The effect of mesenchymal stromal cell administration on porcine kidneys during normothermic machine perfusion

Kaithlyn Mirugia Rozenberg

The effect of mesenchymal stromal cell administration on porcine kidneys during normothermic machine perfusion



Thesis submitted to the Examination Schools for the degree of Doctor of Philosophy by

Kaithlyn Mirugia Rozenberg

Corpus Christi College

Nuffield Department of Surgical Sciences

University of Oxford

Trinity 2021

Supervisors

Professor Rutger J. Ploeg
Professor of Transplant Biology
Principal Investigator with NHS Blood and Transplant
Director of Clinical and Translational Research
Nuffield Department of Surgical Sciences
University of Oxford

Mr James P. Hunter
Academic Clinical lecturer in Transplant
Nuffield Department of Surgical Sciences
University of Oxford

Dedica na mi kerido mayornan Rita y Edwin Rozenberg,
cu hopi amor y cariño.

ABSTRACT

Kidney transplantation is the most effective treatment for patients with end stage renal disease. One of the concerns in the transplant community is the persistent donor organ shortage and the lack of suitable organs for transplantation. Marginal kidneys from high-risk donors are increasingly accepted, however a large proportion are discarded due to their susceptibility to ischemia reperfusion injury (IRI) and the risk that the use of these organs may ultimately result in post-transplant complications. Normothermic machine perfusion (NMP) of the donor kidneys provides an opportunity to assess, potentially repair and therapeutically intervene on the organ *ex vivo* prior to transplantation - hereby transforming kidney transplantation by increasing utilisation of marginal organs. Mesenchymal stromal cells (MSCs) can exert regenerative and anti-inflammatory effects in organs and tissues during IRI. This thesis focused on the optimisation of donor kidney NMP conditions and studied the optimal duration of NMP needed to allow homing and initiation of biological effects of infused MSCs on the donor kidney during NMP. Through the work in this thesis we have developed a reproducible kidney NMP slaughterhouse model and demonstrated that stable kidney NMP with a red blood cell-albumin based solution can be performed for a minimum of seven hours. Furthermore, an oxygen carrier is necessary during NMP of healthy porcine kidneys. NMP of kidneys without an oxygen carrier led to significantly impaired renal function and high lactate and aspartate aminotransferase levels, indicating injury and anaerobic metabolism. Administration of MSCs to the donor kidney during NMP is safe and feasible as there were no adverse effects on perfusion characteristics and injury markers. After seven hours of NMP, MSCs were mainly localised in the glomeruli and the addition of MSCs resulted in secretion of prostaglandin E2, vascular endothelial growth factor A, angiopoietin 1 and interleukin 10 in the perfusate. Despite no improvement on early renal function of MSC therapy during NMP delivery prior to porcine auto-transplantation, MSC administration during NMP was successful, proving the feasibility of NMP as a method to deliver cellular therapies.

TABLE OF CONTENTS

Abstract		**7**
Chapter 1	Introduction	**11**
Chapter 2	Aims and rationale of the thesis	**61**
Chapter 3	Development of normothermic machine perfusion of porcine kidneys using a slaughterhouse model	**65**
Chapter 4	Comparison of ex vivo kidney perfusion using red blood cells, a synthetic haemoglobin-based oxygen carrier and without an oxygen carrier	**135**
Chapter 5	Dose and tracking of mesenchymal stromal cells during normothermic machine perfusion of porcine kidneys	**175**
Chapter 6	Mesenchymal stromal cell treatment during ex vivo normothermic kidney perfusion: A porcine renal auto-transplantation study	**239**
Chapter 7	Summary	**277**
	General Discussion	**281**
	Future perspectives	**286**
Appendix	COVID-19 impact statement	**294**
	Chapter involvement	**295**
	List of publications	**297**
	About the author	**298**

Chapter 1
Introduction

1.1 CHRONIC KIDNEY DISEASE

Kidney failure represents a major healthcare burden worldwide. In the United Kingdom, chronic kidney disease (CKD), characterised by gradual loss of kidney function, affects over 3 million people and 30,000 people are being treated for kidney failure.[1] CKD is classified based on cause, glomerular filtration rate and albuminuria category.[2] Major risk factors for developing CKD are diabetes mellitus, elevated blood pressure and atherosclerosis, explaining the higher prevalence of CKD with age.[3] Progression of CKD finally leads to kidney failure, called end-stage renal disease, where patients require renal replacement therapy in the form of dialysis or transplantation.[4]

1.2 KIDNEY TRANSPLANTATION

Since the first successful clinical case in 1954, kidney transplantation has developed into an established and standardised procedure and is currently the best treatment option for patients with end-stage renal disease. Kidney transplantation offers better life expectancy and improved quality of life when compared to dialysis. Renal replacement therapy in the form of dialysis has significant social, economic and health implications and will not cure the cause of kidney failure.[5, 6] Despite the potential of organ transplantation to save and improve the lives of millions, there is a significant imbalance between organ supply and demand. In the United Kingdom, 2240 kidneys (29%) have been transplanted between April 2019 and February 2020; however, 4841 people (63%) are still on the active waiting list for a kidney only (as of April 2019) and every year people die on the waiting list because a donor kidney arrives too late. The median waiting time for a kidney transplant in the United Kingdom is two years.[7] Recently, countries like the Netherlands and England have changed their organ donation system from an opt-in to an opt-out system in an attempt to increase the number of available organs for transplantation. This means that all adults will be considered as potential organ donors

when they die unless they have made it known that they do not wish to donate their organs.[8, 9] In kidney transplantation, grafts are retrieved from three types of donors: living donors, donation after brain death (DBD) donors and donation after circulatory death (DCD) donors. In addition to DBD and DCD, organ donors are classified into two groups based on variable organ quality: standard criteria donors (SCD) or expanded criteria donors (ECD). ECD organs are from donors aged 60 and over or between the age of 50 and 59 with at least two of the following conditions: a history of hypertension, a cerebrovascular cause of death or a terminal serum creatinine greater than or equal to 133 μmol/L at the time of organ donation.[10] The most frequently used kidneys for transplantation in the United Kingdom are from living and DBD donors.[7] DBD kidneys, much like living donor kidneys, are subjected to minimal or no warm ischaemic injury during the retrieval process. DBD kidneys are retrieved from donors who have sustained irreversible cerebral injury and whose death has been confirmed using the legal neurological criteria but in whom the circulatory system is still functioning. DBD organs are perfused with the donors own oxygenated blood until aortic clamping and cold flush with preservation solution.[10, 11] In an attempt to decrease transplant waiting times, higher-risk donor organs, so-called marginal organs such as those recovered from DCD and ECD, have become an invaluable resource in many transplant centres.[7, 12, 13] DCD donors are patients who died as a result of circulatory arrest, most often after withdrawal of life sustaining treatment at an intensive care unit. In contrast to DBD donors, circulation does not remain intact during the retrieval and the organs inevitably sustain warm ischaemic injury between circulatory arrest and initiation of flush-out when cooling the organs.[14] DCD kidneys are more prone to ischaemia reperfusion injury (IRI) and are associated with an elevated risk of delayed graft function (DGF).[14-16] DGF is when the organ does not function immediately after transplantation and is explained in more detail later in the chapter. As a result, clinicians are uncertain about higher-risk organ quality and 14% retrieved kidneys are discarded. This is despite DBD and DCD kidneys having similar long-term graft survival.[7]

1.3 ISCHAEMIA REPERFUSION INJURY

Ischaemia reperfusion injury (IRI) is defined as a sudden period of disrupted blood flow (ischaemia) followed by the restoration of an oxygenated blood supply (reperfusion) and is inevitable during transplantation.[17] At a cellular level, the main biochemical changes during ischaemia are depletion of ATP and decreased intracellular pH as a result of anaerobic metabolism and lactate accumulation. Consequently, lysosome membranes and the cell structure are damaged and reactive oxygen species (ROS) are generated due to mitochondrial dysfunction.[18, 19] Furthermore, alterations of the Na^+/K^+-ATPase pumps are induced, leading to intracellular sodium and potassium accumulation, causing oedema and apoptosis, respectively.[20, 21] Upon reperfusion, the burst of oxygen returning to the ischaemic areas and normalisation of the intracellular pH leads to a greater overload of intracellular calcium and a larger generation of ROS. ROS trigger endothelial injury and prevent the Na^+/K^+-ATPase from functioning, thereby intensifying oedema.[17, 21, 22] In addition, endothelial injury stimulates a pro-inflammatory environment that activates leucocytes and increases the expression of intracellular adhesion molecules and, in turn, enhance leucocyte-endothelial interaction. This leads to leucocyte infiltration in the ischaemic tissue leading to increased production of cytokines and complement activation, resulting in further inflammation and additional cellular apoptosis and necrosis (Figure 1.1).[23]

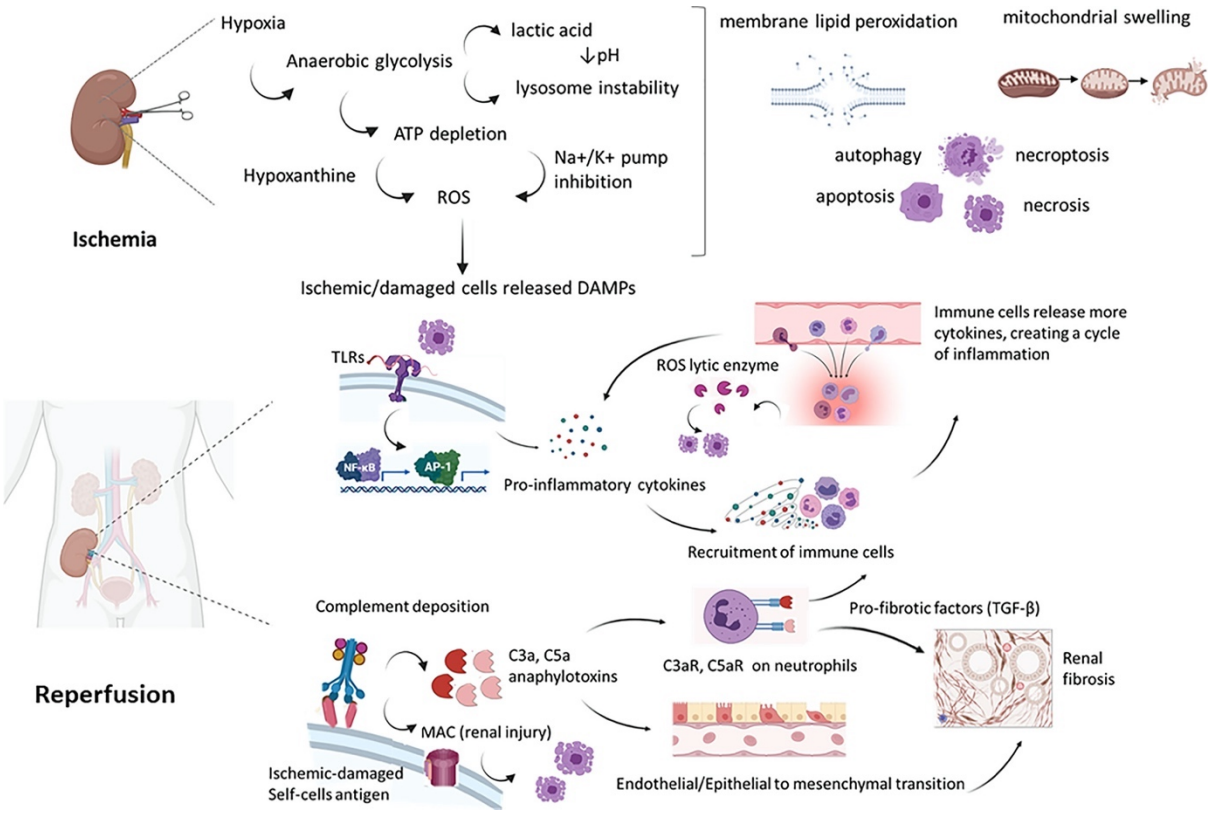


Figure 1.1. Molecular mechanism of kidney ischaemia reperfusion injury. The pathophysiology of ischaemia reperfusion injury is complex. Due to the lack of oxygen, ischaemia result in the predominance of anaerobic processes and subsequent cellular damage via processes such as protease activation and inhibition of Na^+/K^+-ATPase. The associated reperfusion injury occurs due to the restoration of oxygen supply to the kidney, resulting in a mixture of cellular changes including the generation of reactive oxygen species and the upregulation of inflammatory cytokine pathways. TLR, Toll Like receptor; MAC, membrane associated complex; DAMP, Damage-associated molecular patterns; PRR, Pattern Recognition Receptors; ROS, reactive oxygen species. *Figure adapted from Franzin R et al. Renal Delivery of Pharmacologic Agents During Machine Perfusion to Prevent Ischaemia-Reperfusion Injury: From Murine Model to Clinical Trials. Front. Immunol. 12:673562.*

1.3.1 Inflammation, immune cells and adhesion molecules

Inflammation is an inevitable consequence of IRI in kidney transplant and a variety of cell types play an important role in its pathophysiology. Renal IRI triggers an inflammatory cascade that leads to renal damage, so inhibition of inflammatory responses may offer a therapeutic approach to protect renal tissue.[24, 25] The immune response includes a variety of different components including immune receptors, chemokines and cytokines, which are low molecular weight proteins that act as messengers between cells, adhesion molecules and leucocytes.[26] The direct immune

response against the graft is initiated by an inflammatory response that occurs when damaged or dying cells release damage-associated molecular. patterns (DAMPs). Inflammatory DAMPs, including Heat-Shock proteins (HSP), high mobility group box protein 1 (HMGB-1), and DNA fragments, are recognized by specific receptors such as Toll-like receptors (TLR-2 and TLR-4), which, in turn, trigger inflammatory and cytotoxic responses.[27] Pro-inflammatory cytokines and cytokines such as interleukin 1 (IL-1) interleukin 6 (IL-6), tumour necrosis alpha (TNFα), interleukin 11 (IL-11) and interleukin 8 (IL-8) play a major role in renal dysfunction during IRI and are responsible for mediating numerous different pathways.[28-30] One such example is activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway which can compound the severity of IRI by inducing transcription of pro-inflammatory genes.[31] Furthermore pro-inflammatory cytokines such as TNFα and IL-1 play a major role in augmenting endothelial permeability and facilitating leucocyte infiltration. [32] Cell adhesion molecules are typically expressed on endothelial cells and cells of the immune system and are involved in recruitment to sites of injury. These molecules are always expressed in low levels but are upregulated following ischaemia and other forms of organ, tissue and/or cellular injury. Following IRI adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and P-selectin are highly expressed. These signals result in recruitment of leucocytes, in particular, neutrophils which migrate through capillaries into post ischaemic tissue. Neutrophils and tissue macrophages enhance pro-inflammatory cytokine release leading to increased leucocyte-endothelial interactions and direct tissue injury. This injury causes swelling of the endothelial cells and creates a physical impediment to blood flow, further aggravates ischaemia.[33-36] Leukotrienes are inflammatory mediators produced within leucocytes by oxidation of arachidonic acid. They have a potent effect on certain cells types, in particular smooth muscle cells, and are responsible for triggering histamine and prostaglandin release. They have been shown to have a role in IRI in both acute and chronic renal failure.[36-39]

