategies in VCA, 3) Expanding graft availability: high subzero ice free graft storage, and 4) Ethical clinical application of penile transplantation.

The history of transplant surgery and plastic surgery is explored in introductory **chapter 1**. Transplantation has a long history in writing and the arts, but only became a viable specialty through innovations in vascular surgery and the discovery of immunology and immunosuppressive drugs in the 19th and 20th centuries. Plastic surgery has been performed for millennia, but saw a big leap in innovation kickstarted by the Napoleontic wars and the first and second world war. The birth of vascularized composite allotransplantation (VCA) out of these two specialties is described and special consideration is given to the role of major armed conflicts in the development of both plastic surgery and the field of VCA. VCA was first unsuccessfully attempted in ancient history, the 19th century and in 1953, but only became a clinical reality with the first successful hand transplants in the late 1990s. The field has then expanded to include face transplants in 2003. Penile loss negatively influences mental health and regularly affects single young men. The wars in Iraq and Afghanistan involved a high amount of foot patrols due to difficult terrain and many soldiers returned with penis injuries after being struck by improvised explosive devices, while in South Africa many young men dealt with penile loss after infections due to unsanitary circumcisions. Research programs were then established that resulted in successful penile transplants. Many challenges remain in the field of VCA related to acute and chronic rejection, limited donor availability, limits to tolerated ischemic time, adverse effects of immunosuppressive regimens, and poor nerve function. The thesis contains studies that aim to 1) objectify rejection patterns in hand face, and penis transplants, 2) improve candidate access through the treatment of patients that have been sensitized by earlier exposure to foreign DNA, 3) expand graft availability and quality through high subzero ice free graft preservation, and 4) provide an ethical framework for the application of urogenital transplantation in a clinical setting and describe the prerequisite steps needed to perform a penile transplant in a clinical setting.

Part I: Objectifying clinical and histological graft rejection in VCA

Chapter 2 describes a penile transplant model in the rat. Earlier models lacked reliable blood supply to study graft preservation and rejection and were difficult to monitor due to their placement. The model described has been used in over 80 transplants with a >90% success rate. The penile graft is obtained from the donor by dissection into the pelvis to include the proximal urethra, dorsal penile nerves, dorsal penile vein and both internal pudendal arteries. Grafts are placed in the recipient groin and vascular coaptation to the superficial femoral artery and superficial epigastric artery and vein is obtained with a non-suture cuff technique. Syngeneic transplants survived long term, and the model design allows for standardized graft monitoring.

This model was used in **chapter 3** to establish a rejection classification for rat penile transplants. Penile transplantation lacked such a classification, hampering comparison between different studies into penile rejection. Of particular interest is the relative immunogenicity of skin in a penile graft. If the skin is the most immunogenic component of the graft (as it is in other types of VCA), possible rejection can be monitored by monitoring the skin as a sentinel for the entire transplant. Follow up of 25 allogeneic transplants shows that penile rejection follows a 4-stage clinical progression with complete rejection seen between post-operative days 14 and 16. Histological samples were used to develop a specific 4-grade rejection classification analogous to the 2007 Banff Criteria for skin-containing human allografts. The histological analysis specifies the rejection grades for four tissue types: glans skin, urethra, corpus and vessel. Urethra and glans skin are found to be the most immunogenic, thus making the skin a good sentinel for graft rejection in penile transplantation.

Analogous to the study described in chapter 3, **chapter 4** aims to provide a skin rejection grading system in a swine hindlimb transplant model. Like in rat penis transplants, there is no standardized skin rejection grading system available for swine hindlimb grafts. To provide this grading system, 214 skin samples were analyzed and graded for rejection by a veterinary pathologist and graded in a blinded fashion. Clinical imaging of the grafts linked to the analyzed samples were similarly graded for clinical signs of rejection such as edema, erythema, skin sloughing, and necrosis. Based on the findings a Banff 2007 inspired grading system was developed that specifies dermal and epidermal involvement related to leukocyte infiltration and cell necrosis. The rejection grading systems from chapter 3 and chapter 4 can be used in future studies to provide an objective measure of graft rejection.

