Thesis

Graft Optimization during Ex Vivo Organ Perfusion: the Potential of Genetic Modulation

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Graz, 04 September 2024

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Zusammenfassung

Dynamische Organpräservationsmethoden wie normotherme, subnormotherme und hypotherme Maschinenperfusion haben sich als Alternativen zur konventionellen statischen kalten Lagerung etabliert. Diese Organperfusionstechniken dienen nicht nur als Präservationsmethoden, sondern ermöglichen auch die Beurteilung und Rekonditionierung von Organen vor der Transplantation. Gentherapie stellt eine neuartige Strategie dar, welche das Potenzial hat den Bereich der Transplantatoptimierung und -behandlung zu revolutionieren. Dadurch können spezifische Pathomechanismen, die am Transplantationsprozess beteiligt sind, gezielt angesprochen und modifiziert werden. Diese Übersichtsarbeit zielt darauf ab, einen Überblick über die Methoden der Genübertragung während der ex vivo Maschinenperfusion von Nieren- und Lebertransplantaten zu geben. Aktuelle Literatur zu gentherapeutischen Ansätzen während der ex vivo Organerhaltung, insbesondere in Hinblick auf den Ischämie-Reperfusionsschaden, sowie akute und chronische Transplantatabstoßung, wurde analysiert. Darüber hinaus werden potenzielle Herausforderungen aufgezeigt, welche die weitere Verfeinerung dieser therapeutischen Modalität beeinflussen könnten.

Abstract

Dynamic preservation methods such as normothermic, sub-normothermic and hypothermic machine perfusion circuits have emerged as viable alternatives to conventional static cold storage (SCS). These organ perfusion technologies not only serve as preservation methods, but also enable organ assessment, reconditioning, and repair prior to transplantation. Gene therapy is a novel strategy with the potential to transform the field of graft optimization and treatment. Thereby specific pathways involved in the transplantation process can be targeted and modified. This review aims to provide an overview of gene delivery methods during ex vivo machine perfusion of kidney and liver grafts. Recent literature on state-of-the-art gene therapy approaches during ex situ organ preservation, especially with respect to ischemia-reperfusion injury (IRI), as well as acute and chronic graft rejection, has been analyzed. Additionally, potential challenges that could affect further refinement of this therapeutic modality are outlined.

Keywords:

Kidney transplantation, liver transplantation, ischemia-reperfusion injury (IRI), graft rejection, ex vivo organ preservation, machine perfusion, genetic therapy, RNA interference, antisense oligonucleotides, antagomir, adeno-associated virus, adenovirus, lentivirus, nanoparticles

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Review

OPEN

Genetic Modulation: Future Trends Toward Graft Optimization During Machine Perfusion

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Abstract. Dynamic preservation methods such as normothermic, subnormothermic, and hypothermic machine perfusion circuits have emerged as viable alternatives to conventional static cold storage. These organ perfusion technologies serve as preservation methods and enable organ assessment, reconditioning, and repair before transplantation. Gene therapy is a novel strategy with the potential to transform the field of graft optimization and treatment. Thereby specific pathways involved in the transplantation process can be targeted and modified. This review aims to provide an overview of gene delivery methods during ex vivo machine perfusion of kidney and liver grafts. Recent literature on state-of-the-art gene therapy approaches during ex situ organ preservation, especially with respect to ischemia–reperfusion injury, as well as acute and chronic graft rejection have been analyzed. Additionally, potential challenges that could affect further refinement of this therapeutic modality are outlined.

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INTRODUCTION

Solid organ transplantation is the only effective treatment option for many end-stage organ diseases. As the discrepancy between patients requiring organ transplantation and available organs is increasing, the utilization of grafts from extended criteria donors (ECDs) and donations after circulatory death (DCD) has gained importance.[1] These grafts are considered suboptimal for transplantation as they are more likely to come to harm, especially in terms of ischemia–reperfusion injury (IRI). This results in a higher risk of primary nonfunction or delayed graft function.[2,3]

Static cold storage (SCS) after organ procurement is considered the standard preservation method for bridging the time until transplantation.[4] The lack of oxygen and reduced metabolism contribute to IRI and organ dysfunction. Because of the higher vulnerability of ECD and DCD grafts and the associated challenges, more gentle preservation methods are needed to use such marginal organs.[5] Several clinical studies have evidenced improved transplant outcomes and feasibility of both hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) methods compared with SCS.[5-8] However, organ transplantation still faces challenges in the development of new therapies to attenuate IRI, increase graft tolerance and patient survival, and reduce the systemic side effects of current immunosuppressive treatment strategies.[9] Another major hurdle in transplant medicine is the persistent organ shortage, despite the increasing utilization of ECD and DCD grafts. Therefore, alternative approaches to human organ transplantation, such as xenotransplantation, are currently being explored. However, the success of xenografts is limited by several obstacles, such as biological disparities, graft-versus-host disease, and the risk of zoonosis.[10] Genetic modulation is a promising approach to address the challenges in solid organ transplantation and contribute to the expansion of the donor pool.[11,12]

The feasibility of genetic modulation in kidney and liver transplant settings has been demonstrated in several in vivo animal studies and comprehensively summarized elsewhere.[13-15] One major drawback of in vivo gene modulation is the possibility of delivering therapeutic genes to cells other than those intended, thus causing

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off-target effects.[16] Therefore, especially in the setting of multiorgan donations, in vivo gene delivery may not be attainable, and concomitant logistic and ethical obstacles pose additional challenges. In recent years, dynamic preservation methods have emerged as suitable platforms for the ex vivo delivery of treatments. Various therapeutic agents have been tested in preclinical and clinical settings under different temperature settings during organ perfusion.[17] This review focuses specifically on gene delivery methods and recent approaches during ex vivo perfusion of kidney and liver grafts (Figure 1) and their challenges.

MACHINE PERFUSION METHODS

Ex vivo machine perfusion techniques have been established as an alternative to SCS to assess grafts under mimicked physiological conditions, maintain organ function and viability, remove metabolic waste products, or deliver therapeutics ex vivo. Amidst this transition, novel preservation solutions are emerging with the potential to surpass the efficacy of existing solutions.[18] Determined by a specific goal, the temperature range is one of the crucial aspects to be controlled and is often used to subdivide the perfusion methods into hypothermic (4–12 °C), normothermic (35–37 °C), and subnormothermic (temperature in between hypothermic and normothermic). However, there is no consensus on the temperature range of each term. Machine perfusion strategies will be briefly discussed for brevity, as comprehensive reviews of kidney[19-21] and liver[20-22] preservation methods are available.