1.3.2 Oxidative stress and lipid peroxidation

In addition to immune cell mediated tissue injury, direct cellular damage during IRI is the result of ROS production. ROS occur following the reperfusion phase of IRI when oxygen is reintroduced and there is alteration in the charge of the mitochondrial membrane and electron transport chain. Disruption in oxidative phosphorylation, ATP depletion, increase in intracellular calcium and activation of membrane phospholipids proteases are all pathological aspects that lead to ROS production.[40-42] ROS cause direct damage to DNA, proteins, lipids and cell organelles. Lipid peroxidation is the result of injury to lipids and can result in disruption to the phospholipid bilayer and the cell membrane which can cause apoptosis and necrosis.[43, 44] Direct damage to DNA, cytosolic and membrane bound proteins disrupts cellular and mitochondrial function. Furthermore, downregulation of antioxidant enzymes systems including catalase, superoxide dismutase, and glutathione peroxidase compounds the injury and contributes to the pathophysiology of IRI.[45] Supporting the cells antioxidant capacity and preventing free radical production would be the ideal strategy to protect the tissue during IRI. Studies have shown beneficial effects of free radical scavengers and antioxidants on IRI, through protective effects on IRI induced oxidative stress.[46-51]

1.3.3 Mitochondrial dysfunction

During ischemia, mitochondrial oxidative phosphorylation is suppressed by a lack of oxygen. This results in an alteration in membrane potential, and electron transport chain dysfunction which results in impaired ATP synthesis. The reduction in ATP and therefore diminished activity of cellular energy-dependent processes affects many cellular functions and contributes to cell death. Many of the membrane ion channels are ATP-dependent and the reduction in ATP results in an alteration in ion transport. The most notable is the Na^+/K^+-ATPase which actively removes sodium from within the cell at the expense of potassium. Failure of this ion channel leads to the passive movement of sodium and water into the cell, leading to oedema. The intracellular sodium concentration

is further increased by dysfunction of the Na^+/Ca^{2+} antiporter channel, which results in calcium and sodium accumulation in the cell.[52] In addition, intracellular calcium overload occurs from calcium redistribution from endoplasmic reticulum stores and this increase can activate calcium-dependent phospholipase A2, endonuclease and proteases within the cell leading to apoptosis.[17, 53] As discussed above, following IRI, mitochondria are the major source of intracellular ROS, and are also the primary target for ROS. The prolonged exposure to large amounts of calcium and oxygen free radicals contribute to the progressive functional deterioration of mitochondria during the reperfusion phase [54] It has also been shown that succinate, an intermediate of the tricarboxylic acid (TCA) cycle and substrate of the electron transport chain, accumulates in the mitochondria during ischaemia.[55] At time of reperfusion, the accumulated succinate mediates the reverse transfer of electrons back to complex I in the mitochondria, causing production of ROS and further damage to the organelle.

1.3.4 The complement system

The complement system is a major component of the innate immune system and has a central element in host defence, clearance of immune complexes, and tissue homeostasis. There are three main complement pathways, the classical pathway, lectin pathway and alternative pathway. Traditionally, the classical pathway has been thought to be largely responsible for the pathogenesis of complement driven IRI[56], however this might not be the case, with multiple pathways involved. Studies in rodent models of renal IRI show that the alternative pathway has a major role and the classical pathway does not seem to have an effect on reperfusion injury when tested in C4-deficient mice or RAG-1-deficient animals. These two mouse genotypes are unable to generate IgM or IgG which are essential in the classical pathway.[56,58] However, Factor B-deficient mice known to be defective in alternative pathway activation, showed a notable reduction in functional injury and histological findings induced by ischaemia and reperfusion.[59] More recently, the lectin pathway has gained attention and has been shown to play a pivotal

role in the pathophysiology of ischaemic kidney damage.[60] Following IRI, the system of complement proteins is activated by endogenous ligands that trigger proteolytic cleavage of complement components. The result is the formation of terminal complement components C3a, C5a, and the membrane attack complex (C5b-9 or MAC), all of which play essential roles in the propagation of the inflammatory response, chemotaxis, immune cell recruitment and activation, and direct tubular cell injury. Other studies have shown that the activation of the complement system in various organs following IRI results in release of a number of biologically active products.[61-65] In addition to C4a, C3a, and C5a, C5b-9, anaphylatoxins with pro-inflammatory actions and adhesion molecules are also upregulated.[66] C5a and C5b-9 have been shown to stimulate endothelial cell expression of selectins and ICAM-1.[67, 68]

1.3.5 Transplant related ischaemia reperfusion injury

Successful organ function requires adequate perfusion. Transplantation related IRI is characterised by a variable amount of warm ischaemic time (WIT). WIT is the duration from when the circulation ceases to the time of cold flushing. In addition to the WIT, the organs, once retrieved are subject to a period of cold ischaemic time (CIT), which is the duration of hypothermic kidney storage. CIT alone is an independent risk factor for graft failure and is directly associated with DGF.[69-71] During these periods of ischaemia, damage occurs as the tissue responds in various ways to the hypothermia, lack of oxygen and nutrients. The reperfusion at the time of transplantation further aggravates the state of the tissue by oxidation and inflammation “primed” by ischaemia.[20, 22] This is particularly relevant in DCD organs, which have a period of WIT between the withdrawal of medical support and initiation of cold perfusion and organ retrieval.[72] Renal transplantation IRI leads to loss of functional tubular epithelial cells contributing to acute kidney injury, DGF, acute rejection and reduced graft survival.[27, 73]

Delayed Graft Function (DGF) is the clinical manifestation of IRI and is when the organ does not function immediately after transplantation, which is recoverable. Several

classifications are used to define DGF but the most commonly used is 'failure of the renal transplant to function immediately with the need for dialysis within the first 7 days after kidney transplantation'. DGF can persist for a number of weeks and only after 3 months does the classification change to primary non function (PNF), which is when the organ is considered to have not worked.

1.4 ORGAN PRESERVATION

Preserving the function of kidney grafts is of vital importance for effective transplantation. The main goal of organ preservation is to maintain the organ and tissue viable during storage and transportation so that the graft will function at reperfusion in the recipient; thus, maintenance of cellular energy must be accomplished. For decades, donor organs have preserved using static cold storage (SCS). However, there is an increased interest in alternative dynamic preservation methods, such as hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP), aiming to reduce IRI and increase and optimise utilisation of marginal donor organs and previously discarded organs for transplantation.

1.4.1 Static cold storage

To date, most European transplant centres still use conventional static cold storage (SCS) to preserve donor organs due to its simplicity, accessibility, simple logistics, ease of transport and cost-effectiveness. SCS involves cold flush of organs with a preservation solution designed primarily to prevent cell swelling after retrieval. Organs are subsequently stored in preservation solution on melting ice at 4°C until time of implantation.[74] Lowering temperature slows the metabolism down, however at 4°C 10-12% of basal metabolic activity remains. This residual metabolism while under oxygen-deprived circumstances causes depletion of ATP and accumulation of toxic metabolites that contribute to IRI after reperfusion in the recipient, causing inflammation and organ

damage.[20] Over the last years, SCS has been reconsidered as the donors are gradually getting older and they have more pre-existing medical co-morbidities (e.g. hypertension, diabetes and cardiovascular disease). Although SCS may be adequate for preserving young and high-quality organs, its use has been limited with higher-risk organs as these organs are associated with an increased risk of DGF and reduced long-term graft survival.[75] Therefore, SCS may limit the expansion of the donor pool and lack the ability to assess and repair the damage that has occurred during the retrieval process of marginal organs prior to transplantation.

1.4.2 Dynamic preservation: machine perfusion

Machine perfusion of donor kidneys involves connecting the organ to an *ex vivo* circuit after retrieval and cold flush of the organ. A bespoke perfusion solution is pumped either by roller or centrifugal pumps through the organ vasculature and can generate either a continuous or a pulsatile flow. Pulsatile flow during hypothermic machine perfusion (HMP) has been associated with improved microcirculation and organ function, expression of flow-dependent vasoprotective endothelial genes and reduced expression of inflammatory genes compared with continuous flow.[76-80] Furthermore, the perfusion can be either pressure- or flow-controlled. HMP and NMP studies showed that pressure-controlled perfusion improved renal perfusion and better preserved the tissue's structural integrity than flow-controlled perfusion.[81-83] All commercially available CE marked HMP devices are pulsatile and pressure-controlled systems. The only commercially available CE marked NMP kidney device is the Kidney Assist from XVIVO based in Groningen, the Netherlands and is a pulsatile and pressure-controlled system. Other reported systems are in-house adaptations that use cardiopulmonary bypass technology and are continuous and pressure-controlled systems (Appendix, Table S1.1). The perfusion solution provides the required components needed to maintain viability, while removing waste products released as a result of the metabolism and perfusion of the organ. The

perfusion solution can be non-oxygenated or oxygenated. An oxygenator is responsible for the gas exchange of carbon dioxide and oxygen and delivers oxygen-rich perfusion solution to the organs when applicable. During HMP, the organ and reservoir are cooled with melting ice without the need for a heat exchanger, unlike NMP, where a heat exchanger is necessary to regulate the perfusate and organ temperature. The organ sits in an organ chamber that is connected to the rest of the circuit.

1.4.3 Hypothermic machine perfusion

HMP is a preservation technique that has been re-visited in the past decade to try to limit IRI and improve outcomes of marginal organs. Similarly to SCS, HMP slows down the metabolism, reducing oxygen requirement and ATP depletion. However, during HMP, cold preservation fluid is pumped continuously through the renal vasculature at temperatures between 1 and 10°C while removing some of the accumulating metabolites and can also simultaneously provide substrates during organ storage. HMP has been hypothesised to work by maintaining the expression of genes like eNOS and krüppel-like factor 2 that improve circulation during perfusion and have anti-inflammatory properties.[76-78] Endothelial nitric oxide synthase is activated by a small degree of shear stress necessary for normal vascular function, and releases the vasodilator nitric oxide which in turn improves circulation during perfusion, preserving endothelial function.[76, 77] Large clinical trials and meta-analyses have shown that non-oxygenated HMP is superior to SCS, reducing DGF in all types of deceased donors[84-86], which has prompted centres in some countries to adopt non-oxygenated HMP as their new standard.[87] However, while better able to maintain viability than simple SCS, there is only limited evidence that HMP can improve long-term graft function of DCD kidneys.[88, 89]

Despite reduced metabolic needs, there is residual ongoing metabolism during HMP. Oxygenation can be added during HMP to allow a low level of aerobic respiration and restore some ATP levels and maintain cellular processes.[90-92] A recent international randomised controlled trial compared oxygenated HMP and non-oxygenated HMP in

DCD kidneys above 50 years old. This study demonstrated that oxygenated HMP improved one-year kidney graft function when including all grafts that failed and reduced rejection one year post-transplant compared to non-oxygenated HMP. Furthermore, there were no significant differences in DGF and primary non-function.[92] Independent of the beneficial effects HMP has shown to date; there is still no means of assessing viability of injured donor organs and utilisation of marginals organs that have been previously discarded. Furthermore, prolonged CIT (more than 10 hours) is still an independent risk factor for DGF in non-oxygenated HMP preserved kidneys.[93] This is where NMP may provide an important alternative to reduce ischaemic injury associated with hypothermic preservation and assess organ viability prior to transplantation.

1.4.4 Normothermic machine perfusion

Preserving organs on normothermia is not a new concept. The first NMP and thus organ device predates the first successful kidney transplantation and was designed in 1935 by Alexis Carrel and Charles Lindbergh.[94] They demonstrated viability of several organs, including kidneys, for various durations using oxygenated serum; however, this was never used in clinical transplantation due to its complexity. NMP provides a near-physiological environment and involves circulating warm (36–38°C) oxygenated and nutrient-enriched perfusion solution through the organ vasculature allowing the restoration of cellular metabolism and replenishment of ATP. [95] NMP could maintain kidney function, assess organ viability while avoiding or minimising cold ischaemic injury. Furthermore, NMP offers a unique opportunity for active cellular interventions to an isolated organ *ex vivo* under near-physiological conditions prior to transplantation.[96-98] There are an increasing number of studies by different groups focusing on the development of NMP technology in kidney transplantation. However, the application into clinical practice has been limited and it is primarily used as a research tool.[95, 99] There is currently no consensus on the best NMP technique, perfusion duration or optimal perfusate composition.

1.5 NORMOTHERMIC MACHINE PERFUSION OF THE KIDNEY IN 2020

1.5.1 Parameters in normothermic machine perfusion

1.5.1.1 Arterial pressure

There is currently no consensus on the optimal arterial pressure during NMP generated either by a roller or centrifugal pumps and whether a continuous or pulsatile flow should be applied. Similar to cardiopulmonary bypass procedures[100], although slightly higher, during NMP, mean arterial pressure (MAP) between 40 and 100 mmHg are chosen to obtain sufficient tissue perfusion and avoid mechanical injury of tissue and blood.[98, 101-105] Pre-clinical studies by the Cambridge group showed less tubular damage and less endothelial injury during *ex vivo* reperfusion in kidneys perfused at a MAP of 75 and 95 mmHg compared with kidneys perfused at 55 mmHg.[103, 106]

1.5.1.2 Temperature

Different temperatures have been used in normothermic kidney perfusion, ranging from 32°C, sub-normothermia, to physiological body temperature 37°C. Brasile *et al*. performed early studies of perfusion of canine kidneys at 32°C for 24 hours with an acellular solution and showed superior post-transplant serum-creatinine, resuscitation of oxidative metabolism and recovery of cytoskeletal integrity compared to SCS kidneys.[107, 108] The Cambridge group showed during simulated reperfusion that renal and tubular function were better preserved during one-hour perfusion with a blood-based perfusate at 37°C rather than 32°C or SCS.[109] Another important question with regards to temperature is whether rewarming during NMP should be abrupt or gradual. Schopp *et al.* proposed that the abrupt rewarming of the kidney after cold storage could lead to mitochondrial dysfunction and published a new strategy to rewarm the kidneys, called control oxygenated rewarming (COR).[110] Minor *et al*. compared porcine kidneys undergoing NMP after SCS with or without COR (8°C to 35°C during the first 90 min, accompanied by an adapted pressure increase from 30 to 75 mmHg) and showed that

COR improved mitochondrial recovery and oxygen utilisation efficiency compared to SCS and NMP kidneys. Furthermore, a 2–3 fold improvement in renal function was seen after COR, which also significantly improved aerobic efficiency (total Na absorption/VO_2) upon simulated reperfusion.[111] The first clinical case describing COR with STEEN solution for 90 minutes of an ECD kidney was shown to be safe and feasible.[112]

1.5.1.3 Oxygenation

The majority of the NMP protocols in the literature use high levels of oxygen concentration. Administered oxygen during NMP is usually either 100% or a mixture of 95% and 5% carbon dioxide (carbogen) to maintain acid-base balance in the perfusate. Reported partial pressures of oxygen during NMP are usually supraphysiological, 70 to 85 kPa, however, the Oxford group described perfusion of kidneys at physiological oxygen partial pressures, 26 kPa.[98] The Cambridge group described a study comparing different levels of oxygen partial pressures during one hour of NMP in a simulated reperfusion model.[113] Reduction of oxygen concentrations led to a decrease in oxygen kinetics (oxygen consumption, delivery and extraction) but no difference in creatinine clearance, tubular function or kidney biomarkers of injury. However, future studies should investigate the membrane potential to assess mitochondrial function as well as assessment of mitochondrial DNA to determine oxidative damage and whether the tissue has taken up oxygen.[114]

1.5.2 Perfusate composition

The perfusate composition is of vital importance to ensure optimal organ preservation. An optimal NMP protocol should address the metabolic requirements of the kidney and compensate the absence of the body's homeostatic system that would normally replenish nutrients and dispose of waste products. The intended duration of NMP is tightly linked with the perfusate composition; a prolonged period to assess and condition

the donor kidney will require a more complex perfusate to allow adequate metabolism than a short period of NMP to merely resuscitate the organ immediately prior to transplantation. The majority of current published renal NMP protocols are all with red blood cells (RBCs) or an alternative oxygen carrier and an isotonic and/or colloid solution (Table 1.1).