Part II: Improving transplant candidate access: desensitization strategies in VCA

Sensitization is a major problem in transplantation. Patients that have been exposed to foreign HLA develop memory to foreign tissues and rapidly reject allografts. Such patients are considered to be bad candidates for transplantation, as the chance of failure is significant. In VCA this problem is particularly pressing, since many patients are sensitized due to the necessary care they received as part of the injury that led to the tissue loss that necessitates a transplant. Current strategies aimed at mitigating the effects of donor specific antibodies produced by sensitized recipients yield mixed results.

In **chapter 5** a novel approach was tested in rat hind limb transplants that aims to specifically target B-cell memory responsible for the accelerated production of donor specific antibodies. For this aim an autologous bone marrow transplant after total body irradiation and fludarabine was tested for its effect on allogeneic hind limb transplant survival after earlier sensitization of recipient rats with skin grafts of the same donor. Bone marrow transplant treated animals showed significant declines in circulating donor specific antibodies and allowed for long term graft survival in sensitized rat hind limb recipients, proving the effectiveness of the approach.

Part III: Expanding graft availability: high subzero ice free graft storage.

In addition to sensitization, extended graft preservation is an enduring challenge in transplantation. In the current clinical practice still utilizes static cold storage as the gold standard, where grafts are flushed with HTK or University of Wisconsin solution before being put on ice. This approach only allows for graft survival of 4-24 hours before quality has deteriorated enough to make proper functioning after reperfusion impossible. The associated time constraints provide limitations in donor matching and recipient pretreatment. Approaches for extended preservation are normo- and hypothermic machine perfusion, cryopreservation and high subzero ice free preservation.

In **chapter 6** an approach to high subzero ice free graft preservation is described that uses peptoids that are based upon naturally occurring peptides in arctic animal species. These peptoids prevent ice formation and depress the freezing point of the surrounding water. Rat penile grafts were perfused with peptoid solution and stored at -5°C for 24, 48, or 72 hours, after which they were transplanted using the model described in chapter 2. Grafts were followed up clinically for signs of inflammation and necrosis. They were histologically assessed for inflammatory markers and cell death. Peptoid perfused grafts turned out to be able to sustain up to 72 hours of ischemia and fully recover.

Part IV: Ethical clinical application of penile transplantation

The do no harm principle that is a cornerstone of medicine is inevitably broken with the performance of allotransplantation, as the necessary immunosuppression has a wide range of adverse effects on the recipient's health. Over their lifetimes graft recipients can expect to suffer from kidney failure, neurological problems, cardiovascular disease and malignancies. In vascularized composite allotransplantation there thus is a trade-off by the improvement of quality of life provided by the transplant and the expected reduction in quantity of life due to immunosuppression side effects. The ethical considerations that are necessitated by this trade-off are applied to the clinical implementation of a penile transplant program in **chapter 7.** Penile transplants are justified in light of their considerable improvements over conventional reconstructive options concerning erectile function, voiding, erogenous function and donor site morbidity. For the clinical implementation, the importance of rigorous patient selection, prudent informed consent, donor & recipient privacy and ensuring lifelong care is highlighted. Based on the ethical considerations made it can be concluded that penile transplants can be performed in an ethically sound fashion when patients are selected rigorously both for their indication as well as their mental fitness to receive a transplant and commit to lifelong clinical visits and use of immunosuppressants.