HMP leads to a considerable decline in cellular metabolism, eventually slowing down to 10%.[22-24] Perfusion can be performed either oxygenated or nonoxygenation, whereas oxygenation is considered superior as the succinate metabolism pathway remains active and oxidative damage upon reperfusion is mitigated.[22,25] The absence of oxygen leads to a shift toward anaerobic metabolism and the aggravation of cellular processes, such as inflammation or immune responses, thus contributing to graft dysfunction.[26] Additionally, during

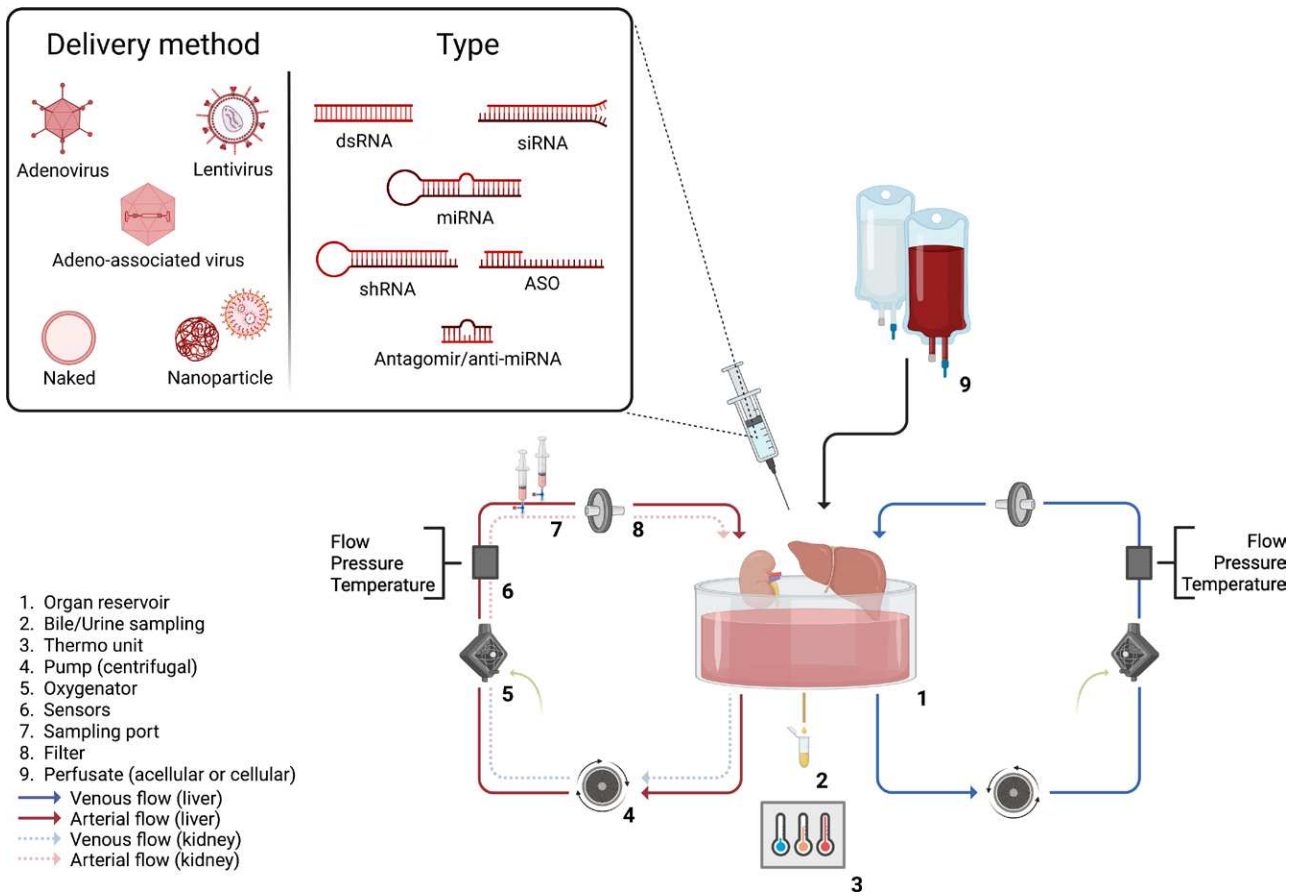

FIGURE 1. Overview of gene modulation techniques currently applied during ex vivo kidney or liver preservation and a schematic illustration of the perfusion set up of both the liver and kidney assist device (XVIVO Perfusion AB). Ex vivo machine perfusion devices usually consist of an organ reservoir, filled with acellular or cellular perfusion solutions, depending on hypothermic or normothermic conditions, respectively. The organs are connected via cannulas to the tubing of the device. More precisely, the liver is connected via the hepatic artery and the portal vein, and the kidney via the renal artery. Centrifugal pumps enable the circulation of the perfusate through the organ. Via the arterial arm the organ is perfused in a pulsatile manner, whereas the recirculation into the vein is continuous. The pressure and flow rate are permanently measured and adjustable depending on the perfusion temperature. The temperature is measured via sensors along the tubing and can be controlled via the thermo unit. This enables temperatures ranging from 12 °C to 37 °C, and thus hypothermic, subnormothermic, or normothermic perfusion. Perfusate samples can be taken from the sampling ports and cannulation of the bile duct or ureter enables collection of bile or urine, respectively. anti-miRNA, anti-micro-RNA; ASO, antisense oligonucleotide; dsRNA, double-stranded RNA; miRNA, micro-RNA; shRNA, short hairpin RNA; siRNA, small interfering RNA.

hypothermic preservation, the organ is protected from further injury by reconditioning mitochondrial function and diminishing levels of damage-associated molecular patterns and other detrimental agents, such as toxins and proinflammatory cytokines. Several clinical studies have proven the feasibility of organ preservation under hypothermic conditions, with the effect that this method is not inferior to SCS.[22] Furthermore, dynamic hypothermic perfusion mitigates cellular damage and improves graft function and overall patient survival. Additionally, after liver transplantation, biliary-related complications can be reduced.[24] HMP before kidney transplantation resulted in a decline in delayed graft function.[6] These findings have been strengthened by a recently conducted clinical trial showing that machine perfusion outperforms SCS despite therapeutic hypothermia of the donor.[27]

During subnormothermic machine perfusion, the temperature is usually adjusted to 20 °C to 25 °C. With this technique, organ injury caused by low preservation temperatures can be counteracted, and the metabolism is prevented from slowing down completely but not increased to the extent that an oxygen carrier is needed, as is the case with long-term normothermic preservation methods.[28]