1.5.2.1 Oxygen carriers

Red blood cell-based perfusion solutions, either packed RBCs or whole blood, are most commonly used during kidney NMP studies to deliver oxygen to the tissues.[98, 101, 112, 115-118] Whole blood has been used in NMP systems as a surrogate for reperfusion and the inevitable IRI in the recipient and as a mean to assess function and viability of the treated graft. [82, 117, 119] Other NMP protocols used autologous whole blood depleted of leucocytes and platelets to develop NMP techniques.[103, 120-122] Perfusion in the absence of leucocytes will limit an inflammatory response and vasoconstriction. Studies have shown that the use of leucocyte depleted blood in the perfusate leads to superior post-ischaemic renal function compared to whole blood.[116, 123] Recently the use of artificial oxygen carriers during NMP has been studied in porcine and human kidneys and demonstrated equivalent oxygen-carrying capacity to RBCs.[124, 125]

1.5.2.2 Supplements

NMP perfusate solutions are supplemented with many essential ingredients to support the metabolic processes and maintain kidney viability. Specific perfusion protocols were developed depending on the intended NMP duration and aim of its clinical application. A detailed overview of the published perfusate protocols for NMP as a preservation method in pre-clinical and clinical models are provided in Table 1.1. The Cambridge group developed a perfusate based on Ringer's solution with a low oncotic pressure for 1–2 hours of kidney NMP.[101, 105] The Toronto group developed a perfusate with STEEN

solution, a solution with a high concentration of albumin and dextran, to maintain oncotic pressure within physiological range and prevent cellular oedema supporting stable kidney NMP for 16 hours.[126-128] The Oxford group has also developed a kidney NMP perfusate for 24 hours perfusions with human serum albumin as the main component. Rather than replacing the urine with Ringer's solution, the urine was re-circulated in the circuit maintaining better homeostasis and acid-base balance.[98] Other groups in Sydney, Brussels, Cleveland, California, Rotterdam and Leiden performed NMP with perfusates based on the Cambridge, Toronto and Oxford groups.[104, 129135]

Other components that are included in the perfusate protocols are: mannitol for its osmotic effect and antioxidative role, vasodilators such as sodium nitroprusside, epoprostenol sodium and verapamil to increase renal blood flow, protective additives such as dexamethasone to reduce inflammation and antibiotics such as cefuroxime and amoxicillin-clavulanate to prevent bacterial or fungal contamination.[91, 98, 116, 136] Bacterial contamination in the organ donor's perfusion fluid is common and contamination during the procurement seemed to be the primary cause.[137-139] However, the pharmacology of antibiotics during NMP is currently unknown and this needs to be taken into consideration. Furthermore, nutrition supplements such as glucose and amino acids are added as energy source. Most groups add insulin in various concentrations to facilitate glucose absorption.

Table 1.1. Overview of perfusate protocols used during NMP of kidneys in clinical and pre-clinical studies. The protocols described are aimed at NMP as a preservation method/model rather than a simulated reperfusion model.

Group	Model*	Time on NMP (hours)	Priming solution	Colloid	Oxygen carrier	Fluid replacement	Nutrient/supplementation	Anti-coagulant	Vasodilator	Antibiotics
Cambridge, UK [101, 105]	Human clinical	1	400 mL Ringers solution, 25 mL 10% mannitol, 8 mg dexamethasone, 8.4% sodium bicarbonate	—	RBCs, 1 unit	Ringers solution	Nutriflex + 25 mL 8.4% sodium bicarbonate + 100 IU insulin + multivitamins, rate 20 mL/h. 5% glucose, rate 7 mL/h	2000 IU heparin	0.5 mg epoprostenol sodium, rate 4 mL/h	—
Rotterdam, Netherlands [134]	Human clinical	2	500–1000 mL Sterofundin solution, 10–20 mL 15% mannitol, 2 mL 4 mg dexamethasone, 10-40 mL 8.4% sodium bicarbonate	—	RBCs, 250 mL	Urine recirculation	Nutriflex + 25 mL 8.4% sodium bicarbonate + 100 IU insulin + 5 mL multivitamins, rate 20 mL/h. 5% glucose, rate 7 mL/h	2-4 mL 1000 IU/mL heparin	0.5 mg prostacyclin, rate 4 mL/h	1.2 g augmentin
Oxford, UK [98]	Human pre-clinical	24	250 mL 5% human serum albumin solution, 10 mL of 10% mannitol, 5–15 mL 8.4% sodium bicarbonate, 10 mL 10% calcium gluconate	Human serum albumin	RBCs, 1 unit	Urine recirculation or ringers lactate	2.5 mL bolus total parental nutrition-lipid-free solution when perfusate glucose levels dropped below 4 mmol/L	—	0.5 mg epoprostenol sodium, rate 4 ug/h	750 mg cefuroxime
California, USA [133]	Human pre-clinical	3	250 mL Plasma-Lyte, 0.06 g anhydrous creatinine	—	RBCs, 250 mL	Plasma-Lyte A	Parental nutrition (CLINIMIX E 2.75/10) + 26 ml/L 8.4% sodium bicarbonate + 100 IU insulin + 5 mL multivitamins, rate 20 mL/h	2000 IU heparin	—	—
Boston, USA [124]	Human pre-clinical	6	1500 mL of Williams E Media, 8 mg/mL dexamethasone, 8.4% sodium bicarbonate, calcium chloride, 1000 umol/L creatinine, 5 U/L insulin	—	RBCs, 500 mL or Hemopure, 500 mL	Ringers lactate	Dextrose throughout perfusion to maintain perfusate glucose levels at 100–200 mg/dL	1000 IU/L heparin	—	
Sydney, Australia [129, 130]	Human pre-clinical, Porcine Animal house	1–3	150 mL Hartmann's solution, 250 mL gelofusine, 50 mL 10% mannitol, 15 mL 8.4% sodium bicarbonate, 5 mL 10% calcium gluconate, 25 mL water for injection, 1000 umol/L creatinine	Gelofusine	RBCs, 250 mL	Hartmann's solution	M199 with ultraglutamine nutrient solution, rate 20 mL/h. 5% dextrose, rate 5 mL/h	2000 IU heparin	2.5 mg verapamil, rate 5 mL/h	—

(Continues)

Table 1.1. (Continued)

Group	Model*	Time on NMP (hours)	Priming solution	Colloid	Oxygen carrier	Fluid replacement	Nutrient/supplementation	Anti-coagulant	Vasodilator	Antibiotics
Leiden, Netherlands [135]	Human pre-clinical	6	100 mL 20% human serum albumin, 500 mL sodium chloride, 11.1 mmol/L 10% mannitol, 20–30 mL 8.4% sodium bicarbonate, 4.4 mmol/L calcium gluconate	Human serum albumin	RBCs, 500 mL	Urine recirculation	2.33 g/h 10% aminoplasmal, 2.22 mmol/h glucose, Cerenevit	—	0.0009 mg/h prostacyclin	—
Toronto, Canada [97, 102, 126-128]	Porcine Animal house	1–16	150 mL STEEN solution, 200 mL Ringers lactate, 8 mL 8.4% sodium bicarbonate, 1.8 mL 10% calcium gluconate, 27 ml double reverse osmosis water	STEEN solution	RBCs, 125 mL	Ringers lactate	Amino acids + glucose, rate 1 mL/h and adjusted if necessary to maintain perfusate glucose levels between 5–15 mmol/L. Insulin, rate 5 IU/h	1000 IU heparin	0.25 mg/h verapamil	—
Cleveland, USA [132]	Porcine Animal house	12–24	STEEN solution, 20 mL 8.4% sodium bicarbonate, 20 mL 10% calcium gluconate, 50 mg methylprednisolone	STEEN solution	RBCs, whole blood	—	1.5 IU/h insulin lispro, 500 IU/h heparin and vitamins, rate 0.25 mL/h . 50 mg/dL 50% dextrose supplementation as needed.	4000 IU heparin and 500 IU/h	25 mg/h nitroprusside, 10 mg bolus ± 1 mg/h verapamil or cocktail	1 g cefotaxime
Cleveland, USA [104]	Porcine - Slaughterhouse	8	22.5 g bovine serum albumin, 282 mL Ringers lactate, 322 mL Krebs-Henseleit buffer, 10 mg dexamethasone, 3 mL 10% calcium gluconate, 44 mL water	Bovine serum albumin	RBCs, 202 mL	Urine recirculation	5 IU/h insulin lispro + 0.05 g/h amino acids	2000 IU heparin	0.25 mg/h verapamil	1 g ampicillin and 1 g cefotaxime
Groningen, Netherlands [140-142]	Porcine - Slaughterhouse	7	40 g bovine serum albumin and 500 mL Williams' Medium E	Bovine serum albumin	RBCs, 350 mL	—	—	heparin	—	1000/200 mg amoxicillin-clavulanate

*Model: ‘Human clinical’ refers to kidneys that were transplanted and ‘human pre-clinical’ refers to experimental non-transplanted kidneys.

1.5.3 Normothermic machine perfusion strategies

Different strategies have been proposed to implement NMP in the clinical setting:

1. NMP for the entire preservation period
2. A short period of NMP at the donor hospital followed by SCS for transportation to the recipient centre
3. End NMP at the recipient centre
4. An intermittent period of NMP which may be in an organ hub or at the recipient centre. After NMP, kidneys are placed back in SCS.[143]

The Cambridge group have demonstrated clinical feasibility and safety of one hour of NMP for graft reconditioning/resuscitation of ECD kidneys after prolonged hypothermic storage, not aiming to replace the entire preservation period with warm perfusion.[105] In addition, they showed a reduction in DGF of marginal donor kidneys undergoing NMP compared to historical controls[105], which led to a UK multicentre phase II randomised controlled trial (ISRCTN15821205) to assess the efficacy of 1-hour normothermic reconditioning after SCS in DCD kidneys.[101] The trial was completed in February 2020 and the results are eagerly awaited. In another study, they have also demonstrated that NMP can potentially rescue DCD kidneys previously deemed un-transplantable due to inadequate cold flush with preservation solution, thereby reducing their discard rate.[96, 144] Hosgood *et al*. also demonstrated successful transplantation of a kidney subjected to a second period of cold storage, 5 hours and 21 minutes, after NMP.[145] This intermediate NMP might facilitate the development of organ perfusion hubs.

While this brief period will resuscitate the donor kidney and its energy metabolism (ATP repletion), it may be too short to fully assess kidney quality and potentially repair the kidney. Prolonged perfusion, which may be required for organ assessment and repair of injured kidneys, was first described by Brasile *et al*. In these studies, oxidative metabolism of canine and human kidneys could be maintained up to 48 hours at 32°C.[107]

The Toronto group studied NMP (at 37°C) and the combination of different hypothermic (SCS and HMP) and normothermic durations in a DCD pig auto-transplant model.[127, 128, 146] Kidneys perfused with 8 and 16 hours of NMP had less tubular injury and better function compared to a brief 1 hour period of NMP. Interestingly, one hour of NMP only resulted in worse allograft function after transplantation when compared to SCS kidneys alone, suggesting that a prolonged period of NMP was necessary to observe the presumed benefit of reconditioning and repair.[127] The Oxford group published the first report of prolonged normothermic human kidney preservation without transplantation and showed that perfusion of discarded kidneys up to 24 hours was feasible.[98] The Toronto group (NEVKP, NCT03136848), Oxford group (NKP1, ISRCTN13292277) and Leiden group (PROPER, NCT04693325) have all registered single centre phase I pilot studies to assess the safety and feasibility of prolonged periods (1–10 hours, 2–24 hours, 1–6 hours, respectively) of kidney NMP prior to transplantation.[147-149] The results of these studies are awaited with interest.

1.5.4 Normothermic machine perfusion as a delivery platform for therapeutic agents

NMP presents a unique opportunity to deliver potential treatments to an isolated organ *ex vivo* under near-physiological conditions to further reduce IRI and may help condition or repair the organ prior to transplantation. The administration of therapeutic agents during NMP allows direct contact with the organ vasculature, reducing the risk of most complications from systemic delivery. NMP conditions could be more beneficial for the delivery of therapies compared to HMP. Hypothermic conditions may impede the uptake of therapeutic agents, cellular signalling cascades may be altered, and the effector mechanisms blocked until the organ is re-warmed.[95] Therapies that have been investigated in pre-clinical kidney NMP models to reduce IRI include hydrogen sulfide

[150, 151], carbon monoxide[152], argon gases[153], nanoparticles[154-156], gene therapy (short hairpin RNA)[157], monoclonal antibody[158] and cellular therapies[140, 159, 160].

1.6 MESENCHYMAL STROMAL CELLS

In recent years, cellular therapy with mesenchymal stromal cells (MSCs) has been evaluated as a new strategy to achieve immune tolerance in kidney transplantation due to the cells ability to suppress the immune system at the site of tissue or cellular injury.[161, 162] As marginal kidneys from high-risk donor kidneys with inferior quality are increasingly accepted, research has slowly shifted towards improving preservation techniques and treating the kidneys to promote tissue repair prior to transplantation. MSCs have also been shown to possess reparative and regenerative properties, with different small and large animal studies reporting a beneficial effect on IRI with the possibility of acute repair in the kidney.[163, 164] This thesis focusses on MSC therapy during NMP prior to transplantation.