The findings of the preclinical studies in this thesis are then applied in a novel clinical case of penile transplantation that is included in **chapter 8.** A penile transplant is described that includes the complete penis shaft, scrotum and part of the abdominal wall. The recipient is a young soldier that lost both his legs along with his penis and partial abdominal wall as a result of an improvised explosive device blast sustained while employed. Extensive analysis of the defect and remaining structures was performed with a focus on corpora, nerves and vasculature. The surgery was extensively practiced before being clinically performed. Vascular anastomoses were performed ensuring the perfusion of both the penis shaft as well as the different skin flaps. The recipient was treated with an immunomodulatory regimen consisting of monoclonal antibody induction, calcineurin inhibitor (tacrolimus) monotherapy maintenance, and a donor bone marrow cell infusion. Clinical outcomes were excellent. There was no skin necrosis and a year post transplant the patient can void standing, have erections and also orgasm through the transplant. Most importantly, the patient is very happy with the outcome and experiences a great improvement in quality of life.

Finally, **chapter 9** features a discussion of the main findings of the thesis and relates them to the current literature in VCA and organ transplantation. Limitations of the studies are related to the findings and the clinical applicability of the studies is discussed. The positive early clinical outcomes found in penile transplantation combined with the innovations the field has seen since its inception provide hopeful signs for further expansion of this experimental procedure to standard care for complete penile loss.

Nederlandse samenvatting

Dit proefschrift behelst studies gerelateerd aan transplantaatafstoting, desensibilisatie van transplantatiekandidaten, transplantaat preservatie en transplantatie-ethiek in relatie tot gevascularisseerde samengestelde donorweefseltransplantatie (Vascularized Composite Allotransplantation, VCA) en in het bijzonder penistransplantatie. In dit deel van het proefschrift worden de belangrijkste bevindingen samengevat volgens de structuur van het complete werk. Het betreft hierbij vier onderdelen waarin onderzoek wordt beschreven met betrekking tot: (1) het objectiveren van klinische en histologische transplantaatafstoting in VCA, (2) het vergroten van de toegankelijkheid van VCA voor patienten door het verbeteren van desensibilisatiestrategieen in VCA, (3) het vergroten van het aantal beschikbare weefsels voor transplantatie door ijsvrije transplantaatpreservatie bij temperaturen vlak onder het vriespunt, (4) de ethische klinische implementatie van penistransplantatie.

De geschiedenis van transplantatiechirurgie en plastische chirurgie worden beschreven in **hoofdstuk 1**. Transplantatie heeft een lange historie in kunst en literatuur, maar is pas een medisch specialisme geworden in de 19e en 20e eeuw door innovaties op het gebied van vasculaire chirurgie en immunosuppresiva. Plastische chirurgie wordt reeds sinds millennia beoefend en maakte een grote ontwikkeling door die werd aangewakkerd door de Napoleontische oorlogen en WO1&2. Gevascularisseerde samengestelde donorweefseltransplantatie (VCA) ontstond uit deze twee specialismes, waarbij oorlog wederom een rol speelde in deze ontwikkeling. In de 19e eeuw en in 1953 werden onsuccesvolle pogingen tot VCA gedaan. De eerste handtransplantaties in de late jaren 90 waren de eerste succesvolle VCA en in 2003 werd gelaatstransplantatie voor het eerst succesvol uitgevoerd.

Het verlies van de penis heeft een negatief effect op mentaal welbevinden en komt vaak voor bij jonge mannen zonder partner. De oorlogen in Irak en Afghanistan leidden tot relatief veel penisverwondingen bij soldaten en traditionele besnijdenissen in Zuid-Afrika leidden regelmatig tot het verlies van de penisschacht. In dit kader opgerichtte onderzoeksgroepen hebben recent succesvolle penistransplantaties uitgevoerd.