During NMP physiological conditions are mimicked to address the limitations of hypothermic and subnormothermic preservation methods.[22] Perfusion is performed with cellular perfusion solutions, usually based on red blood cells mixed with colloid or crystalloid solutions.[23] Cell viability, and thus organ function, can be supported by adding various of nutrients and medications, such as antioxidants, glucose, sodium, amino acids, vitamins, and vasoactive agents. Also, pure oxygen or gas mixtures, such as oxygen and carbon dioxide, maintain organ metabolism.[22,23,29] One of the key benefits of NMP is the potential to replenish and maintain ATP levels and thus mitigating IRI.[5] Furthermore, the near physiological conditions enable a viability and quality assessment of the organ before transplantation.[22]

In the process of organ storage, abrupt temperature changes caused by transferring the organ from cold to warm and vice versa can result in a thermal shock, which can aggravate cellular damage and disrupt mitochondrial function. By increasing the temperature gradually and controllingly, organs can adjust to temperature changes and the risk of injury can be minimized.[22,28] Recent clinical research has proven the feasibility of using oxygenated controlled rewarming with a maximum temperature of 35 °C to preserve ECD kidneys after cold storage before transplantation.[30] Another innovative approach toward ischemia-free transplantation is to perform organ cannulation for ex vivo machine perfusion in such a manner that dynamic preservation can be continued concurrently with surgical anastomosis.[31,32] Thereby, organs preserved with normothermic perfusion methods only need to be cooled once. This is especially beneficial for high-risk organs, which are more susceptible to IRI. Recently, a pilot study confirmed the practicability of this approach by preserving and transplanting human liver grafts to the effect that outcomes were comparable with standard NMP but superior to SCS.[31]

GENETIC MODULATION IN ORGAN PRESERVATION

The introduction or modulation of genes can be accomplished through different biochemical mechanisms. Approaches based on messenger RNA use antisense oligonucleotides (ASOs) or antagomirs to introduce or modify genes. Another mechanism of gene regulation is RNA interference (RNAi), which uses RNA molecules such as micro-RNA (miRNA), short hairpin RNA (shRNA), or small interfering RNA (siRNA; Figure 2). RNAi is a promising approach for organ transplantation to mitigate IRI or counteract acute and chronic graft rejection by silencing the respective genes.[13] Different biosynthetic pathways are involved depending on whether miRNAs, shRNAs, or siRNAs are used.[13,14] siRNA has been demonstrated to be safe for use in humans and has been approved by the European Medicines Agency and the Food and Drug Administration (FDA) for the treatment of acute hepatic porphyria, transthyretin amyloidosis, hypercholesterolemia, and primary hyperoxaluria type 1.[33] Another possibility for therapeutic RNA modification are ASOs, which are similar to siRNAs in terms of production and FDA approval in the clinical setting. However, the mechanism of action differs from that of RNAi in that ASOs can exert their effects both in the cytoplasm and nucleus, thus not only downregulating gene expression but also enhancing the production of target proteins.[33] Antagomirs/anti-miRNAs are chemically modified ASOs that specifically target mature miRNAs in the cytoplasm and block protein translation.[34]

GENE DELIVERY METHODS

One of the major challenges in gene therapy is the efficient and safe delivery of genetic materials into target cells. This can be either viral or nonviral vector-mediated. Most vectors successfully used in gene therapy in preclinical and clinical settings are based on adenoviruses (Ads), adeno-associated viruses (AAVs), or lentiviruses.[35] However, immunogenicity, mutagenesis, and limited viral payload capacity still pose challenges.[35,36] Therefore, nonviral vectors have emerged with the potential to overcome some viral vector-associated challenges. Techniques applied in the context of organ perfusion are, among others, nanoparticles, polymers, lipids, or chemical modification of oligonucleotides.[13,14] Another strategy to deliver genetic material is either naked[35] or gymnotic, which could be beneficial for patients undergoing organ transplantation and associated immunosuppressive therapy because vector-mediated delivery methods can aggravate the adverse effects of gene therapy, such as carcinogenesis and inflammation.[11] Each delivery method entails varying advantages and disadvantages, as summarized in Table 1.

Ads and AAV Vectors

Ads offer several unique advantages that enhance their suitability as therapeutic agents for gene therapy. Firstly, stable gene expression can be achieved in both dividing and resting cells. Secondly, Ads can infect a wide range of cells within the host organism. Furthermore, large quantities of adenoviral vectors can be inexpensively produced.[36] However, this type of virus can be involved in human diseases owing to its high immunogenicity—to which extent mainly depends on the immunocompetence of the