1.6.1 Characterisation of mesenchymal stromal cells

Mesenchymal stromal cells are fibroblast shaped adult stromal cells with the ability to differentiate into many different cell lineages.[165, 166] They are characterized by their ability to adhere to plastic, their ability to express cell surface markers CD105, CD90, and CD73 along the absence of haematopoietic stem cell and endothelial markers CD11b, CD14, CD19, CD34, CD31 CD45, CD79α and HLA-DR.[167] MSCs express low levels of MHC class I and co-stimulatory molecules such as CD80, CD86 and CD40 and lack the expression of MHC class II and are unlikely to be detected by the adaptive immune system.[168]

1.6.2 Sources of mesenchymal stromal cells

MSCs can be harvested from multiple locations in the body, such as bone marrow, adipose tissue and cord blood and can secrete different factors having different therapeutic potential.[169-171] Kusama *et al*. and Tsuji *et al*. suggested that this is most likely caused by the different native microenvironments the cells reside in and the different signals they receive. During culture, the cells reside in additional '*in vitro*' microenvironments and corresponding conditions (e.g. oxygen tension) and this can also influence the characteristics of the MSCs.[172, 173] The number of MSCs that can be acquired per isolation varies depending on the source.[174] Bone marrow-MSCs (BM-MSCs) currently represent the predominant source of MSCs used in kidney transplantation[175] however, an increasing numbers of studies are reported that use umbilical cord derived MSCs and adipose derived MSCs (A-MSCs).[166] *In vitro* studies comparing A-MSCs and BM-MSCs showed that A-MSCs might have a stronger immunosuppressive potential than BM-MSCs.[170, 171, 176] Another advantage of A-MSCs is that they are more easily accessible, the harvesting approach is considered to be safe and a significantly high number of cells can be obtained from the same amount of tissue compared to MSCs harvested from other sources.[177, 178]

Most studies administered autologous MSC to recipients after transplantation, however in deceased organ transplantation the use of allogenic MSC is inevitable.[161, 179] Treatment with allogenic MSCs offer the advantage that they can be easily standardised and immediately available as an 'off-the-shelf' clinical GMP grade product. In addition, MSCs can be isolated from young donors as studies showed that the MSC number and functionality decrease with donor age.[180-183] Despite some concerns regarding cryopreserved MSCs[184] with studies showing that fresh cultured MSCs have elevated MSC activity compared to cryopreserved MSCs[185-187], cryopreserved MSCs are more in line with eventual clinical reality. Results of the NEPTUNE study showed safety of allogenic MSC administration after kidney transplantation.[188]

1.6.3 MSC immunomodulatory and regenerative mechanism of action

Initially, MSCs were thought to mediate tissue repair by replacing damaged cells due to their multilineage differentiation capacity.[161] The paradigm shifted and it is now believed that MSCs immunomodulatory and regenerative capacities are encouraged via interaction with host immune and progenitor cells, driven by their produced soluble factors (secretome) which change the microenvironment at sites of injury leading to anti-inflammatory, anti-apoptotic, pro-angiogenic and anti-fibrotic effects (Figure 1.2).[189-192] The MSC secretome constitutes of trophic and regulatory proteins including growth factors, cytokines, chemokines, and adhesion molecules.

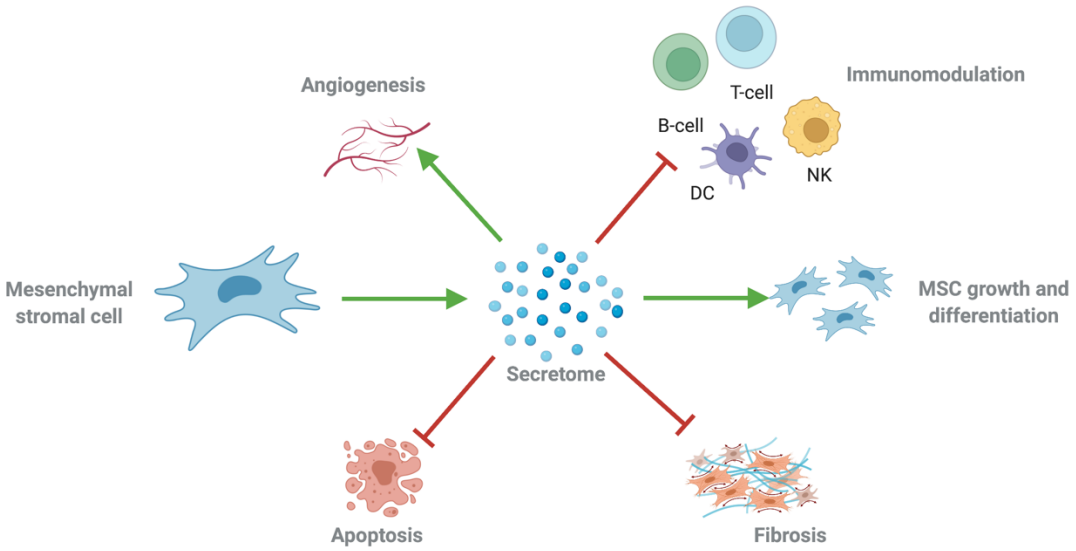


Figure 1.2. Mechanism of action of mesenchymal stromal cells. Mesenchymal stromal cell (MSCs) have the ability to modulate the immune response at the site of tissue or cellular injury. In addition, MSCs have been shown to possess reperative and regenerative properties via interaction with host immune and progenitor cells, driven by the soluble factors they produce (secretome), leading to anti-inflammatory, anti-fibrotic, anti-apoptotic and pro-angiogenic influences.

MSCs can suppress the immune system by secretion of molecules and by direct cell contact. There is mounting evidence of a relationship between MSC soluble factors and immune cells such as lymphocytes, dendritic cells, natural killer cells and macrophages.[193] Interferon-γ (IFN-γ) is one of the main molecules eliciting immune suppression, often in combination with other pro-inflammatory cytokines like TNFα and IL-1.[194] MSCs modulate both the adaptive and innate host immune systems by preventing T-cell and B-cell activation and proliferation.[195] Secreted factors affecting T-cell biology include hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF-β1), indoleamine 2,3 dioxygenase (IDO), prostaglandin E2 (PGE2) and IL-10.[194,196] Furthermore, MSCs impair dendritic cell maturation [197, 198] and reprogram monocytes and macrophages from a pro-inflammatory to an anti-inflammatory state.[199, 200] Macrophage anti-inflammatory phenotype is defined as macrophages with high secretion of IL-10 and IL-6 and low secretion of IL-12 and TNFα.[201] This could influence allograft rejection and therefore, also might contribute to an alleviation of IRI.[202] In addition, MSCs have the potential to suppress oxidative stress and trigger tubular cell proliferation, which may minimise tissue injury and stimulate repair.[192] MSCs produce a number of antioxidants, including hemeoxygenase-1 (HO-1)[203] and so far, have been shown to suppress oxidative stress and inflammation in IRI models *in vivo*.[192, 204-206] This is a result of increased expression of HO-1, IL-10 and HGF and a decreased expression of the pro-inflammatory cytokines IL-1β, TNFα, IFN-γ as well as of reduced apoptosis, number of activated T-cells and immune cells.[192, 205, 206, 208, 209] Sierra-Parraga *et al.* recently showed in an *in vitro* model that MSCs decrease injury markers and oxidative stress of endothelial cells after hypoxia-reoxygenation, as well as improve wound healing and angiogenic function on injured human endothelial cells via a mechanism which requires both physical and paracrine interaction.[210] MSCs exert angiogenic effects on injured tissue by secreting paracrine factors such as VEGF, basic fibroblast growth factor 2 (FGF2), HGF, stromal cell-derived factor-1, TGF-β, platelet-derived growth factor (PDGF), angiopoietin-1 and monocyte chemotactic protein-1 (MCP-1).[164, 211] These

factors are capable of ameliorating tissue vascularity and enhance angiogenesis.[212, 213] Moreover, vasculature integrity is protected through angiopoietin-1 interaction with endothelial cells.[214] MSCs also help to repair wounds and have anti-fibrotic effects that avoid the formation of scar tissue via paracrine secretion of pro-angiogenic factors[215, 216] and increasing keratinocyte and fibroblast migration towards injured tissue.[217] In a porcine model, the injection of autologous amniotic fluid-derived MSCs in the renal artery 6 days after transplantation resulted in improved function of glomeruli and tubules, as well as significantly less fibrosis after 3 months.[218] Less fibrosis and less atrophy of the tubules were also observed when injecting MSCs 11 week postoperatively in a rat transplant model. [219]

1.6.4 Migration and route of administration

The exact mechanism of how MSCs migrate to the damaged tissue, a process termed 'homing', is not fully understood. Following injury, MSCs preferentially home to inflammatory sites where they migrate across the endothelium and enter the injured tissue bed. [220] The adhesion molecule CD44 expression on MSCs seems to play a vital role in migration of MSCs as well as the chemokines and receptors expressed on the MSC cell surface. Specifically stromal cell-derived factor 1, secreted by endothelial cells during hypoxic conditions, which binds to CXCR4 and CXCR7 receptors on the MSC cell surface enhance MSC migration.[193, 210, 221, 222] However, MSC migration also depends on the administration route. Chamberlain *et al.* proposed two different mechanisms of how MSCs migrate to the tissue. The first one involves active homing, in which MSCs actively tether to and roll on the activated vasculature leading to arrest and firm adhesion. The second mechanism is a passive mechanism where MSCs are passively retained in the narrow capillaries due to their large size.[223] The latter being confirmed by Sierra-Parraga *et al.*, who showed similar living and dead MSC retention in the kidney tissue after intra-arterial administration.[224] Intravenous (IV) infusion of MSCs to transplant recipients has been the route of administration for most clinical studies and appears to be safe.[161, 225-

[227] However, this mode of systemic administration is associated with off-target distribution where IV infused MSCs are largely trapped in the lungs.[228, 229] Furthermore studies showed that MSCs are generally short-lived after IV administration.[228-231] Intra-arterial administration of MSCs could improve the migration of MSCs to the site of injury and prolong the survival of MSCs.[232-236]

REFERENCES

1. Kidney Research 2. Chronic Kidney Disease . 2020.

2. Levin A, Stevens PE, Bilous RW, et al. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *Kidney Int. Suppl.* 2013;3:1-150.

3. Jha V, Garcia-Garcia G, Iseki K, et al. Chronic kidney disease: global dimension and perspectives. *Lancet*. 2013;382:260-272.

4. Schena PF. Epidemiology of end-stage renal disease: International comparisons of renal replacement therapy. *Kidney International*. 2000;57:S39-S45.

5. Tonelli M, Wiebe N, Knoll G, et al. Systematic review: kidney transplantation compared with dialysis in clinically relevant outcomes. *Am J Transplant*. 2011;11:2093-2109.

6. Wolfe RA, Ashby VB, Milford EL, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med*. 1999;341:1725-1730.

7. National Health Service Blood and Transplant. Organ Donation and Transplantation Activity Report 2019/20. United Kingdom: Statistics and Clinical Studies, NHS Blood and Transplant; 2020.

8. National Health Service Blood and Transplant. UK laws: Organ donation law in England. 2020.

9. Government of the Netherlands. Organ and Tissue Donation: The new Donor Act (active donor registration). 2020.

10. Rao PS, Ojo A. The alphabet soup of kidney transplantation: SCD, DCD, ECD--fundamentals for the practicing nephrologist. *Clin J Am Soc Nephrol*. 2009;4:1827-1831.

11. Manara AR, Thomas I. Current status of organ donation after brain death in the UK. *Anaesthesia*. 2020;75:1205-1214.

12. Eurotransplant International Foundation. Eurotransplant Annual Report 2018. Eurotransplant International Foundation; 2018.

13. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2016 Annual Data Report: Kidney. *Am J Transplant*. 2018;18 Suppl 1:18-113.

14. Heylen L, Jochmans I, Samuel U, et al. The duration of asystolic ischemia determines the risk of graft failure after circulatory-dead donor kidney transplantation: A

Eurotransplant cohort study. *Am J Transplant*. 2018;18:881-889.

15. Singh RP, Farney AC, Rogers J, et al. Kidney transplantation from donation after cardiac death donors: lack of impact of delayed graft function on post-transplant outcomes. *Clin Transplant*. 2011;25:255-264.

16. Tennankore KK, Kim SJ, Alwayn IP, Kiberd BA. Prolonged warm ischemia time is associated with graft failure and mortality after kidney transplantation. *Kidney Int*. 2016;89:648-658.

17. Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant Proc*. 2008;40:3279-3288.

18. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol*. 2015;6:524-551.

19. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417:1-13.

20. Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. *Nephrol Dial Transplant*. 2014;29:1134-1140.

21. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol*. 2012;298:229-317.

22. Nieuwenhuijs-Moeke GJ, Pischke SE, Berger SP, et al. Ischemia and Reperfusion Injury in Kidney Transplantation: Relevant Mechanisms in Injury and Repair. *J Clin Med*. 2020;9:10.3390/jcm9010253.

23. Kezić A, Stajic N, Thaiss F. Innate Immune Response in Kidney Ischemia/Reperfusion Injury: Potential Target for Therapy. *J Immunol Res*. 2017;2017:6305439.

24. Jang HR, Ko GJ, Wasowska BA, Rabb H. The interaction between ischemia-reperfusion and immune responses in the kidney. *J Mol Med (Berl)*. 2009;87:859-864.

25. Stroo I, Stokman G, Teske GJ, et al. Chemokine expression in renal ischemia/reperfusion injury is most profound during the reparative phase. *Int Immunol*. 2010;22:433-442.

26. Furuichi K, Wada T, Yokoyama H, Kobayashi KI. Role of Cytokines and Chemokines in Renal Ischemia-Reperfusion Injury. *Drug News Perspect*. 2002;15:477-482.

27. Zhao H, Alam A, Soo AP, George AJT, Ma D. Ischemia-Reperfusion Injury Reduces Long Term Renal Graft Survival: Mechanism and Beyond. *EBioMedicine*. 2018;28:31-42.

28. Voss A, Bode G, Kerkhoff C. Double-stranded RNA induces IL-8 and MCP-1 gene

expression via TLR3 in HaCaT-keratinocytes. *Inflamm Allergy Drug Targets*. 2012;11:397-405.

29. Patel NS, Chatterjee PK, Di Paola R, et al. Endogenous interleukin-6 enhances the renal injury, dysfunction, and inflammation caused by ischemia/reperfusion. *J Pharmacol Exp Ther*. 2005;312:1170-1178.

30. Donnahoo KK, Shames BD, Harken AH, Meldrum DR. Review article: the role of tumor necrosis factor in renal ischemia-reperfusion injury. *J Urol*. 1999;162:196-203.

31. Yang N, Luo M, Li R, et al. Blockage of JAK/STAT signalling attenuates renal ischaemia-reperfusion injury in rat. *Nephrol Dial Transplant*. 2008;23:91-100.

32. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest*. 2011;121:4210-4221.