Er zijn nog vele uitdagingen voor het vakgebied van VCA op het vlak van acute en chronische afstoting, beperkte beschikbaarheid van donorweefsel, beperkingen in door transplantaten verdragen ischemietijd, ernstige bijwerkingen van immunosuppresiva, en beperkte motorische en sensorische functie van transplantaten. Dit proefschrift behandelt studies gericht op (1) Het objectiveren van afstotingspatronen in hand-, gelaat-, en penistransplantatie, (2) Het door middel van desensibilisatie verbeteren van toegang tot transplantatie voor kandidaten die door eerdere blootstelling aan vreemd DNA gesensibiliseerd zijn geraakt, (3) Het vergroten van het aantal

beschikbare transplantaten door middel van ijsvrije transplantaatpreservatie bij temperaturen vlak onder het vriespunt, en (4) Het vervaardigen van een ethisch raamwerk voor de toepassing van urogenitale transplantatie in de kliniek en het vaststellen van de vereiste stappen om penistransplantatie in de kliniek mogelijk te maken.

Deel I: Objectiveren van klinische en histologische transplantaatafstoting in VCA.

Hoofdstuk 2 beschrijft een penistransplantatiemodel in de rat. Eerdere modellen misten betrouwbare bloedvoorziening van het transplantaat die noodzakelijk is om preservatie en rejectie te kunnen onderzoeken en waren tevens lastig klinisch te vervolgen als gevolg van hun positionering. Het nieuw beschreven model is gebruikt in meer dan 80 transplantaties met een succespercentage van 90%. Het transplantaat wordt bij de donor gedissecteerd tot in het kleine bekken en bevat de proximale urethra, dorsale peniszenuwen, de vena dorsalis penis en de arteria pudenda interna beiderzijds. Het transplantaat wordt in de lies van de ontvanger geplaatst en geanastomoseerd aan de arteria femoris superficialis en de arteria en vena epigastrica superficialis door middel van een hechtingvrije manchet techniek. Syngene transplantaten bleven levensvatbaar op de lange termijn en het model maakt het mogelijk om het transplantaat op gestandaardiseerde wijze te monitoren.

Het model uit hoofdstuk 2 wordt in **hoofdstuk 3** gebruikt om een afstotingsclassificatie te ontwikkelen voor penistransplantaties in de rat. Een dergelijke classificatie bestond nog niet voor penistransplantaties, waardoor het vergelijken tussen studies met betrekking tot afstoting van penisweefsel belemmerd werd. Specifiek werd gekeken naar de immunogeniciteit van de huid in een penistransplantaat. Wanneer de huid het meest immunogene onderdeel van het transplantaat is (zoals bij andere VCA) kunnen klinische tekenen van afstoting van de huid gebruikt worden als indicator voor afstoting van het gehele transplantaat. Het vervolgen van 25 transplantaten liet zien dat de afstoting van penisweefsel een in vier stadia op te delen klinische progressie volgt waarbij volledige afstoting geobserveerd wordt tussen postoperatieve dagen 14 en 16. Histologische monsters werden gebruikt om een specifieke 4-stadia classificatie van de afstoting van penisweefsel te maken analoog aan de 2007 Banff criteria voor huid-bevattende humane allotransplantaties. De histologische analyse specificeert de afstotingsgraden voor vier verschillende weefseltypes: huid van de glans penis, de bekleding van de urethra, weefsel van de corpora en vaatweefsel. De urethra en de huid van de glans penis werden het meest immunogeen bevonden, waarmee geconcludeerd werd dat de huid een goede schildwachter is voor afstoting van een penistransplantatie.

Analoog aan de studie beschreven in hoofdstuk 3, heeft **hoofstuk 4** als doel om een gradatie voor huidafstoting in een achterpoot-transplantatiemodel in het minia-

tuurvarken vast te stellen. Net als in rattenpenistransplantaties is er geen gestandaardiseerde huidafstotingsclassificatie voor het achterpootmodel in het miniatuurvarken. Voor de ontwikkeling van een dergelijke classificatie werden 214 huidmonsters geanalyseerd en geclassificeerd met betrekking tot afstoting door een geblindeerde veterinaire patholoog. Klinische foto's van de achterpoottransplantaties die tegelijk werden genomen met de huidmonsters werden op eenzelfde wijze beoordeeld voor klinische tekenen van afstoting zoals oedeem, erytheem, huidschilfering, en necrose. Gebaseerd op deze bevindingen werd een eveneens op de Banff 2007 classificatie gebaseerde classificatie ontwikkeld die onderscheid maakt met betrekking tot dermale en epidermale betrokkenheid op het gebied van leukocyteninfiltratie en cel necrose. De afstotingsclassificaties uit hoofdstuk 3 en 4 kunnen in toekomstige studies gebruikt worden als objectieve uitkomstmaten voor transplantaatafstoting.