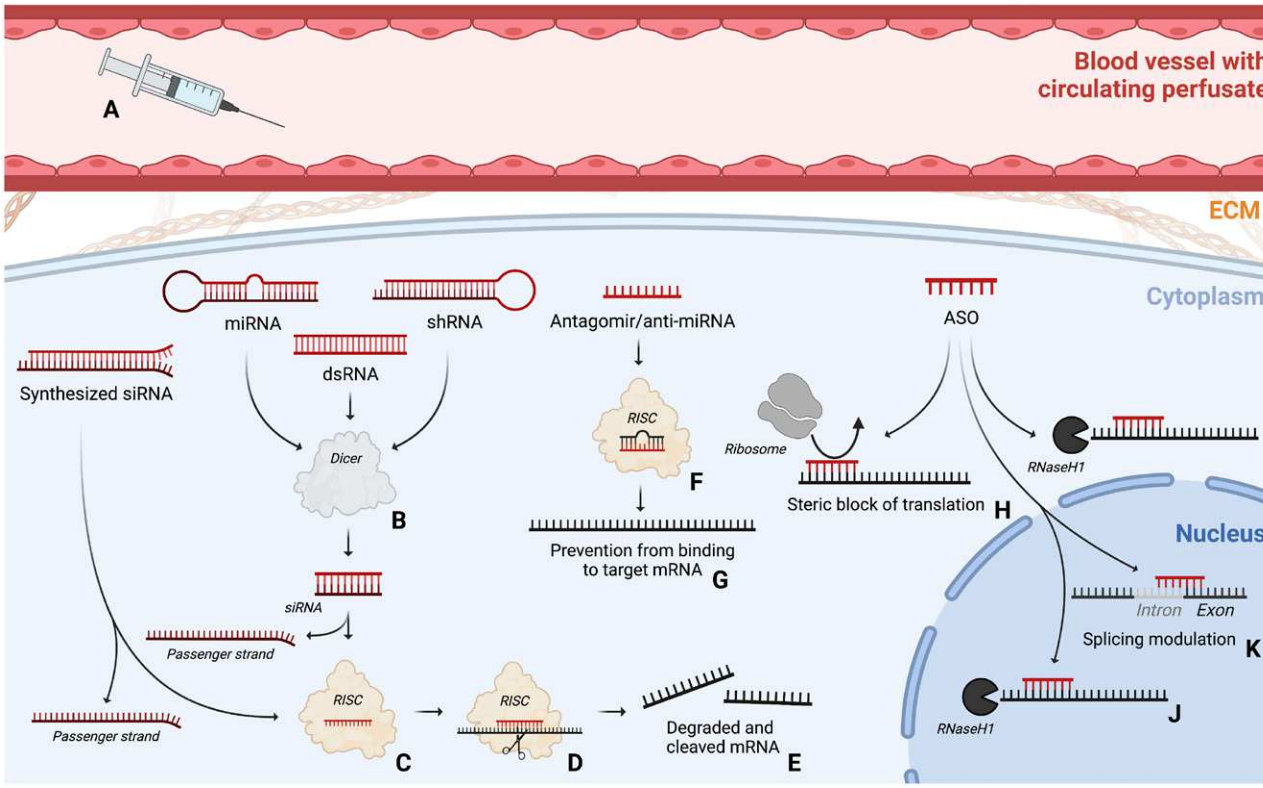

FIGURE 2. Mechanisms of gene modulation techniques applied during ex vivo organ perfusion. Gene therapeutics administered into the perfusion circuit and taken up into the cell to reach the cytoplasm (A). miRNAs, shRNAs, or dsRNAs bind to the dicer (B), which cleaves double-stranded RNA into short double-stranded fragments, also referred to as siRNA. The passenger strand is degraded while the guide strand binds to proteins of the Argonaut family that are, among other proteins, part of the RISC (C). The RISC then binds to mature mRNA complementary to the siRNA sequence (D), which is cleaved, degraded, and thus, the expression of the corresponding protein-coding gene silenced (E).[13,14] Antagomirs/anti-miRNA mimic the structure of miRNAs, thus bind to the RISC complex (F) and thereby supersedes miRNAs. This prevents the RISC complex from binding to the corresponding mRNA, and thus, gene expression is upregulated (G). ASOs bind to mRNA in the cytoplasm and either sterically block ribosomes (H) or induce RNaseH1-mediated cleavage (I). Furthermore, in the nucleus ASOs can target splicing sites to either inhibit or alternate splicing (J). Similarly, to their function in the cytoplasm ASOs can also induce RNaseH1 in the nucleus (K). AAV, adeno-associated virus; Ad, adenovirus; anti-miRNA, anti-micro-RNA; ASO, antisense oligonucleotide; dsRNA, double-stranded RNA; ECM, extracellular matrix; miRNA, micro-RNA; RISC, RNA-induced silencing complex; RNaseH1, ribonuclease H1; shRNA, short hairpin RNA; siRNA, short interfering RNA.

individual. Because of their immunosuppressive medications, transplant patients are at a higher risk of severe disease progression than immunocompetent humans who remain asymptomatic or develop mild symptoms.[36,42]

AAVs are nonpathogenic and can deliver only small amounts of genetic material, which can potentially be increased by delivering 2 vectors with overlapping sequences.[36,45] In comparison with Ad and other viral vectors, AAVs, especially recombinant ones, provoke cytotoxicity and immunogenicity to a much lesser extent. AAVs have been used in various clinical trials to deliver genetic material as a therapeutic approach for several human diseases,[22] especially hemophilia A[46] and B.[47]

In contrast to adenoviral vectors, the genetic material delivered by AAVs is incorporated into the host genome,[36] enabling stable and potentially sustainable expression. Furthermore, these 2 types of vectors differ in their duration of transgene expression. Ad vectors lead to expression within a few hours, whereas the expression of genes delivered by AAV vectors can be detected after 1 to 2 wk.[43] Self-complementary AAVs (scAAVs) can be used to achieve faster transgene expression and increase efficiency. However, 1 major limitation is the resulting reduction in packing capacity by approximately half. Furthermore, scAAVs can induce an immune reaction to a much stronger extent and thus influence gene expression and effectiveness of the therapy; however, the exact mechanisms are not yet fully understood.[36]

Both Ad and AAV vectors have been applied during organ perfusion to prove the feasibility of ex vivo gene transfection. Canine kidneys were transfected with different amounts of Ad5-cytomegalovirus-5-green fluorescent protein (GFP) particles for 24 h using a 32 °C exsanguinous metabolic support perfusion technique and a pyridoxalated hemoglobin polyoxyethylene-based solution.[44,49,50] Either omitting the administration of Ad particles or administration under hypothermic conditions served as controls. Fluorescence microscopy revealed that transfection was dependent on the perfusion temperature and concentration applied. Concerning the latter, the administration of 10^6 or 10^4 Ad particles per gram of tissue detected fluorescence in the intima of small and large kidney vessels. A higher concentration of 10^8 particles per gram of tissue led to the breach of the vascular endothelium and tubular fluorescence detection.[44] This could be explained by the fact that both the classical and alternative complement pathways can be induced by Ad5 vectors, resulting in the release of proinflammatory cytokines, leading to a cytokine storm.[42]

TABLE 1.

Pros and cons of delivery strategies

Delivery strategy	Advantages	Disadvantages	Temperature	References
Nonviral				
Naked (gymnotic)	Low costs Less off-target effects	Limited stability and thus reduced efficiency Susceptible to degradation	Feasibility of active intracellular antagomir uptake during NMP but not during HMP Limited transfection of siRNA during HOPE	11,12
Nanoparticles	Modification of size, shape, chemical, or physical characteristics to prolong circulation time, improve stability Enhancement of delivery with protein-targeting antibodies	Successful delivery is, among others, dependent on particle size, endothelial structure, biodistribution, and clearance mechanism Cytotoxicity (dose-dependent)	Lipid nanoparticles enable siRNA uptake during HMP and NMP PACE nanoparticles facilitate siRNA transfection during NMP	12,35,37-41
Viral				
Ad	Transfection of both dividing and resting cells Infection of a wide range of cells Expression within a few hours	Immunogenicity—the extent depends on the immunocompetence of the individual	Effective transfection at 32 °C after 24 h No transfection during HMP	36,42-44
AAV	Nonpathogenic Lesser cytotoxicity and immunogenicity compared with Ads Incorporations into the host's genome and thus potentially enabling a stable and sustainable expression	Expression after 1–2 wk—faster expression could be achieved with scAAVs; however, posing the risk of immune response activation Limited packaging capacity	Successful transduction during HOPE after 24 h	22,36,45-48
Lentivirus	Transfection of both dividing and resting cells Integration and enabling a sustainable expression Low immunogenicity Several genes can be targeted with 1 vector	Based on the pathogenic HIV 1, however, in the course of vector development, the amount of viral genome was decreased so that they are self-inactivating	Successful delivery and downregulation during SNMP	16,36

AAV, adeno-associated virus; Ad, adenovirus; HIV, human immunodeficiency virus; HMP, hypothermic machine perfusion; HOPE, hypothermic oxygenated machine perfusion; NMP, normothermic machine perfusion; PACE, poly(amine-co-ester); scAAV, self-complementary AAV; siRNA, small interfering RNA; SNMP, subnormothermic machine perfusion.