33. Linas SL, Whittenburg D, Parsons PE, Repine JE. Ischemia increases neutrophil retention and worsens acute renal failure: role of oxygen metabolites and ICAM 1. *Kidney Int*. 1995;48:1584-1591.

34. Linas S, Whittenburg D, Repine JE. Nitric oxide prevents neutrophil-mediated acute renal failure. *Am J Physiol*. 1997;272:48.

35. Sheridan AM, Bonventre JV. Cell biology and molecular mechanisms of injury in ischemic acute renal failure. *Curr Opin Nephrol Hypertens*. 2000;9:427-434.

36. Takada M, Nadeau KC, Shaw GD, Marquette KA, Tilney NL. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J Clin Invest*. 1997;99:2682-2690.

37. Patel NS, Cuzzocrea S, Chatterjee PK, et al. Reduction of renal ischemia-reperfusion injury in 5-lipoxygenase knockout mice and by the 5-lipoxygenase inhibitor zileuton. *Mol Pharmacol*. 2004;66:220-227.

38. Sener G, Sehirli O, Velioğlu-Oğünç A, et al. Montelukast protects against renal ischemia/reperfusion injury in rats. *Pharmacol Res*. 2006;54:65-71.

39. Sener G, Sakarcan A, Sehirli O, et al. Chronic renal failure-induced multiple-organ injury in rats is alleviated by the selective CysLT1 receptor antagonist montelukast. *Prostaglandins Other Lipid Mediat*. 2007;83:257-267.

40. Johnson KJ, Weinberg JM. Postischemic renal injury due to oxygen radicals. *Curr Opin Nephrol Hypertens*. 1993;2:625-635.

41. Paller MS. The cell biology of reperfusion injury in the kidney. *J Investig Med*. 1994;42:632-639.

42. Bonventre JV. Mechanisms of ischemic acute renal failure. *Kidney Int*. 1993;43:1160-1178.

43. Paller MS, Hoidal JR, Ferris TF. Oxygen free radicals in ischemic acute renal failure in the rat. *J Clin Invest*. 1984;74:1156-1164.

44. Kehrer JP. Free radicals as mediators of tissue injury and disease. *Crit Rev Toxicol*. 1993;23:21-48.

45. Singh I, Gulati S, Orak JK, Singh AK. Expression of antioxidant enzymes in rat kidney during ischemia-reperfusion injury. *Mol Cell Biochem*. 1993;125:97-104.

46. Giovannini L, Migliori M, Longoni BM, et al. Resveratrol, a polyphenol found in wine, reduces ischemia reperfusion injury in rat kidneys. *J Cardiovasc Pharmacol*. 2001;37:262-270.

47. Rhoden EL, Pereira-Lima L, Telöken C, Lucas ML, Belló-Klein A, Rhoden CR. Beneficial effect of alpha-tocopherol in renal ischemia-reperfusion in rats. *Jpn J Pharmacol*. 2001;87:164-166.

48. Seth P, Kumari R, Madhavan S, et al. Prevention of renal ischemia-reperfusion-induced injury in rats by picroliv. *Biochem Pharmacol*. 2000;59:1315-1322.

49. Bayati A, Källskog O, Wolgast M. The long-term outcome of post-ischaemic acute renal failure in the rat. I. A functional study after treatment with SOD and sucrose. *Acta Physiol Scand*. 1990;138:25-33.

50. Baker GL, Corry RJ, Autor AP. Oxygen free radical induced damage in kidneys subjected to warm ischemia and reperfusion. Protective effect of superoxide dismutase. *Ann Surg*. 1985;202:628-641.

51. Greenwald RA. Superoxide dismutase and catalase as therapeutic agents for human diseases. A critical review. *Free Radic Biol Med*. 1990;8:201-209.

52. Devarajan P. Cellular and molecular derangements in acute tubular necrosis. *Curr Opin Pediatr*. 2005;17:193-199.

53. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol*. 2003;4:552-565.

54. Malis CD, Bonventre JV. Susceptibility of mitochondrial membranes to calcium and reactive oxygen species: implications for ischemic and toxic tissue damage. *Prog Clin Biol Res*. 1988;282:235-259.

55. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. 2014;515:431-435.

56. Weiser MR, Williams JP, Moore FD,Jr, et al. Reperfusion injury of ischemic skeletal

muscle is mediated by natural antibody and complement. *J Exp Med*. 1996;183:2343-2348.

57. Park P, Haas M, Cunningham PN, Bao L, Alexander JJ, Quigg RJ. Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes. *Am J Physiol Renal Physiol*. 2002;282:352.

58. Lin T, Zhou W, Farrar CA, Hargreaves RE, Sheerin NS, Sacks SH. Deficiency of C4 from donor or recipient mouse fails to prevent renal allograft rejection. *Am J Pathol*. 2006;168:1241-1248.

59. Thurman JM, Ljubanovic D, Edelstein CL, Gilkeson GS, Holers VM. Lack of a functional alternative complement pathway ameliorates ischemic acute renal failure in mice. *J Immunol*. 2003;170:1517-1523.

60. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol*. 2004;165:1677-1688.

61. Kilgore KS, Friedrichs GS, Homeister JW, Lucchesi BR. The complement system in myocardial ischaemia/reperfusion injury. *Cardiovasc Res*. 1994;28:437-444.

62. Arumugam TV, Shiels IA, Woodruff TM, Granger DN, Taylor SM. The role of the complement system in ischemia-reperfusion injury. *Shock*. 2004;21:401-409.

63. De Vries B, Matthijsen RA, Wolfs TG, Van Bijnen AA, Heeringa P, Buurman WA. Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation. *Transplantation*. 2003;75:375-382.

64. Williams JP, Pechet TT, Weiser MR, et al. Intestinal reperfusion injury is mediated by IgM and complement. *J Appl Physiol (1985)*. 1999;86:938-942.

65. Bonventre JV. Complement and renal ischemia-reperfusion injury. *Am J Kidney Dis*. 2001;38:430-436.

66. Foreman KE, Glovsky MM, Warner RL, Horvath SJ, Ward PA. Comparative effect of C3a and C5a on adhesion molecule expression on neutrophils and endothelial cells. *Inflammation*. 1996;20:1-9.

67. Foreman KE, Vaporciyan AA, Bonish BK, et al. C5a-induced expression of P-selectin in endothelial cells. *J Clin Invest*. 1994;94:1147-1155.

68. Hattori R, Hamilton KK, McEver RP, Sims PJ. Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein

GMP-140 to the cell surface. *J Biol Chem*. 1989;264:9053-9060.

69. Debout A, Foucher Y, Trébern-Launay K, et al. Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation. *Kidney Int*. 2015;87:343-349.

70. Summers DM, Johnson RJ, Hudson A, Collett D, Watson CJ, Bradley JA. Effect of donor age and cold storage time on outcome in recipients of kidneys donated after circulatory death in the UK: a cohort study. *Lancet*. 2013;381:727-734.

71. Ponticelli CE. The impact of cold ischemia time on renal transplant outcome. *Kidney Int*. 2015;87:272-275.

72. Thuong M, Ruiz A, Evrard P, et al. New classification of donation after circulatory death donors definitions and terminology. *Transpl Int*. 2016;29:749-759.

73. Saat TC, van den Akker, E. K., IJzermans JN, Dor FJ, de Bruin RW. Improving the outcome of kidney transplantation by ameliorating renal ischemia reperfusion injury: lost in translation? *J Transl Med*. 2016;14:20-2.

74. O'Callaghan JM, Knight SR, Morgan RD, Morris PJ. Preservation solutions for static cold storage of kidney allografts: a systematic review and meta-analysis. *Am J Transplant*. 2012;12:896-906.

75. Gavriilidis P, Inston NG. Recipient and allograft survival following donation after circulatory death versus donation after brain death for renal transplantation: A systematic review and meta-analysis. *Transplant Rev (Orlando)*. 2020;34:100563.

76. Gallinat A, Fox M, Lüer B, Efferz P, Paul A, Minor T. Role of pulsatility in hypothermic reconditioning of porcine kidney grafts by machine perfusion after cold storage. *Transplantation*. 2013;96:538-542.

77. Chatauret N, Coudroy R, Delpech PO, et al. Mechanistic analysis of nonoxygenated hypothermic machine perfusion's protection on warm ischemic kidney uncovers greater eNOS phosphorylation and vasodilation. *Am J Transplant*. 2014;14:2500-2514.

78. Mas VR, Archer KJ, Dumur CI, et al. Reduced expression of inflammatory genes in deceased donor kidneys undergoing pulsatile pump preservation. *PLoS One*. 2012;7:e35526.

79. von Horn C, Minor T. Isolated kidney perfusion: the influence of pulsatile flow. *Scand J Clin Lab Invest*. 2018;78:131-135.

80. Kim HK, Son HS, Fang YH, Park SY, Hwang CM, Sun K. The effects of pulsatile flow upon renal tissue perfusion during

cardiopulmonary bypass: a comparative study of pulsatile and nonpulsatile flow. *ASAIO J*. 2005;51:30-36.

81. Wszola M, Kwiatkowski A, Diuwe P, et al. One-year results of a prospective, randomized trial comparing two machine perfusion devices used for kidney preservation. *Transpl Int*. 2013;26:1088-1096.

82. Mancina E, Kalenski J, Paschenda P, et al. Determination of the preferred conditions for the isolated perfusion of porcine kidneys. *Eur Surg Res*. 2015;54:44-54.

83. Jochmans I, Akhtar MZ, Nasralla D, et al. Past, Present, and Future of Dynamic Kidney and Liver Preservation and Resuscitation. *Am J Transplant*. 2016;16:2545-2555.

84. Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study Group. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med*. 2012;366:770-771.

85. Jochmans I, Moers C, Smits JM, et al. Machine perfusion versus cold storage for the preservation of kidneys donated after cardiac death: a multicenter, randomized, controlled trial. *Ann Surg*. 2010;252:756-764.

86. Tingle SJ, Figueiredo RS, Moir JA, et al. Hypothermic machine perfusion is superior to static cold storage in deceased donor kidney transplantation: A meta-analysis. *Clin Transplant*. 2020;34:e13814.

87. Brat A, van Heurn E, Huurman V, et al. Hypothermic Machine Perfusion as a National Standard Preservation Method for Deceased Donor Kidneys . *Transpl Int.* 2019;32.

88. Summers DM, Ahmad N, Randle LV, et al. Cold pulsatile machine perfusion versus static cold storage for kidneys donated after circulatory death: a multicenter randomized controlled trial. *Transplantation*. 2019.

89. Watson CJ, Wells AC, Roberts RJ, et al. Cold machine perfusion versus static cold storage of kidneys donated after cardiac death: a UK multicenter randomized controlled trial. *Am J Transplant*. 2010;10:1991-1999.

90. Patel K, Smith TB, Neil DAH, et al. The Effects of Oxygenation on Ex Vivo Kidneys Undergoing Hypothermic Machine Perfusion. *Transplantation*. 2019;103:314-322.

91. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation*. 2019;103:2057-2064.

92. Jochmans I, Brat A, Davies L, et al. Oxygenated versus standard cold perfusion preservation in kidney transplantation

(COMPARE): a randomised, double-blind, paired, phase 3 trial. *Lancet*. 2020;396:1653-1662.

93. Kox J, Moers C, Monbaliu D, et al. The Benefits of Hypothermic Machine Preservation and Short Cold Ischemia Times in Deceased Donor Kidneys. *Transplantation*. 2018;102:1344-1350.

94. Carrel A, Lindbergh CA. The Culture of Whole Organs. *Science*. 1935;81:621-623.

95. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? *Transpl Int*. 2015;28:657-664.

96. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable but Resuscitated by Ex Vivo Normothermic Machine Perfusion. *Am J Transplant*. 2016;16:3282-3285.

97. Hamar M, Urbanellis P, Kaths MJ, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. *Transplantation*. 2018;102:1262-1270.

98. Weissenbacher A, Lo Faro L, Boubriak O, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. *Am J Transplant*. 2019;19:178-192.

99. Kaths JM, Paul A, Robinson LA, Selzner M. Ex vivo machine perfusion for renal graft preservation. *Transplant Rev (Orlando)*. 2018;32:1-9.

100. Murphy GS, Hessel EA,2nd, Groom RC. Optimal perfusion during cardiopulmonary bypass: an evidence-based approach. *Anesth Analg*. 2009;108:1394-1417.

101. Hosgood SA, Saeb-Parsy K, Wilson C, Callaghan C, Collett D, Nicholson ML. Protocol of a randomised controlled, open-label trial of ex vivo normothermic perfusion versus static cold storage in donation after circulatory death renal transplantation. *BMJ Open*. 2017;7:e012237-012237.

102. Kaths JM, Spetzler VN, Goldaracena N, et al. Normothermic Ex Vivo Kidney Perfusion for the Preservation of Kidney Grafts prior to Transplantation. *J Vis Exp*. 2015;(101):e52909. doi:e52909.

103. Hosgood S, Harper S, Kay M, Bagul A, Waller H, Nicholson ML. Effects of arterial pressure in an experimental isolated haemoperfused porcine kidney preservation system. *Br J Surg*. 2006;93:879-884.

104. Blum MF, Liu Q, Soliman B, et al. Comparison of normothermic and hypothermic perfusion in porcine kidneys

donated after cardiac death. *J Surg Res*. 2017;216:35-45.

105. Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: the first clinical study. *Am J Transplant*. 2013;13:1246-1252.

106. Patel M, Hosgood S, Nicholson ML. The effects of arterial pressure during normothermic kidney perfusion. *J Surg Res*. 2014;191:463-468.

107. Brasile L, Stubenitsky BM, Booster MH, Haisch C, Kootstra G. NOS: the underlying mechanism preserving vascular integrity and during ex vivo warm kidney perfusion. *Am J Transplant*. 2003;3:674-679.

108. Brasile L, Stubenitsky BM, Booster MH, et al. Overcoming severe renal ischemia: the role of ex vivo warm perfusion. *Transplantation*. 2002;73:897-901.

109. Adams TD, Patel M, Hosgood SA, Nicholson ML. Lowering Perfusate Temperature From 37 degrees C to 32 degrees C Diminishes Function in a Porcine Model of Ex Vivo Kidney Perfusion. *Transplant Direct*. 2017;3:e140.

110. Schopp I, Reissberg E, Lüer B, Efferz P, Minor T. Controlled Rewarming after Hypothermia: Adding a New Principle to Renal Preservation. *Clin Transl Sci*. 2015;8:475-478.

111. von Horn C, Minor T. Improved approach for normothermic machine perfusion of cold stored kidney grafts. *Am J Transl Res*. 2018;10:1921-1929.

112. Minor T, von Horn C, Gallinat A, et al. First-in-man controlled rewarming and normothermic perfusion with cell-free solution of a kidney prior to transplantation. *Am J Transplant*. 2020;20:1192-1195.

113. Adams TD, Hosgood SA, Nicholson ML. Physiological effects of altering oxygenation during kidney normothermic machine perfusion. *Am J Physiol Renal Physiol*. 2019;316:F823-F829.