Deel II: Verbeteren van toegang tot VCA: desensibilisatiestrategieën.

Sensibilisatie is een belangrijk probleem in transplantatie. Patienten die zijn blootgesteld aan vreemd DNA ontwikkelen geheugen tegen vreemd weefsel dat leidt tot versnelde afstoting van allotransplantaties. Dergelijke patienten worden gezien als slechte kandidaten voor transplantatie daar de kans op transplantaatfalen aanzienlijk is. Voor VCA is dit een extra relevant probleem omdat veel patienten gesensibiliseerd zijn door de noodzakelijke behandelingen die zij ondergingen ten gevolge van het trauma dat de transplantatie noodzakelijk maakt. Huidige strategieën gericht op het mitigeren van de effecten van donor-specifieke antilichamen die geproduceerd worden door gesensibiliseerde ontvangers leveren wisselende resultaten op.

In **hoofdstuk 5** wordt een nieuwe desensibilisatiestrategie getest in de transplantatie van rattenpoten gericht op het B-cel geheugen dat verantwoordelijk is voor de versnelde productie van donor-specifieke antilichamen. Met dit doel werd een autologe beenmergtransplantatie uitgevoerd na bestraling van het complete rattenlichaam en toediening van Fludarabine. Hierna werd gekeken naar de overleving van deze allogene achterpoottransplantaties nadat de ontvangers eerder waren gesensibiliseerd met huidtransplantaten van dezelfde donor. Dieren die een beenmergtransplantatie hadden ondergaan vertoonden een siginificante afname van circulerende donor-specifieke antilichamen en lange-termijn overleving van achterpoottransplantaten in gesensibiliseerde ontvangers bleek mogelijk, waarmee de effectiviteit van de interventie werd aangetoond.

Deel III: Weefselbeschikbaarheid voor transplantatie vergroten: ijsvrije lage-temperatuurs transplantaatpreservatie.

Net als sensibilisatie is lange termijn transplantaatpreservatie een voortdurende uitdaging in de transplantatiegeneeskunde. In de huidige klinische praktijk is het op ijs opslaan van met HTK of University of Wisconsin oplossing geperfundeerde transplantaten nog immer de gouden standaard. Deze aanpak maakt het mogelijk om weefsels voor 4 tot 24 uur te bewaren voordat de kwaliteit van het weefsel zo sterk verslechterd is dat functieherstel na reperfusie niet mogelijk is. Deze tijdslimiet leidt tot beperkingen op het gebied van het koppelen van donors en ontvangers en het van tevoren behandelen van ontvangers. Technieken voor het verlengen van preservatietijd zijn normo- en hypotherme machine perfusie, cryopreservatie en ijsvrije preservatie bij temperaturen kort onder het vriespunt.

In **hoofdstuk 6** wordt een techniek voor ijsvrije weefselpreservatie beschreven die peptoïdes gebruikt die gebaseerd zijn op natuurlijk voorkomende peptides die gevonden worden in diersoorten die leven in poolgebieden. Deze peptoïdes voorkomen ijsformatie en verlagen hiermee het vriespunt van het water in hun omgeving. Penistransplantaten van ratten werden geperfundeerd met peptoïdeoplossing en opgeslagen bij -5°C voor 24, 48, of 72 uur, waarna zij werden getransplanteerd volgens het in hoofdstuk 2 beschreven model. Deze transplantaten werden klinisch vervolgd voor tekenen van inflammatie en necrose. Histologisch werden inflammatoire markers en celdood vervolgd. Met peptoïden geperfundeerde transplantaten bleken volledig te kunnen herstellen na 72 uur van ischemie te hebben doorstaan.