The endothelial barrier function of the kidneys could have been modified by this cytokine storm, disrupting endothelial integrity. Furthermore, no fluorescence was detectable in the cold-perfused kidneys, similar to that observed in the nontransfected organs.[44] In another study, rat liver grafts were subjected to hypothermic oxygenated machine perfusion (HOPE) supplemented with 4×10^8 plaque-forming units/mL of AAV8 vectors to transfect GFP for 2 h before transplantation. Twenty-four hours posttransplantation analysis confirmed the successful transfection of liver grafts during HOPE.[48]

Lentiviral Vector

Lentiviral vectors are based on the pathogenic human immunodeficiency virus 1; however, in the course of vector development, the amount of viral genome decreased to the extent that they are self-inactivating and thus safer. A major advantage of lentiviral vectors is their ability to effectively transfect dividing and nondividing cells. Furthermore, genetic material is integrated into the host genome, allowing a stable expression.[36]

Lentiviruses have not only been deployed in the production of immunotherapies based on chimeric antigen receptor regulatory T cells to treat cancer but have also been admitted to several clinical trials in the fields of genetic or metabolic disorders, malignancies, and immune diseases.[36] In the setting of organ transplantation and cell therapy, alloantigen-specific chimeric antigen receptor regulatory T cells have the potential to promote transplant acceptance and lower the risk of graft-versus-host disease. Although several preclinical animal studies have demonstrated its feasibility, more research is needed to ensure the efficiency and safety of this therapeutic option in the posttransplant setting.[51] Lentiviruses have become promising tools for ex vivo applications and gene modulation.[36] In preclinical hepatocyte transplantation, lentiviral vectors have been used effectively to transduce human hepatocytes and achieve a persistent gene expression.[52] Similarly, beta-2-microglobulin-targeting shRNA (shβ2m), delivered with lentiviral vectors, decreased gene expression levels in primary human hepatocytes while maintaining metabolism.[53] Recently, lentiviral vectors have been successfully used under subnormothermic conditions to silence gene expressions of major histocompatibility complex (MHC) I and MHC II in kidneys. Utilization during ex vivo machine perfusion caused no additional organ damage; however, it resulted in modification of cytokine expression.[16]

Nanoparticles

Nanoparticles are nonviral vectors that can be used to deliver gene therapeutics to specific organs. Successful delivery is mainly contingent on the size of the particles

as well as the endothelial structure, biodistribution, and clearance mechanism of the organ. Furthermore, warm ischemia, cold ischemia, and ex vivo machine perfusion techniques can influence the accumulation of nanoparticles in organs because IRI alters the structure of the endothelium. In addition to the size of the nanoparticles, other properties such as shape, chemical or physical characteristics, and the charge or composition of the surface can be modified to prolong circulation time, improve stability or accelerate internalization,[37] and overcome the risk of endonuclease degradation.[35]

The interaction of circulating nanoparticles with endothelial cells can cause cytotoxicity through several mechanisms, including the formation of reactive oxygen species, leading to inflammation or apoptosis, alterations in the integrity and permeability of the endothelial membrane, or activation of the autophagy lysosomal pathway.[38] Therefore, microvascular perfusion of the organ is an important aspect to consider when applying nanoparticles during ex vivo machine preservation. Inhomogeneous perfusion can lead to the accumulation of nanoparticles in certain areas and failure to reach others. Using antibodies to target proteins expressed on the surface of endothelial cells, such as anti-CD31, can enhance their delivery to endothelial cells. Normothermic perfusion of human kidneys with nanoparticles and anti-CD31 antibodies resulted in at least a 10-fold increase in endothelial cell targeting.[39] Another important aspect related to cellular toxicity is the surface charge of nanoparticles. Transfection of cultured human umbilical vein endothelial cells with poly(amine-co-ester) (PACE) 90 nanoparticles, exhibiting highly positive surface charges, showed higher cytotoxicity compared with neutrally charged nanoparticles (PACE 50). Notably, the administration of lipofectamine nanoparticles loaded with 200 nM siRNA resulted in a considerably higher rate of cell death than all dosage variations of PACE nanoparticles. Transfection with a class II MHC transactivator (CIITA) targeting siRNA, using either PACE 50, 60, or 70 nanoparticles or lipofectamine, led to >90% transfection efficiency.[41] The delivery of 38 nM Fas (CD95) siRNA to rat livers via invivofectamine nanoparticles during an HMP circuit increased transaminase levels, indicating intoxication, presumably because of excessive doses of invivofectamine.[12] Contrary results showed that rat livers could be successfully transfected with lipofectamine nanoparticles loaded with 50 nM Fas-targeting siRNA under HMP and NMP conditions.[40] However, the liver enzyme levels were not evaluated in this study. To overcome the limitations of conventional synthetic nanoparticles, extracellular vesicles (EVs), which are naturally released by eukaryotic cells, are a promising and novel approach as they can act as intercellular transporters for delivery of therapeutics.[54,55] Despite the rapid advancements and successes in EV research some of the underlying biological processes are yet not fully understood, and more research is needed to translate the findings into clinical applications.[54] In the transplant setting, the analysis of EVs released from discarded human kidney grafts during 6-h NMP provided the first insights into their role during machine perfusion. It revealed that it inferred the kidney quality from the subset of EVs released. This suggests that EVs not only hold the potential to deliver therapeutics but also serve as pretransplant biomarkers.[56]

APPLICATIONS DURING EX VIVO MACHINE PERFUSION

Only a limited number of genetic modulation approaches have been conducted during the preservation of kidneys or livers in an ex vivo machine perfusion circuit. Attempts aimed at mitigating IRI, acute and chronic graft dysfunction, and antiviral treatment (Table 2).