114. Kezic A, Spasojevic I, Lezaic V, Bajcetic M. Mitochondria-Targeted Antioxidants: Future Perspectives in Kidney Ischemia Reperfusion Injury. *Oxid Med Cell Longev*. 2016;2016:2950503.

115. Kaths JM, Hamar M, Echeverri J, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. *Am J Transplant*. 2018;18:580-589.

116. Harper S, Hosgood S, Kay M, Nicholson M. Leucocyte depletion improves renal function during reperfusion using an experimental isolated haemoperfused organ preservation system. *Br J Surg*. 2006;93:623-629.

117. Hameed AM, Lu DB, Patrick E, et al. Brief Normothermic Machine Perfusion Rejuvenates Discarded Human Kidneys. *Transplant Direct*. 2019;5:e502.

118. Kabagambe SK, Palma IP, Smolin Y, et al. Combined Ex Vivo Hypothermic and Normothermic Perfusion for Assessment of High-risk Deceased Donor Human Kidneys for Transplantation. *Transplantation*. 2019;103:392-400.

119. Hosgood SA, Shah K, Patel M, Nicholson ML. The effect of prolonged of warm ischaemic injury on renal function in an experimental ex vivo normothermic perfusion system. *J Transl Med*. 2015;13:207-4.

120. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation*. 2019;103:2057-2064.

121. Hosgood SA, Barlow AD, Yates PJ, Snoeijs MG, van Heurn EL, Nicholson ML. A pilot study assessing the feasibility of a short period of normothermic preservation in an experimental model of non heart beating donor kidneys. *J Surg Res*. 2011;171:283-290.

122. Weissenbacher A, Voyce D, Ceresa CDL, et al. Urine Recirculation Improves Hemodynamics and Enhances Function in Normothermic Kidney Perfusion. *Transplant Direct*. 2020;6:e541.

123. Yang B, Hosgood SA, Harper SJ, Nicholson ML. Leucocyte depletion improves renal function in porcine kidney hemoreperfusion through reduction of myeloperoxidase+ cells, caspase-3, IL-1β, and tubular apoptosis. *J Surg Res*. 2010;164:315.

124. Aburawi MM, Fontan FM, Karimian N, et al. Synthetic hemoglobin-based oxygen carriers are an acceptable alternative for packed red blood cells in normothermic kidney perfusion. *Am J Transplant*. 2019;19:2814-2824.

125. Bhattacharjee RN, Patel SVB, Sun Q, et al. Renal Protection Against Ischemia Reperfusion Injury: Hemoglobin-based Oxygen Carrier-201 Versus Blood as an Oxygen Carrier in Ex Vivo Subnormothermic Machine Perfusion. *Transplantation*. 2020;104:482-489.

126. Kaths JM, Echeverri J, Linares I, et al. Normothermic Ex Vivo Kidney Perfusion Following Static Cold Storage-Brief, Intermediate, or Prolonged Perfusion for Optimal Renal Graft Reconditioning? *Am J Transplant*. 2017;17:2580-2590.

127. Kaths JM, Cen JY, Chun YM, et al. Continuous Normothermic Ex Vivo Kidney Perfusion Is Superior to Brief Normothermic Perfusion Following Static Cold Storage in Donation After Circulatory Death Pig Kidney

Transplantation. *Am J Transplant*. 2017;17:957-969.

128. Urbanellis P, Hamar M, Kaths JM, et al. Normothermic Ex Vivo Kidney Perfusion Improves Early DCD Graft Function Compared With Hypothermic Machine Perfusion and Static Cold Storage. *Transplantation*. 2020;104:947-955.

129. Hameed AM, Lu DB, Patrick E, et al. Brief Normothermic Machine Perfusion Rejuvenates Discarded Human Kidneys. *Transplant Direct*. 2019;5:e502.

130. Hameed AM, Miraziz R, Lu DB, et al. Extra-corporeal normothermic machine perfusion of the porcine kidney: working towards future utilization in Australasia. *ANZ J Surg*. 2018;88:E429-E434.

131. Darius T, Gianello P, Vergauwen M, et al. The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model. *Am J Transplant*. 2019;19:752-762.

132. Urcuyo D, Blum MF, Liu Q, et al. Development of a prolonged warm ex vivo perfusion model for kidneys donated after cardiac death. *Int J Artif Organs*. 2017;40:265-271.

133. Kabagambe SK, Palma IP, Smolin Y, et al. Combined Ex Vivo Hypothermic and Normothermic Perfusion for Assessment of High-risk Deceased Donor Human Kidneys for Transplantation. *Transplantation*. 2019;103:392-400.

134. Rijkse E, IJzermans JN, Minnee RC. Machine perfusion in abdominal organ transplantation: Current use in the Netherlands. *World J Transplant*. 2020;10:15-28.

135. Arykbaeva AS, de Vries DK, Doppenberg JB, et al. Metabolic needs of the kidney graft undergoing normothermic machine perfusion. *Kidney Int*. 2021;100:301-310.

136. Hosgood SA, Barlow AD, Dormer J, Nicholson ML. The use of ex-vivo normothermic perfusion for the resuscitation and assessment of human kidneys discarded because of inadequate in situ perfusion. *J Transl Med*. 2015;13:329-x.

137. Yahav D, Manuel O. Clinical relevance of preservation-fluid contamination in solid-organ transplantation: a call for mounting the evidence. *Clin Microbiol Infect*. 2019;25:536-537.

138. Corbel A, Ladrière M, Le Berre N, et al. Microbiological epidemiology of preservation fluids in transplanted kidney: a nationwide retrospective observational study. *Clin Microbiol Infect*. 2020;26:475-484.

139. Oriol I, Sabé N, Tebé C, Veroux M, Boin, I. F. S. F., Carratalà J. Clinical impact of culture-positive preservation fluid on solid organ transplantation: A systematic review and meta-analysis. *Transplant Rev (Orlando)*. 2018;32:85-91.

140. Pool M, Eertman T, Sierra Parraga J, et al. Infusing Mesenchymal Stromal Cells into Porcine Kidneys during Normothermic Machine Perfusion: Intact MSCs Can Be Traced and Localised to Glomeruli. *Int J Mol Sci*. 2019;20:10.3390/ijms20143607.

141. Pool MBF, Hartveld L, Leuvenink HGD, Moers C. Normothermic machine perfusion of ischaemically damaged porcine kidneys with autologous, allogeneic porcine and human red blood cells. *PLoS One*. 2020;15:e0229566.

142. Pool MBF, Vos J, Eijken M, et al. Treating Ischemically Damaged Porcine Kidneys with Human Bone Marrow- and Adipose Tissue-Derived Mesenchymal Stromal Cells During Ex Vivo Normothermic Machine Perfusion. *Stem Cells Dev*. 2020;29:1320-1330.

143. Jochmans I, Nicholson ML, Hosgood SA. Kidney perfusion: some like it hot others prefer to keep it cool. *Curr Opin Organ Transplant*. 2017;22:260-266.

144. Hosgood SA, Thompson E, Moore T, Wilson CH, Nicholson ML. Normothermic machine perfusion for the assessment and transplantation of declined human kidneys from donation after circulatory death donors. *Br J Surg*. 2018;105:388-394.

145. Hosgood SA, Nicholson ML. The first clinical case of intermediate ex vivo normothermic perfusion in renal transplantation. *Am J Transplant*. 2014;14:1690-1692.

146. Kaths JM, Echeverri J, Chun YM, et al. Continuous Normothermic Ex Vivo Kidney Perfusion Improves Graft Function in Donation After Circulatory Death Pig Kidney Transplantation. *Transplantation*. 2017;101:754-763.

147. Dumbill R. Investigation of the safety and feasibility of preservation of kidneys for up to 24 hours at normal body temperature prior to transplant. Available at: https://doi.org/10.1186/ISRCTN1329227720 21.

148. Grant D. The Feasibility and Safety of Normothermic ex Vivo Kidney Perfusion (NEVKP). Available at: https://clinicaltrials.gov/ct2/show/study/NCT 031368482020.

149. Alwayn IP. PROlonged Ex-vivo Normothermic Machine PERfusion for Kidney Regeneration (PROPER). Available at: https://clinicaltrials.gov/ct2/show/NCT04693 3252021.

150. Hosgood SA, Nicholson ML. Hydrogen sulphide ameliorates ischaemia-reperfusion injury in an experimental model of non-heart-beating donor kidney transplantation. *Br J Surg*. 2010;97:202-209.

151. Maassen H, Hendriks KDW, Venema LH, et al. Hydrogen sulphide-induced hypometabolism in human-sized porcine kidneys. *PLoS One*. 2019;14:e0225152.

152. Bagul A, Hosgood SA, Kaushik M, Nicholson ML. Carbon monoxide protects against ischemia-reperfusion injury in an experimental model of controlled nonheartbeating donor kidney. *Transplantation*. 2008;85:576-581.

153. Smith SF, Adams T, Hosgood SA, Nicholson ML. The administration of argon during ex vivo normothermic perfusion in an experimental model of kidney ischemia-reperfusion injury. *J Surg Res*. 2017;218:202-208.

154. Tietjen GT, Hosgood SA, DiRito J, et al. Nanoparticle targeting to the endothelium during normothermic machine perfusion of human kidneys. *Sci Transl Med*. 2017;9:10.1126/scitranslmed.aam6764.

155. DiRito JR, Hosgood SA, Reschke M, et al. Lysis of cold-storage-induced microvascular obstructions for ex vivo revitalization of marginal human kidneys. *Am J Transplant*. 2021;21:161-173.

156. Woud WW, Merino A, Hoogduijn MJ, et al. Nanoparticle Release by Extended Criteria Donor Kidneys During Normothermic Machine Perfusion. *Transplantation*. 2019;103:e110-e111.

157. Yuzefovych Y, Valdivia E, Rong S, et al. Genetic Engineering of the Kidney to Permanently Silence MHC Transcripts During ex vivo Organ Perfusion. *Front Immunol*. 2020;11:265.

158. Rogers NM, Zhang ZJ, Wang JJ, Thomson AW, Isenberg JS. CD47 regulates renal tubular epithelial cell self-renewal and proliferation following renal ischemia reperfusion. *Kidney Int*. 2016;90:334-347.

159. Brasile L, Henry N, Orlando G, Stubenitsky B. Potentiating Renal Regeneration Using Mesenchymal Stem Cells. *Transplantation*. 2019;103:307-313.

160. Thompson ER, Bates L, Ibrahim IK, et al. Novel delivery of cellular therapy to reduce ischemia reperfusion injury in kidney transplantation. *Am J Transplant*. 2020.

161. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation*. 2018;102:35-43.

162. Morigi M, Benigni A. Mesenchymal stem cells and kidney repair. *Nephrol Dial Transplant*. 2013;28:788-793.

163. Furuichi K, Shintani H, Sakai Y, et al. Effects of adipose-derived mesenchymal cells on ischemia-reperfusion injury in kidney. *Clin Exp Nephrol*. 2012;16:679-689.

164. Togel F, Zhang P, Hu Z, Westenfelder C. VEGF is a mediator of the renoprotective effects of multipotent marrow stromal cells in acute kidney injury. *J Cell Mol Med*. 2009;13:2109-2114.

165. Beyer Nardi N, da Silva Meirelles L. Mesenchymal stem cells: isolation, in vitro expansion and characterization. *Handb Exp Pharmacol*. 2006;(174):249-282.

166. Pittenger MF, Discher DE, Péault BM, Phinney DG, Hare JM, Caplan AI. Mesenchymal stem cell perspective: cell biology to clinical progress. *NPJ Regen Med*. 2019;4:22-6. eCollection 2019.

167. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315-317.

168. Jacobs SA, Roobrouck VD, Verfaillie CM, Van Gool SW. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol Cell Biol*. 2013;91:32-39.

169. Wilson A, Hodgson-Garms M, Frith JE, Genever P. Multiplicity of Mesenchymal Stromal Cells: Finding the Right Route to Therapy. *Front Immunol*. 2019;10:1112.

170. Mattar P, Bieback K. Comparing the Immunomodulatory Properties of Bone Marrow, Adipose Tissue, and Birth-Associated Tissue Mesenchymal Stromal Cells. *Front Immunol*. 2015;6:560.

171. Najar M, Raicevic G, Fayyad-Kazan H, et al. Impact of different mesenchymal stromal cell types on T-cell activation, proliferation and migration. *Int Immunopharmacol*. 2013;15:693-702.

172. Kusuma GD, Carthew J, Lim R, Frith JE. Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect. *Stem Cells Dev*. 2017;26:617-631.

173. Tsuji K, Kitamura S, Wada J. Secretomes from Mesenchymal Stem Cells against Acute Kidney Injury: Possible Heterogeneity. *Stem Cells Int*. 2018;2018:8693137.

174. Hass R, Kasper C, Böhm S, Jacobs R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal*. 2011;9:12-12.

175. Klingemann H, Matzilevich D, Marchand J. Mesenchymal Stem Cells - Sources and Clinical Applications. *Transfus Med Hemother*. 2008;35:272-277.

176. Melief SM, Zwaginga JJ, Fibbe WE, Roelofs H. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. *Stem Cells Transl Med*. 2013;2:455-463.

177. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*. 2002;13:4279-4295.

178. Czapla J, Matuszczak S, Kulik K, et al. The effect of culture media on large-scale expansion and characteristic of adipose tissue-derived mesenchymal stromal cells. *Stem Cell Res Ther*. 2019;10:235-9.

179. Erpicum P, Weekers L, Detry O, et al. Infusion of third-party mesenchymal stromal cells after kidney transplantation: a phase I-II, open-label, clinical study. *Kidney Int*. 2019;95:693-707.

180. Liu M, Lei H, Dong P, et al. Adipose-Derived Mesenchymal Stem Cells from the Elderly Exhibit Decreased Migration and Differentiation Abilities with Senescent Properties. *Cell Transplant*. 2017;26:1505-1519.

181. Stolzing A, Jones E, McGonagle D, Scutt A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev*. 2008;129:163-173.

182. Rando TA. Stem cells, ageing and the quest for immortality. *Nature*. 2006;441:1080-1086.

183. Beane OS, Fonseca VC, Cooper LL, Koren G, Darling EM. Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. *PLoS One*. 2014;9:e115963.

184. Galipeau J, Sensébé L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell*. 2018;22:824-833.

185. Sierra Parraga JM, Rozenberg K, Eijken M, et al. Effects of Normothermic Machine Perfusion Conditions on Mesenchymal Stromal Cells. *Front Immunol*. 2019;10:765.

186. Hoogduijn MJ, de Witte SF, Luk F, et al. Effects of Freeze-Thawing and Intravenous Infusion on Mesenchymal Stromal Cell Gene Expression. *Stem Cells Dev*. 2016;25:586-597.