Deel IV: Ethische klinische implementatie van penis transplantatie.

Het primum non nocere principe dat een van de hoekstenen van de geneeskunde is wordt onvermijdelijk gebroken met het uitvoeren van een transplantatie, daar de noodzakelijke immunosuppressie een groot aantal negatieve effecten heeft op de gezondheid van de ontvanger. Gedurende hun leven kunnen ontvangers van transplantaten te maken krijgen met nierfalen, neurologische problemen, cardiovasculaire aandoeningen en maligniteiten. Voor VCA is er derhalve een afweging te maken tussen het verbeteren van de kwaliteit van leven door een transplantatie en de te verwachten afname van de levensduur gerelateerd aan de bijwerkingen van de bij de transplantatie noodzakelijke immunosuppressie. De ethische vraagstukken die opgeworpen worden door deze afweging worden toegespitst op de klinische implementatie van een penistransplantatieprogramma in **hoofdstuk 7**. Penistransplantaties zijn gerechtvaardigd in het kader van de grote verbetering die zij vertegenwoordigen over de uitkomsten van conventionele reconstructieve opties met betrekking tot erectie, blaaslediging, erogene functies en schade aan plaatsen waar autoloog weefsel anders verwijderd

zou worden. Voor de klinische implementatie van penistransplantatie wordt het belang benadrukt van grondige patiëntselectie, adequaat verkregen informed consent, anonimiteit van donor en ontvanger en het garanderen van levenslange zorg voor ontvangers van penistransplantaties. Gebaseerd op geldende ethische normen kan geconcludeerd worden dat penistransplantaties op ethisch verantwoorde wijze kunnen worden uitgevoerd wanneer patiënten een grondige selectie ondergaan met betrekking tot hun indicatie en hun mentale gesteldheid die het mogelijk maakt om zich te committeren aan het levenslang innemen van immunosuppresiva en het levenslang ondergaan van klinische controles.

De bevindingen van de preklinische studies uit dit proefschrift worden vervolgens toegepast in een klinische casus van penistransplantatie in **hoofdstuk 8**. Een penistransplantatie wordt beschreven waarbij de complete schacht, het scrotum en een deel van de buikwand zijn getransplanteerd. De ontvanger is een jonge soldaat die beide benen, zijn penis en een deel van zijn buikwand verloor als gevolg van een bom waar hij door getroffen werd gedurende een uitzending. Een uitvoerige analyse van de uitgebreidheid van het defect en de nog aanwezige structuren werd uitgevoerd met een focus op de corpora, zenuwen en vaatvoorziening. De operatie werd langdurig geoefend voordat deze klinisch werd uitgevoerd. Vaatverbindingen werden gelegd om de doorbloeding te garanderen van zowel de penisschacht als de verschillende huidflappen die onderdeel waren van het transplantaat. De ontvanger werd behandeld met een immunomodulerend protocol dat bestond uit inductietherapie met monoclonale antilichamen, donor beenmerginfusie en onderhoudstherapie met calcineurineremmer (tacrolimus) monotherapie. De klinische resultaten waren uitstekend. Er was geen huidnecrose en één jaar na de transplantatie kon de patient staand urineren, had hij spontane erecties en tevens orgasmes door het transplantaat. Bovenal is de patient zelf zeer tevreden over de uitkomst en ervaart een grote verbetering in zijn kwaliteit van leven.

Tot slot bevat **hoofdstuk 9** een discussie van de bevindingen uit dit proefschrift en relateert deze aan de huidige literatuur op het gebied van VCA en orgaantransplantatie. De beperkingen van de studies worden gerelateerd aan de bevindingen en de klinische toepasbaarheid van de studies wordt besproken. De uitstekende vroege uitkomsten die gevonden zijn voor penistransplantaties, gecombineerd met de innovaties die in het veld van VCA hebben plaatsgevonden sinds de geboorte ervan bieden hoop voor verdere ontwikkeling van deze experimentele procedure om ooit standaardbehandeling te worden bij compleet verlies van de penis.