IRI

After organ transplantation, IRI and concomitant cellular damage are decisive factors contributing to primary nonfunction or graft dysfunction and to acute or chronic rejection.[60,61] During the transplantation process, both cold ischemic conditions, in terms of organ procurement, and warm ischemic conditions, such as surgical anastomosis time, cause cellular damage. Damage-associated molecular patterns are released because of either necrosis, necroptosis, ferroptosis, or pyroptosis. This causes noninfectious sterile inflammation involving both the innate and adaptive immune systems.[60,61] Furthermore, intrinsic mitochondria-dependent or extrinsic pathways are involved in apoptosis, leading to noninflammatory cell death and activating caspases.[60] The extrinsic pathway is based on extracellular cell death receptor signaling.[61] These signals are, for example, transduced via Fas, which is expressed on the surface of hepatocytes and are ultimately related to apoptosis.[14] The influence of Fas silencing on rat liver grafts was evaluated using a transplant model. Naked Fas siRNA was administered during either 1-h HOPE, after 4-h cold storage, or in vivo, 2 h before liver explantation and subsequent 22-h cold storage. Liver transplantation was performed after both types of organ preservation. This revealed that prolonged cold storage time (22 h) before HOPE caused death within 24 h; therefore, cold ischemia was reduced to 4 h. In the cold storage group, administration of naked Fas siRNA resulted in a significant decrease in transaminase levels, concomitant with a significant reduction in proinflammatory cytokines, such as interleukin (IL)-1a, IL-2, tumor necrosis factor, monocyte chemoattractant protein 1, and C-X-C motif chemokine 10. Compared with the administration of Fas siRNA in vivo, treatment with Fas siRNA during HOPE led to a lower decrease. Levels of anti-inflammatory cytokines increased during Fas siRNA-treated machine perfusion, with IL-10 increasing significantly and IL-4 showing no significant increase. Furthermore, there was no significant difference in transcription levels in all study groups, and the extent of apoptosis and necrosis of hepatic cells remained similar.[12] In contrast, rat livers were subjected to approximately 25 min of warm ischemia followed by either HMP or NMP with a Williams' Media E-based solution for 4 h. Invivofectamine lipid nanoparticles loaded with Fas-targeting siRNA or invivofectamine alone as control were transfected during perfusion. Confocal fluorescence microscopy showed that successful transfection, and therefore, Fas silencing, could be achieved under both temperature conditions.[40]

Additionally, various other miRNA pathways play important roles in the mechanisms underlying IRI.[62] To assess the influence of miRNA-182-5p suppression on posttransplant acute kidney injury, pig kidneys were perfused for 6 h at 34 °C with acellular oxygenated solution and 25 mg of miRNA-182-5p inhibiting ASO. Administration into the perfusion

TABLE 2.

Gene modulation approaches during ex vivo organ perfusion

Author, year	Organ	Species	Machine perfusion method (temperature)	Oxygenation	Perfusion solution	Perfusion time, h	Delivery method	Target	RNA type	Dose	Main outcomes in treatment groups
Yuzefovych et al, 2020[16]	Kidneys	Rat	SNMP with COR (31–32 °C)	Oxygen saturation in perfusion solution: 65%–70%	WME	2	Lentivirus	β2m, CIITA	shRNA	1.5×10^{11} particles	(1) ↓ β2m and CIITA transcript levels (2) Preserved structural integrity (3) Lentiviral vectors inflicted no additional damage (4) Alteration in cytokine profile (5) Vector specificity for kidneys
Moser et al, 2016[57]	Kidneys	Rat	HMP (3–5 °C)	No	KPS-1	22	NA	MMP-2	siRNA	50 nM	(1) ↓ mitochondrial damage, LDH activity, and NGAL levels after 22 h of perfusion
Wilflingseder et al, 2017[58]	Kidneys	Pig	NMP (34 °C)	95% O_2 and 5% CO_2	UW solution	6	NA	miRNA-182-5p	miRNA	25 mg	(1) Persistent downregulation of miRNA-182-5p and increased target genes expression
Thompson et al, 2022[11]	Kidneys	Human	NMP (36.5 °C), HMP (<8 °C)	NMP: 95% O_2 and 5% CO_2 HMP: no	RBC-based perfusate	6	Naked (gymnotic)	miRNA-24-3p	miRNA	1 mg	(1) NMP facilitates active intracellular uptake of antagomirs (2) Inhomogeneous distribution of antagomirs within the kidneys (3) Specific inhibition of miRNA-24-3p function
Brasile et al, 2002[44]	Kidneys	Canine	EMS (32 °C), HMP (4 °C)	NA	Acellular solution based on perflubron[50]	24	Ad5	GFP delivery	–	1×10^4, 1×10^6, 1×10^8, or per g kidney	(1) Successful transfection during EMS (2) Dose-dependent transfection
Gillooly et al, 2019[40]	Liver	Rat	NMP (37 °C), HMP (4 °C)	NA	WME	4	Nanoparticles (lipid)	Fas	siRNA	50 nM	(1) Nonspecific uptake during NMP and HMP, however, more distinct during HMP
Bonaccorsi-Riani et al, 2022[12]	Liver	Rat	HOPE	100% O_2	UW solution	1	Nanoparticles (lipid), naked	Fas	siRNA	38 nmol	(1) ↑ Anti-inflammatory cytokines during HOPE
Goldaracena et al, 2017[59]	Liver	Pig	NMP (37 °C)	95% O_2 and 5% CO_2	Steen solution and washed RBCs	4 or 12	NA	miRNA-122	miRNA	100 μM	(1) NMP resulted in significant miR-122 sequestration
Bonaccorsi-Riani et al[48]; 2021	Liver	Rat	HOPE	Yes	UW solution	2	AAV8	GFP delivery	–	4×10^8 PFU/mL	(1) Successful transduction during HOPE
Cui et al, 2017[41]	Vessels	Human	NMP (37 °C)	NA	M199 medium	6	Nanoparticles (PACE)	CIITA	siRNA	0.1 mg/mL	(1) Flow-dependent nanoparticle uptake (2) Specific downregulation of gene expression