187. Chinnadurai R, Garcia MA, Sakurai Y, et al. Actin cytoskeletal disruption following cryopreservation alters the biodistribution of

human mesenchymal stromal cells in vivo. *Stem Cell Reports*. 2014;3:60-72.

188. Reinders ME, Dreyer GJ, Bank JR, et al. Safety of allogeneic bone marrow derived mesenchymal stromal cell therapy in renal transplant recipients: the neptune study. *J Transl Med*. 2015;13:344-0.

189. Reinders ME, Leuning DG, de Fijter JW, Hoogduijn MJ, Rabelink TJ. Mesenchymal stromal cell therapy for cardio renal disorders. *Curr Pharm Des*. 2014;20:2412-2429.

190. Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int*. 2006;70:121-129.

191. Ueno T, Nakashima A, Doi S, et al. Mesenchymal stem cells ameliorate experimental peritoneal fibrosis by suppressing inflammation and inhibiting TGF-beta1 signaling. *Kidney Int*. 2013;84:297-307.

192. Chen YT, Sun CK, Lin YC, et al. Adipose-derived mesenchymal stem cell protects kidneys against ischemia-reperfusion injury through suppressing oxidative stress and inflammatory reaction. *J Transl Med*. 2011;9:51-51.

193. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol*. 2014;5:148.

194. Chen C, Hou J. Mesenchymal stem cell-based therapy in kidney transplantation. *Stem Cell Res Ther*. 2016;7:16-6.

195. Perico N, Casiraghi F, Remuzzi G. Clinical Translation of Mesenchymal Stromal Cell Therapies in Nephrology. *J Am Soc Nephrol*. 2018;29:362-375.

196. Yagi H, Soto-Gutierrez A, Parekkadan B, et al. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplant*. 2010;19:667-679.

197. English K, Barry FP, Mahon BP. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Lett*. 2008;115:50-58.

198. Chiesa S, Morbelli S, Morando S, et al. Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells. *Proc Natl Acad Sci U S A*. 2011;108:17384-17389.

199. Németh K, Leelahavanichkul A, Yuen PS, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*. 2009;15:42-49.

200. Chiossone L, Conte R, Spaggiari GM, et al. Mesenchymal Stromal Cells Induce Peculiar

Alternatively Activated Macrophages Capable of Dampening Both Innate and Adaptive Immune Responses. *Stem Cells*. 2016;34:1909-1921.

201. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. *Exp Hematol*. 2009;37:1445-1453.

202. Souidi N, Stolk M, Seifert M. Ischemia-reperfusion injury: beneficial effects of mesenchymal stromal cells. *Curr Opin Organ Transplant*. 2013;18:34-43.

203. Mougiakakos D, Jitschin R, Johansson CC, Okita R, Kiessling R, Le Blanc K. The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stem cells. *Blood*. 2011;117:4826-4835.

204. Chen S, Chen L, Wu X, et al. Ischemia postconditioning and mesenchymal stem cells engraftment synergistically attenuate ischemia reperfusion-induced lung injury in rats. *J Surg Res*. 2012;178:81-91.

205. Sun CK, Yen CH, Lin YC, et al. Autologous transplantation of adipose-derived mesenchymal stem cells markedly reduced acute ischemia-reperfusion lung injury in a rodent model. *J Transl Med*. 2011;9:118-118.

206. Du T, Cheng J, Zhong L, et al. The alleviation of acute and chronic kidney injury by human Wharton's jelly-derived mesenchymal stromal cells triggered by ischemia-reperfusion injury via an endocrine mechanism. *Cytotherapy*. 2012;14:1215-1227.

207. Zhang ZH, Zhu W, Ren HZ, et al. Mesenchymal stem cells increase expression of heme oxygenase-1 leading to anti-inflammatory activity in treatment of acute liver failure. *Stem Cell Res Ther*. 2017;8:70-3.

208. Raffaghello L, Bianchi G, Bertolotto M, et al. Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells*. 2008;26:151-162.

209. Hara Y, Stolk M, Ringe J, et al. In vivo effect of bone marrow-derived mesenchymal stem cells in a rat kidney transplantation model with prolonged cold ischemia. *Transpl Int*. 2011;24:1112-1123.

210. Sierra-Parraga JM, Merino A, Eijken M, et al. Reparative effect of mesenchymal stromal cells on endothelial cells after hypoxic and inflammatory injury. *Stem Cell Res Ther*. 2020;11:352-3.

211. Tao H, Han Z, Han ZC, Li Z. Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. *Stem Cells Int*. 2016;2016:1314709.

212. Madrigal M, Rao KS, Riordan NH. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med*. 2014;12:260-8.

213. Burlacu A, Grigorescu G, Rosca AM, Preda MB, Simionescu M. Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis in vitro. *Stem Cells Dev*. 2013;22:643-653.

214. Zacharek A, Chen J, Cui X, et al. Angiopoietin1/Tie2 and VEGF/Flk1 induced by MSC treatment amplifies angiogenesis and vascular stabilization after stroke. *J Cereb Blood Flow Metab*. 2007;27:1684-1691.

215. Qi Y, Jiang D, Sindrilaru A, et al. TSG-6 released from intradermally injected mesenchymal stem cells accelerates wound healing and reduces tissue fibrosis in murine full-thickness skin wounds. *J Invest Dermatol*. 2014;134:526-537.

216. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells*. 2007;25:2648-2659.

217. Lee DE, Ayoub N, Agrawal DK. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. *Stem Cell Res Ther*. 2016;7:37-6.

218. Baulier E, Favreau F, Le Corf A, et al. Amniotic fluid-derived mesenchymal stem cells prevent fibrosis and preserve renal function in a preclinical porcine model of kidney transplantation. *Stem Cells Transl Med*. 2014;3:809-820.

219. Franquesa M, Herrero E, Torras J, et al. Mesenchymal stem cell therapy prevents interstitial fibrosis and tubular atrophy in a rat kidney allograft model. *Stem Cells Dev*. 2012;21:3125-3135.

220. Peired AJ, Sisti A, Romagnani P. Mesenchymal Stem Cell-Based Therapy for Kidney Disease: A Review of Clinical Evidence. *Stem Cells Int*. 2016;2016:4798639.

221. Tögel F, Isaac J, Hu Z, Weiss K, Westenfelder C. Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. *Kidney Int*. 2005;67:1772-1784.

222. Rüster B, Göttig S, Ludwig RJ, et al. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood*. 2006;108:3938-3944.

223. Chamberlain G, Smith H, Rainger GE, Middleton J. Mesenchymal stem cells exhibit firm adhesion, crawling, spreading and

transmigration across aortic endothelial cells: effects of chemokines and shear. *PLoS One*. 2011;6:e25663.

224. Sierra-Parraga JM, Munk A, Andersen C, et al. Mesenchymal Stromal Cells Are Retained in the Porcine Renal Cortex Independently of Their Metabolic State After Renal Intra-Arterial Infusion. *Stem Cells Dev*. 2019;28:1224-1235.

225. Franquesa M, Hoogduijn MJ, Reinders ME, et al. Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: lessons learned from first clinical trials. *Transplantation*. 2013;96:234-238.

226. Lalu MM, McIntyre L, Pugliese C, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One*. 2012;7:e47559.

227. Thompson M, Mei SHJ, Wolfe D, et al. Cell therapy with intravascular administration of mesenchymal stromal cells continues to appear safe: An updated systematic review and meta-analysis. *EClinicalMedicine*. 2020;19:100249.

228. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol*. 2012;3:297.

229. de Witte, S. F. H., Luk F, Sierra Parraga JM, et al. Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. *Stem Cells*. 2018;36:602-615.

230. Saat TC, van den Engel S, Bijman-Lachger W, et al. Fate and Effect of Intravenously Infused Mesenchymal Stem Cells in a Mouse Model of Hepatic Ischemia Reperfusion Injury and Resection. *Stem Cells Int*. 2016;2016:5761487.

231. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. *Stem Cells Dev*. 2013;22:2825-2835.

232. Moghadasali R, Azarnia M, Hajinasrollah M, et al. Intra-renal arterial injection of autologous bone marrow mesenchymal stromal cells ameliorates cisplatin-induced acute kidney injury in a rhesus Macaque mulatta monkey model. *Cytotherapy*. 2014;16:734-749.

233. Zonta S, De Martino M, Bedino G, et al. Which is the most suitable and effective route of administration for mesenchymal stem cell-based immunomodulation therapy in experimental kidney transplantation: endovenous or arterial? *Transplant Proc*. 2010;42:1336-1340.

234. Eirin A, Zhu XY, Krier JD, et al. Adipose tissue-derived mesenchymal stem cells improve revascularization outcomes to restore renal function in swine atherosclerotic renal artery stenosis. *Stem Cells*. 2012;30:1030-1041.

235. Eirin A, Zhu XY, Ferguson CM, et al. Intra-renal delivery of mesenchymal stem cells attenuates myocardial injury after reversal of hypertension in porcine renovascular disease. *Stem Cell Res Ther*. 2015;6:7.

236. Lee KW, Kim TM, Kim KS, et al. Renal Ischemia-Reperfusion Injury in a Diabetic Monkey Model and Therapeutic Testing of Human Bone Marrow-Derived Mesenchymal Stem Cells. *J Diabetes Res*. 2018;2018:5182606.

APPENDIX

Table S1.1. Commercially available and reported hypothermic machine perfusion and normothermic machine perfusion devices and features.

Preservation strategy	Device	CE marked	Pump	Perfusion	Operating temperature	Studies
HMP	Kidney Assist Transporter, XVIVO	Yes	Centrifugal	Pulsatile and pressure controlled	4–10ºC	Clinical and experimental
	Lifeport Kidney Transporter, Organ Recovery systems	Yes	Roller	Pulsatile and pressure controlled	4–8ºC	Clinical and experimental
	Waves, Institute George Lopez, Waters Medical System	Yes	Roller	Pulsatile and pressure controlled	2–8ºC	Clinical and experimental
	RM3 Kidney Perfusion System, Waters Medical System	Yes	Roller	Pulsatile and pressure controlled	3–10ºC	Clinical and experimental
NMP	Kidney Assist, XVIVO	Yes	Centrifugal	Pulsatile and pressure controlled	10–38ºC	Clinical and experimental
	NMP transportable prototype, OrganOx Limited	No	Centrifugal	Continuous and pressure controlled	—	Experimental
	In-house NMP device, Cambridge, UK	No	Centrifugal	Continuous and pressure controlled	—	Clinical and experimental
	In-house NMP device, Toronto, Canada	No	Centrifugal	Continuous and pressure controlled	—	Experimental

HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

Chapter 2

Aims and outline of the thesis

2.1 THE MEPEP CONSORTIUM

This project is part of the work taken on by the MePEP Consortium sponsored by the Danish Lundbeck Foundation. MePEP stands for kidney transplant regeneration with **Me**senchymal stem cells in normothermic *ex situ* **PE**rfusion in **P**igs. MePEP is a collaboration between Aarhus University in Denmark, Erasmus University Rotterdam in The Netherlands and Oxford University in the United Kingdom. This collaboration aimed to explore (i) the effect of normothermic machine perfusion (NMP) on pig kidneys injured by warm ischaemia, simulating the donation after circulatory death donor (DCD), and (ii) the additional effect of administration of pig mesenchymal stromal cells (MSCs) to the injured pig kidney while perfused on NMP. Rotterdam focused on MSCs preparation and optimisation. Oxford established a sustainable and reproducible NMP pig kidney model, where MSCs were infused to evaluate the behaviour and interaction of MSCs with the injured kidney. Subsequently, in the next stage, the Rotterdam and Oxford experience translated to NMP and NMP with administration of MSCs in an auto-transplantation model of pig kidneys in Aarhus.

2.2 AIM AND OUTLINE OF THE THESIS

This thesis concerns work how to optimise the conditions and perfusate of NMP of the donor pig kidney and study the duration of NMP needed to allow homing and initiation of biological effects of infused MSCs on the donor pig kidney during NMP. Therefore, we optimised and developed a reproducible slaughterhouse pig kidney model of prolonged NMP to successfully administer MSCs which is detailed in **Chapter 3**. Here we mainly focussed on the optimisation of a red blood cell-based perfusion fluid. The optimised perfusate in this chapter was used for the subsequent studies in this thesis. In **Chapter 4,** the need for an oxygen carrier in the perfusion fluid during NMP was studied, where red blood cells, an artificial oxygen carrier (Hemopure® (HBOC-201)) and no oxygen

carrier were compared. **Chapter 5** aimed to determine the optimum MSC dose for administration to the donor pig kidney during NMP and track the location and survival rate of MSCs in the donor kidney throughout perfusion. Furthermore, we investigated the secretion of cytokines and growth factors in the perfusate during NMP with different doses of MSCs. This study was performed in two phases. First we focussed on a dose-response study to identify which MSC dose could be administered without detriment to perfusion and to identify the location and distribution of MSCs in the kidney during NMP. In the second phase, we selected the optimal dose of MSCs and increased the number of kidneys in the group. In **Chapter 6**, the findings from the dose-response study in Chapter 5, together with Rotterdam's findings, were translated into a porcine auto-transplantation model performed in Aarhus. Here the safety of intra-arterial administration of MSCs during NMP was investigated alongside the potential beneficial effects on kidney function after transplantation, with 14 days of follow up. The findings from this thesis are summarised and discussed, with a brief outlook towards future developments in **Chapter 7.**

Chapter 3

Development of normothermic machine perfusion of porcine kidneys using a slaughterhouse model

ABBREVIATIONS

AST	aspartate aminotransferase
AUC	area under the curve
BD	bolus dose
DCD	donation after circulatory death
HI	haemolysis index
HMP	hypothermic machine perfusion
LD	low dose
LDH	lactate dehydrogenase
MAP	mean arterial pressure
MSC	mesenchymal stromal cells
NMP	normothermic machine perfusion
NO	nitric oxide
PBS	phosphate buffered saline
RBC	red blood cell
RT	room temperature
SCS	static cold storage
SD	standard dose
SNP	sodium nitroprusside
UW-MPS	Belzer MPS® UW Machine Perfusion Solution
QAS	quality assessment score

3.1 INTRODUCTION

In the past years, it has been recognised that *ex situ* normothermic machine perfusion (NMP) of donor kidneys after organ retrieval in the context of organ donation and transplantation can help as a diagnostic and therapeutic tool to restore function, assess organ viability and initiate repair.[1-3] This technology has been adopted by groups in the United Kingdom, continental Europe and North America, and is primarily being used as a research tool. Slaughterhouse porcine kidneys have previously been shown to be a suitable model for donation after circulatory death (DCD) and their use avoids the need for laboratory animals.[4] Porcine kidneys are very similar to human kidneys in terms of size, structure and anatomy, and the pig has a similar immune system and physiology. In addition, pigs have been shown to be one of the best large animal models for surgical studies and particularly suitable in kidney transplantation.[5] Furthermore, the use of slaughterhouse pigs in research is also cost-effective and utilises organs and tissues that would otherwise be wasted. Establishing a robust and reproducible model of prolonged NMP using slaughterhouse pig kidneys provides an invaluable tool for translational research. However, from the literature it appears that currently many research groups that are involved in NMP use their own bespoke perfusion techniques, varied perfusion solution compositions and different durations of perfusion.