PhD portfolio

PhD Candidate: S.A.J. Fidder
Erasmus University Medical Center, Rotterdam
Department: Plastic and Reconstructive Surgery and Hand Surgery
PhD Period: 2017-2021
Promotor: Prof. dr. S.E.R. Hovius
Copromotors: Dr. D. Vasilic, Prof. G. Brandacher, M.D.

	Year(s)	Workload (ECTS)
1. PhD Training		
Courses in immunology		
Principles of Immunology I (auditor) (JHU Bloomberg School of Public Health)	2017	8
Graduate Immunology (auditor) (Johns Hopkins Graduate School of Immunology)	2018	8
Animal Research Courses		
JHU Animal Care and Use Committee Animal handling and care course.	2017	1
General courses		
Prescription for Healthcare Lean Six Sigma (Johns Hopkins Carey Business School)	2017	1
Histories of Public Health in Baltimore, 1750 – present (JHU Bloomberg School of Public Health)	2017	6
Medical skills		
Microsurgery training (20 hours/week)	2017	250hrs
Oral Presentations		
JHH Department of Plastic & Reconstructive Surgery Annual Resident Research Symposium, Baltimore, MD, USA	2017	0.7
JHH Department of Plastic & Reconstructive Surgery Annual Resident Research Symposium, Baltimore, MD, USA	2018	0.7
JHH Department of Plastic & Reconstructive Surgery Annual Resident Research Symposium, Baltimore, MD, USA	2019	0.7
Regenerative Medicine Workshop at Charleston, Charleston, SC, USA	2019	0.7
Plastic Surgery Research Council Annual Meeting, Baltimore, MD, USA	2019	0.7
American Transplant Congress, virtual	2021	0.7
Poster Presentation		
American Transplant Congress, Chicago, IL, USA	2017	0.7
American Transplant Congress, Seattle, WA, USA	2018	0.7
American Transplant Congress, Boston, MA, USA	2019	0.7
American Society of Reconstructive Transplantation Bi-annual Meeting, Chicago, IL, USA	2018	0.7

Attended Seminars and meetings		
Departmental Journal Club, Dept of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA	2017-2019	3
American Transplant Congress, Chicago, IL, USA	2017	1
American Transplant Congress, Seattle, WA, USA	2018	1
American Society of Reconstructive Transplantation bi-annual Meeting, Chicago, IL, USA	2018	1
Regenerative Medicine Workshop at Charleston, Charleston, SC, USA	2019	1
Plastic Surgery Research Council Annual Meeting, Baltimore, MD, USA	2019	1
2. Teaching activities		
Supervision		
Final year medical student supervision in lab projects (3 students)	2017-2019	4
Total		43

Author affiliations

Georg J. Furtmüller
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Andres Matoso
Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
Department of Urology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Joanna W. Etra
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Kara Lombardo
Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Maria Chicco
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Byoung Chol Oh
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Dalibor Vasilic
Department of Plastic and Reconstructive Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands

WP Andrew Lee
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Richard J. Redett III
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Damon S. Cooney
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Gerald Brandacher
Department of Plastic and Reconstructive Surgery, Vascularized Composite Allotransplantation (VCA) Laboratory; Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Brian Simons
Department of Urology, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Cory Brayton
The Department of Molecular & Comparative Pathobiology; Johns Hopkins University School of Medicine, Baltimore, MD, USA

Carisa M. Cooney
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Barbara Kern
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Denver Lough
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Michael J. Grzelak
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Keli Kolegraff
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Steven Bonawitz
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA
Department of Surgery, Cooper Medical School of Rowan University, Camden NJ

Jaimie Shores
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Sarah E. Beck
The Department of Molecular & Comparative Pathobiology; Johns Hopkins University School of Medicine, Baltimore, MD, USA

Howard D. Wang
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Devin T. Miller
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Ali R. Ahmadi
Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD.