AAV, adeno-associated virus; β2m, beta-2-microglobulin; CIITA, class II major histocompatibility complex transactivator; COR, controlled rewarming; EMS, exsanguinous metabolic support; GFP, green fluorescent protein; HMP, hypothermic machine perfusion; HOPE, hypothermic oxygenated machine perfusion; KPS-1, kidney perfusion solution 1; LDH, lactate dehydrogenase; miRNA, micro-RNA; MMP, matrix metalloproteinase; NA, not available; NGAL, neutrophil gelatinase-associated lipocalin; NMP, normothermic machine perfusion; PACE, poly(amine-co-ester); PFU, plaque-forming units; RBC, red blood cell; shRNA, short hairpin RNA; siRNA, small interfering RNA; SNMP, subnormothermic machine perfusion; UW solution, University of Wisconsin solution; WME, Williams' Media E.

circuit took place after 30 min of perfusion, whereas the application of saline served as a control. The miRNA-182-5p targets Dpt, Fam129a, Kcnj10, Ppp11r1a, and TEK were significantly upregulated in the treatment group. The relative miRNA-182-5p expression initially decreased in both groups; however, contrary to the control group, the expression in the treatment group further diminished and remained at a low level. This suggests successful downregulation of miRNA-182 expression. However, the distribution within the kidneys has not yet been evaluated.[58] Several studies have investigated the effects of miRNAs involved in IRI. Some miRNAs exhibit a defensive role against IRI, whereas others such as miRNA-24-3p can aggravate cellular damage.[63] Nontransplantable human kidneys were treated for the first time with anti-miRNA-targeting miRNA24-3p during 6-h NMP. Either 1 mg of anti-miRNA or scrambled sequence oligonucleotide, as control, was initially administered into the perfusion circuit. Furthermore, the effect of antagomir treatment on the kidney was assessed during 6-h HMP. HMOX1 protects against injury caused by reactive oxygen species and inflammation as part of IRI, just as S1PR1, which also plays an important role in sustaining the barrier function of the endothelium. Both are miRNA24-3p targets and were significantly upregulated in the groups treated with antagomir. Additionally, only the expression of genes with miRNA-24-3p–binding sites was affected, and no alterations in the cytokine profile were detected. In contrast to HMP, normothermic conditions led to active endocytic uptake of antagomir. Modifying the antagomirs with fluorescent labels enabled the assessment of treatment distribution within the organ. This revealed that despite the successful downregulation of miRNA-24-3p, antagomirs were not homogenously distributed within the organ.[11] Other important mediators contributing to IRI and acute and chronic graft rejection are the matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9. HMP was used to deliver siRNA targeting MMP-2 to rat kidneys for 22 h. Another group of kidneys received doxycycline, which nonspecifically reduced MMP expression. Mitochondrial and tubular damage significantly decreased in both treatment groups but to a greater extent in the doxycycline group. This demonstrates that several other MMPs are involved in kidney injury and need to be targeted during ex vivo gene modulation.[57]

Graft Rejection

The MHC, in humans referred to as HLA complex, plays an important role in organ transplantation because both antibody-mediated and cellular graft rejection are attributed to mismatches in highly polymorphic MHC encoding genes.[64-66] The host immune system can be activated by the expression of MHC molecules on allograft endothelial cells, eventually leading to transplant rejection.[41] This could be counteracted by downregulating the CIITA, which plays an important role in the expression of MHC class II.[67] The expression of MHC class II in human umbilical veins could be suppressed by ex vivo normothermic transfection of the grafts with PACE nanoparticles loaded with siRNA targeting the CIITA. The expression of MHC class II molecules, which is positively regulated by CIITA, was reduced by >90% in the CIITA siRNA group. This downregulation was limited to MHC class II expression and did not influence the expression of other endothelial cell surface proteins, such as CD31, E-selectin, interferon-γ–responsive MHC class I, and vascular cell adhesion protein-1. Furthermore, human arteries were transplanted into severely combined immunodeficient mice after 6-h ex vivo incubation of the allografts in M199 medium with CIITA siRNA-loaded nanoparticles. A knockdown of >80% of MHC class II expression, restricted to the allografts, which gradually decreased to 20% at 6 wk posttransplantation, could be achieved.[41] Similarly, oxygenated subnormothermic machine perfusion was used to genetically modify rat kidneys toward MHC class I and MHC class II silencing before allogenic transplantation. Lentiviral vector particles encoding CIITA and β2m were injected during 2 h of machine perfusion, and the recipients were followed up for 6 wk. Secreted NanoLuc Luciferase served as a reporter gene, and the resulting posttransplant increase in bioluminescence activity indicated a stable genetic transfer. Furthermore, a biodistribution assay showed no in vivo distribution, indicating that genetic engineering was specific to kidney allografts. Compared with the control group, the group treated with short hairpin CIITA and shβ2m showed 70% and 71% decreases in transcript levels, respectively. Both lactate dehydrogenase activity analysis and histological examination revealed that the application of lentiviral vectors, either in the form of short hairpin CIITA, shβ2m, or nonsense shRNA controls, led to no additional cellular damage compared with a separate group of kidneys perfused without lentiviral vectors. Furthermore, lentiviral vector transduction during perfusion resulted in alterations in the cytokine profile, such that epidermal growth factors, IL-10, interferon-gamma–induced protein 10, macrophage inflammatory protein-1α, macrophage inflammatory protein-2, and tumor necrosis factor-alpha significantly increased in the lentiviral vector particle groups after 2 h of perfusion.[16]

Antiviral Treatment

miRNA-122 is an important regulator of both physiological and pathological processes in the liver; thus, it is considered a biomarker for liver injury and a predictive factor for therapeutic approaches. In hepatitis C virus (HCV) replication, miRNA-122 is a key regulator and potential target for treating HCV infections.[68] Especially after organ transplantation, HCV reinfection tends to be more severe. Therefore, it is reasonable to address the risk of recurrent infection before transplantation. The antagomir miravirsen, targeting miRNA-122, was administered during 12-h NMP or SCS of pig livers. The concentration of miravirsen in the liver was significantly higher in the machine perfusion group compared with the cold storage group. Aldolase B and branched-chain ketoacid dehydrogenase, which are miRNA-122 target genes, were upregulated during NMP, indicating sequestration of miRNA-122. In the second experiment, pig livers were subjected to either 4-h NMP or SCS before liver transplantation. After transplantation, miRNA-122 activity decreased in the miravirsen-treated groups, however, in the NMP group to a significantly greater extent.[59] Considering that miRNA-122 depletion promotes the pathogenesis of several liver diseases and the successful treatment of HCV with either interferon-based or direct-acting antiviral medications, the capabilities of miRNA-122 require further investigation.[68]