Felix Nägele
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Joseph Lopez
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Amy Quan
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Joshua Budihardjo
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Burcu Akpinarli
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Giorgio Raimondi
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Robert A. Montgomery
Department of Surgery, New York University (NYU) Langone Medical Center, NYU Langone Transplant Institute, New York, NY.

Zhaoli Sun
Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD.

Andrea Callegari
X-Therma Inc., Berkeley, California, USA

Adam Childs
X-Therma Inc., Berkeley, California, USA

Franka Messner
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Xiaoxi Wei
X-Therma Inc., Berkeley, California, USA

Mark Kline
X-Therma Inc., Berkeley, California, USA

Jordan L. Coleman
Department of Plastic and Reconstructive Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Frederick S. Berlin
Department of Psychiatry and Behavioral Sciences; Johns Hopkins University School of Medicine, Baltimore, MD, USA

Jeremy Sugarman
Johns Hopkins Berman Institute of Bioethics; Johns Hopkins University School of Medicine, Baltimore, MD, USA

Jeffrey Kahn
Johns Hopkins Berman Institute of Bioethics; Johns Hopkins University School of Medicine, Baltimore, MD, USA

Arthur L. Burnett
Department of Urology, Johns Hopkins School of Medicine, Baltimore MD

Sami H. Tuffaha
Department of Plastic and Reconstructive Surgery, Johns Hopkins School of Medicine, Baltimore MD

Justin M. Sacks
Department of Plastic and Reconstructive Surgery, Johns Hopkins School of Medicine, Baltimore MD

Trinity J. Bivalacqua
Department of Urology, Johns Hopkins School of Medicine, Baltimore MD

Devin M. O'Brien-Coon
Department of Plastic and Reconstructive Surgery, Johns Hopkins School of Medicine, Baltimore MD

Aliaksei Pustavoitau
Department of Anesthesiology, Johns Hopkins School of Medicine, Baltimore MD

Nicole A. Rizkalla
Department of Anesthesiology, Johns Hopkins School of Medicine, Baltimore MD

Annette M. Jackson
Department of Surgery, Duke University School of Medicine, Durham, NC

Vidhi Javia
Department of Plastic and Reconstructive Surgery, Johns Hopkins School of Medicine, Baltimore MD

Janice Davis-Sproul
Cell Therapy Laboratory, Johns Hopkins School of Medicine, Baltimore MD

Daniel C. Brennan
Division of Renal Medicine, Johns Hopkins School of Medicine, Baltimore MD

Inbal B. Sander
Department of Dermatology, Johns Hopkins School of Medicine, Baltimore MD

Shmuel Shoham
Division of Infectious Disease, Johns Hopkins School of Medicine, Baltimore MD

Nikolai A. Sopko
Department of Urology, Johns Hopkins School of Medicine, Baltimore MD

List of publications

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About the author

Samuel Fidder was born and raised in Hilversum, The Netherlands. He graduated from the Gemeentelijk Gymnasium Hilversum (cum laude) and obtained his medical degree from Utrecht University, Utrecht, The Netherlands. During medical school he participated in two honors programs and gained international experience through clinical rotations in San Diego, California, USA, and clinical research in Accra, Ghana. In the final year of his studies, he interned at the Vascularized Composite Allotransplantation Lab of The Johns Hopkins University School of Medicine in Baltimore, Maryland, USA. He later returned to Baltimore and JHU to continue his research as part of the Erasmus University PhD program. After gaining experience in intensive care medicine and general surgery at Erasmus University Medical Center and the Maasstad Ziekenhuis, he currently works as a resident-not-in-training (ANIOS) in the plastic surgery department of the Erasmus University Medical center.