STATE OF THE ART AND CHALLENGES

Stability and Delivery Efficiency

One of the major challenges is ensuring a stable introduction of genetic material into the target organ. The stability, especially of naked delivered RNAs, can be compromised in the extracellular environment to the effect that nucleases or enzymes degrade the genetic material.[13,14] This decreases the efficiency of therapy as reduced amounts of therapeutic gene material are available. To overcome this challenge, various chemical modification techniques have been established, usually targeting the phosphate backbone or ribose.[33] Similarly, the chemical modification of naked oligonucleotides has been investigated to enhance their stability and delivery efficiency. A major challenge remains to ensure specificity of delivery,[11] because the goal is to deliver the therapeutic genes only to the intended cells, to minimize and ideally avoid off-target effects. For example, in liver transplantation, *N*-acetylgalactosamine could play an important role in enhancing hepatocyte-specific targeting and tissue absorption. In vivo and in vitro studies have demonstrated the feasibility of this approach, and the FDA has authorized the subcutaneous use of siRNA conjugated with *N*-acetylgalactosamine.[12,69,70] General approaches to enhance target-specific uptake include the utilization of polymers, liposomes, nanoparticles, or conjugation with a ligand that specifically binds to receptors on target cells.[11,14]

Temperature and Oxygenation

The temperature setting is another important aspect to be considered for successful delivery because the requirements for cellular uptake can differ between the vectors used, but the specific design of the vectors is also important. The near-normal physiological conditions during NMP can support the active cellular uptake of gene therapeutics but pose a risk of inactivation because of the presence of RNAses in cellular perfusates. During HMP, genetic material can be taken up by inactive mechanisms.[11,12] For example, in terms of antagomirs administered during ex vivo liver perfusion, normothermic conditions are required to ensure successful uptake, as hypothermia did not facilitate the uptake of the therapeutic agent.[12,59] In particular, with respect to the temperature settings, it is important to consider the duration of ex vivo perfusion. Short-term hypothermic preservation can pose the risk of insufficient delivery of genetic material into target cells because of reduced metabolism.[12] Similarly, is it the case during preservation at 4 °C in terms of SCS, as cellular activity is slowed down and hypoxic conditions lead to anaerobic metabolism.[2] Pig livers treated with antagomirs for 4 h during SCS were less effective in terms of absorption compared with 4-h NMP. This further suggests that, during NMP, potentially lower doses might be necessary for successful uptake. Furthermore, contrary to NMP, sequestration could not be achieved under 4 °C cold conditions.[59] A few animal studies have investigated the efficiency of gene silencing under static hypothermic conditions, mainly by perfusing the organ back-table with perfusion solution and gene therapeutics. The delivery is performed either as single (artery or vein) or dual (artery and vein) perfusion or in the form of a clamping technique, whereby the desired vessels are clamped before perfusion or after perfusion before preservation. These 3 techniques have been investigated for the delivery of AdLacZ during 3-h SCS of rat livers. The highest transduction efficiency was achieved with the clamping technique, which could be further enhanced by preserving the grafts for 18 h, leading to stable but declining expression for 2 wk. The superiority of the clamping technique might be explained by the fact that the therapeutic gene reaches the microvasculature of the organ.[71] Similarly, rat livers transfected with Ad-enhanced green fluorescent protein and cold stored for 18 h showed a transfection efficiency of 30% after 1 wk posttransplantation, and lower dosages were required to achieve similar results as nonclamping techniques. Furthermore, it could be shown that expression was detectable 12 to 24 h posttransplant, contrary to the in vivo application of Ad-enhanced green fluorescent protein, which led to detection after 3 h.[72] This suggests that cold storage requires longer transfection times and leads to a delayed onset of gene expression. However, depending on the targeted mechanism, this delayed onset could potentially be sufficient if acute graft rejection is targeted, which usually occurs from 1 wk posttransplantation onward.

As part of different temperature settings, adaptations in terms of oxygenation and perfusion pressures are required. Although it is common practice to perform oxygenated NMP,[29] hypothermia does not necessarily require an oxygen supply. However, oxygenation during HMP, especially with respect to high-risk organs, can potentially mitigate inflammation and contribute to preserving metabolism and endothelial integrity.[32] As previously described, cellular integrity is one of the crucial aspects of successful gene therapy. As only a limited number of gene modulation approaches under different temperature and oxygen settings are available (Table 2), further investigations are needed to fully understand the underlying mechanisms.

Duration, Dosage, and Perfusion

Another important aspect to be considered is the duration of gene silencing, especially with respect to the transplantation setting and concomitant time factors because prolonged silencing might be desirable to overcome not only the challenges of IRI but also acute graft rejection during the posttransplant period.[41] One major limitation of the clamping technique is that the expansion of the liver capsule[72] was seen as a marker for sufficient infusion of the therapeutic gene. In this context, machine perfusion methods could be used to assess microperfusion and thus contribute to the successful delivery of therapeutic agents. Another major hurdle in gene therapy is the potential activation of the immune system, which can be influenced by several factors, such as the type of vector, genetic modulation itself, or dosage. Concerning the latter, too high dosages can result in an excessive immune response, eventuating in the destruction of the endothelial barrier function[44] and thus causing side effects. Conversely, too low doses do not lead to the desired effect.[12] Overall, the dosage and perfusion of the microvasculature influence how homogenously the therapeutic is delivered to the organ. Inhomogeneous perfusion can result in the accumulation of therapeutic agents in certain areas of the organ,[11] resulting in harmful effects because of overdosing, whereas gene therapy lacks efficiency in marginally perfused areas. In animal studies, fluorescence microscopy was used to assess distribution after sacrification. However, this may not be feasible in a

clinical setting; therefore, methods are required to assess organ perfusion during machine perfusion.

CONCLUSION

Gene therapy approaches in transplant settings, particularly with respect to in vivo animal models, have emerged. However, the applicability of these models in the clinical setting remains limited because treating a single organ is more feasible than administering gene therapy to donors, especially with respect to logistic and ethical considerations, as well as cost concerns and the potential for off-target effects in the setting of multiorgan donations. During the last few decades, machine perfusion methods have advanced as alternatives to SCS, enabling preservation, monitoring, reconditioning, and treatment of organs before transplantation. Therefore, organ perfusion circuits hold promise as an effective platform for therapeutic gene interventions. Although the number of studies conducted in the field of gene therapy during ex vivo kidney and liver perfusion is limited, preliminary findings have shown encouraging results. However, the limited scope highlights the need for further investigation to refine these novel therapeutic approaches, especially with respect to temperature settings, perfusion duration, and dosage of the therapeutic agent.

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