An important variation applied in NMP studies is the type of NMP circuit. Previous studies showed that both open and closed circuits can be used during NMP.[6-9] In the open circuit, blood returning from the kidney via the renal vein drains directly into a reservoir without any resistance. This circuit is straightforward to assemble in comparison with the closed circuit. In contrast to the open circuit, in the closed circuit the renal vein is cannulated and the blood remains in the circuit under constant positive pressure which might be more physiological. Another key variant is the perfusate composition. The intended duration of NMP is tightly linked with the perfusate composition; a prolonged period

(i.e. >1 hour) to assess and condition the donor kidney will require a more complex perfusate to allow adequate metabolism than a short period of NMP to merely resuscitate the organ immediately prior to transplantation. The perfusion solution's composition is of vital importance to ensure adequate delivery of nutrients and oxygen, to maintain cellular integrity and vascular processes during perfusion. It is important to keep oncotic pressure and osmolarity of the perfusate within a physiological range. In particular, a low oncotic pressure will result in net movement of water out of the vasculature into the cells leading to oedema.[10] During a recent European transplant meeting first results of the PROPER study, a study comparing three and six hours NMP using a previously published one-hour perfusion protocol against a newly developed protocol containing albumin, demonstrated that for prolonged NMP a modified perfusate was required to allow more stable and physiological levels of pH and electrolytes during perfusion.[11] Similar results were observed in a study by Pool *et al*.[12], who compared different perfusion solutions during seven hours of kidney NMP. The authors concluded that the composition of the perfusion solution, specifically the addition of a colloid and vasodilator, significantly impacted the perfusion characteristics and kidney injury markers during NMP. In addition, when we aimed to use the NMP system as a delivery platform for cellular therapy involving mesenchymal stromal cells (MSC) such as for the MePEP project, we needed to investigate the use of albumin in the perfusion solution, as plasma would additionally interfere with the ability to measure proteins produced by MSC administered during NMP.

Previous work demonstrated that the addition of a vasodilator during the NMP of DCD livers was vital for optimal microcirculatory support.[13] It appeared that sodium nitroprusside (SNP) and verapamil were both good options of vasodilators during kidney NMP. [7, 14, 15] Also, the majority of experimental kidney NMP models have included the addition of an antibiotic to the perfusion circuit[8, 9, 15, 16] although, the only ongoing randomised controlled trial of human kidney NMP for one hour does not include the

addition of antibiotics to its perfusion circuit.[6] However, as we intended to use slaughterhouse animals and for a prolonged perfusion, the addition of antibiotics during NMP was considered to be necessary. The pharmacology of antibiotics during NMP is currently unknown, and large doses could be nephrotoxic in an isolated circuit in the absence of breakdown by the liver.[17] It is also important to consider the effect antibiotic doses may have on MSCs. *In vitro* studies by Sierra-Parraga *et al.* (personal communication) showed that standard dose antibiotic concentration of 1000 mg/200 mg amoxicillin-clavulanate, used by other groups during NMP, reduced the viability of MSCs by 50% after 30 minutes suspension of the cells in the perfusion solution. When the concentration of amoxicillin-clavulanate was diluted 100x the MSC viability increased to approximately 90%.

The aim of the work presented in this chapter was to optimise a reproducible slaughterhouse pig kidney model of prolonged NMP to allow successful administration of porcine MSC.

The objectives in our pursuit of developing an optimised kidney perfusion model were:

- To compare the effects of the open versus the closed-circuit configuration in NMP on pig kidney perfusion;
- To develop a red cell based and albumin-based perfusion solution for prolonged slaughterhouse pig kidney perfusion
    - Comparing the effect of the use of a vasodilator and no vasodilator on pig kidney perfusion;
    - Assessing the optimal dose of antibiotics to support prolonged slaughterhouse pig kidney perfusion without excessive microbial contamination while avoiding toxicity.

3.2 MATERIAL AND METHODS

The evolution of the NMP technique in this chapter describes both pre-planned sub-studies and changes made, based on experience gained during the process. In total we performed a series of 55 NMP experiments to develop reproducible NMP conditions for pig kidneys retrieved from a local abattoir and of these 44 NMP experiments are included in this chapter.

3.2.1 Porcine DCD kidney retrieval

Porcine kidneys and autologous pig blood were obtained from a local abattoir. Pigs were terminated following standard Home Office procedures which, in brief, involved stunning with a bi-temporal electric shock, followed by rapid exsanguination and then dehairing in a hot wash at 60°C for five minutes. Autologous blood was collected in a heparinised container (20,000 IU, Wockhardt, Wrexham, United Kingdom) and both kidneys were immediately retrieved including vessels and ureter. Following retrieval, kidneys were flushed with Marshall hypertonic citrate solution (Soltran, Baxter Healthcare, Thetford, United Kingdom) supplemented with 5,000 IU of heparin. Warm ischaemia time was calculated from the time of start of exsanguination to the time of cold flush and was 16 ± 2 minutes (mean ± standard deviation, n=38).

3.2.2 Initiation of pig kidney NMP

3.2.2.1 Static cold storage

The first series of experiments conducted (n=4) were designed to establish a protocol that enabled the successful NMP of pig kidneys from the abattoir. Once retrieved and flushed, kidneys were transported to the laboratory using static cold storage (SCS) to reduce metabolism and maintain viability of the donor kidneys, with both kidneys submerged in Marshall's hypertonic citrate solution (Baxter Healthcare) in an organ bag, and placed on melting ice in a Styrofoam box to ensure a temperature of 0–4°C. After

arrival at the laboratory the NMP perfusion solution was prepared and the kidney was connected to the NMP device (described in paragraph 3.2.4.1) and perfusion commenced. Perfusion parameters including flow and renal resistance from these early experiments were very variable and the kidneys did not perfuse at all due to apparent vasospasm and prohibitively high renal resistance. As a result of these failures, the experimental plan was altered to include a short period of hypothermic machine perfusion (HMP) following the period of SCS. This idea was revisited from the clinical experience described by Belzer *et al.* in the 70s[18, 19] that the perfusion pressure set at the beginning of HMP would help to relax the vasculature of the donor kidney reducing renal resistance and allowing a stable flow during HMP prior to the start of subsequent NMP.

3.2.2.2 Hypothermic machine perfusion

To allow adequate HMP of the pig donor kidney the Kidney Assist device (XVIVO, Groningen, the Netherlands) was used. The circuit was filled and primed with 500 mL cold Belzer MPS® UW Machine Perfusion Solution (UW-MPS, Bridge to Life Ltd., London, United Kingdom). The kidney was prepared so that the renal artery, vein and ureter were identifiable. The arterial cannula was filled with UW-MPS and it was closed with a clamp to prevent entry of air bubbles. The renal artery was then cannulated and secured with a 3.0 ligature. The kidney was connected to the perfusion circuit and perfusion was commenced at a mean arterial pressure (MAP) of 25 mmHg for a median duration of 1.5 hours. Unlike with SCS alone, 1.5 hours of HMP reduced the renal resistance significantly overcoming vascular spasm and led to a concomitant increase in renal blood flow which permitted subsequent NMP. Despite all efforts, we were not able to ever reach the target temperature during HMP < 10°C as described by the manufacturer. In our experiments we attempted to insulate and cool the system by putting the organ chamber in a Styrofoam box filled with ice and water, reaching a mean temperature during HMP of 14 ± 2°C. (n=10, Figure 3.1). Our finding that it was difficult

to further reduce the temperature below 10°C was confirmed by colleagues who had the same experience at the Surgical Research Laboratory at the University of Groningen, the Netherlands (personal communication).

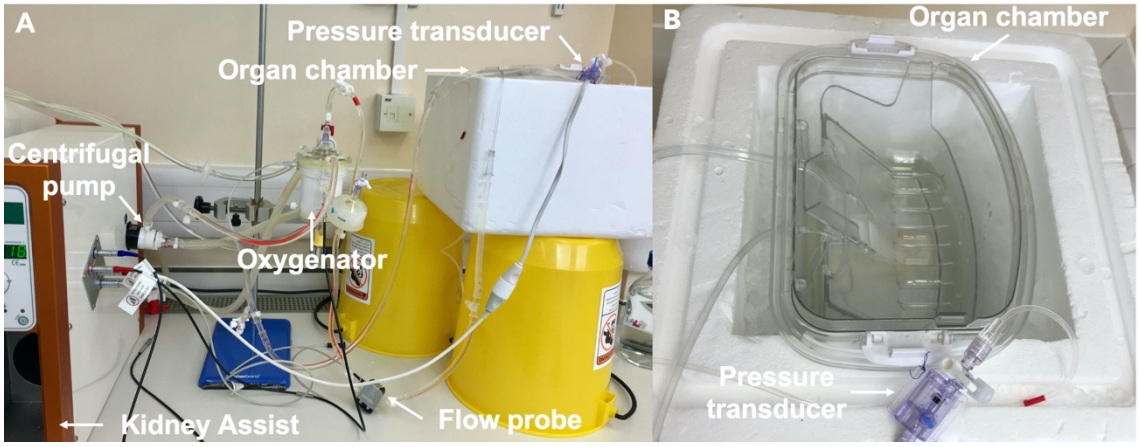


Figure 3.1. Self-assembled hypothermic machine perfusion circuit. (A) The self-assembled HMP circuit contained an oxygenator, centrifugal pump, pressure sensor, flow probe and an organ chamber. (B) The organ chamber was placed in a Styrofoam box and submerged in ice and water.

Following the acquisition of the portable HMP device (Kidney Assist Transport, XVIVO, Groningen, the Netherlands) all kidneys were placed on HMP (n=30) following retrieval at the abattoir (Figure 3.2A). The portable HMP circuit was filled with 500 mL of cold UW-MPS and was set up at a MAP of 25 mmHg. The artery was prepared for connection to the HMP device by putting the aortic patch in the patch holder (XVIVO) and subsequently placing the patch holder in the kidney holder (XVIVO) (Figure 3.2B and 3.2C). Temperature was maintained below 4°C and renal blood flow and renal resistance were favourable for subsequent NMP. Based on these observations all kidneys in subsequent experiments underwent HMP following retrieval unless stated otherwise. Total cold storage time was 4.50 ± 0.75 hours (mean ± standard deviation, n=38 perfusions).

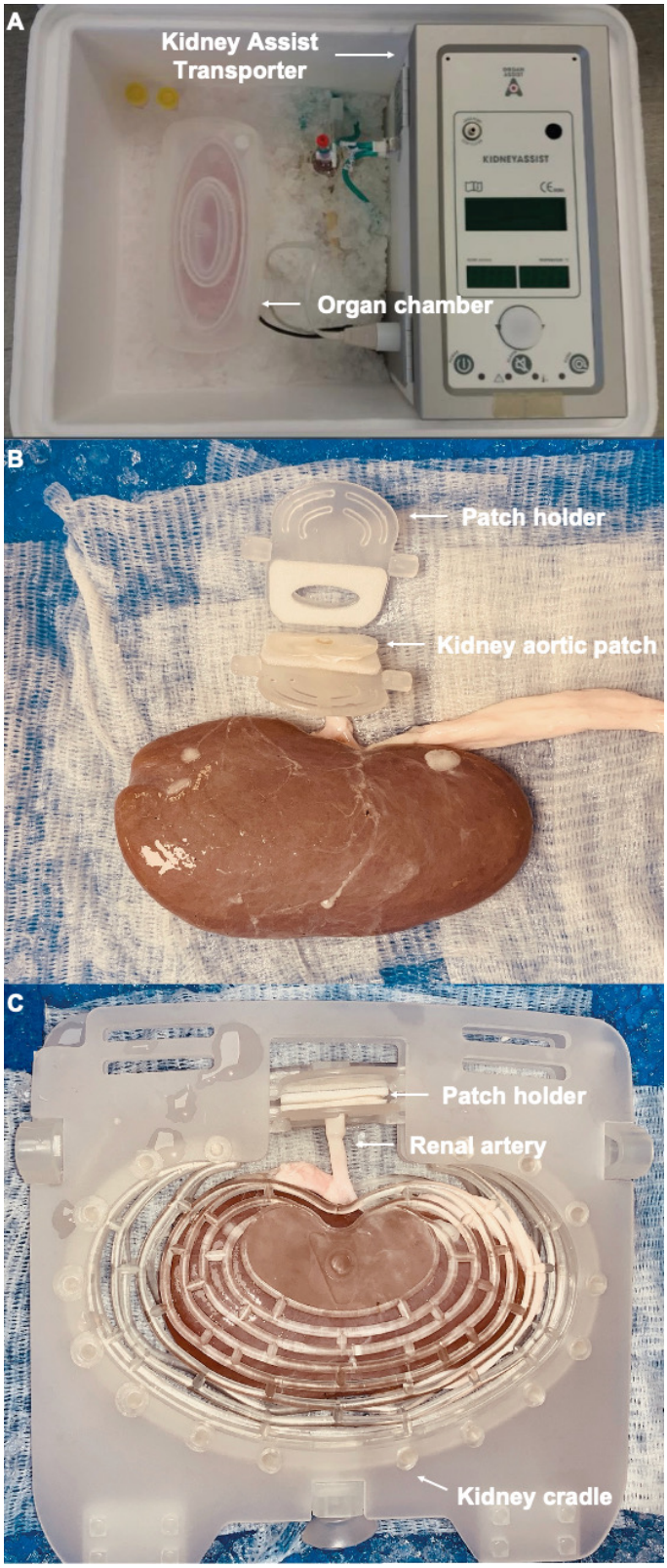


Figure 3.2. Portable hypothermic Machine Perfusion device. (A) Kidney within the organ cradle, connected to the Kidney Assist device and surrounded by crushed ice. (B) Patch holder connected to the aortic patch of the kidney. (C) Placement of kidney with the patch holder in the kidney cradle.

3.2.3 Normothermic machine perfusion

3.2.3.1 Open circuit set-up

In an open circuit set-up the renal artery is cannulated and blood outflow occurs passively via the renal vein that is not cannulated, and drains directly into a reservoir without resistance (Figure 3.3). In the system we used, kidneys were perfused using a pressure-controlled system provided by the Kidney Assist device (XVIVO, Groningen, NL). The circuit included an oxygenator (Hilte Lt 2500, Medos Medizintechnik AG, Stolberg, Germany), a centrifugal pump (DP3, Medos Medizintechnik AG) and a Life Port® organ chamber (Organ Recovery Systems, Illinois, USA). Flow was monitored using an ultrasonic clamp-on flow probe (Em-tec-Gmbh, Finning, Germany). Pressure was measured at the same height as the renal artery using a pressure transducer (TruWave disposable pressure transducer, Edwards Lifesciences, Irvine, California, USA).

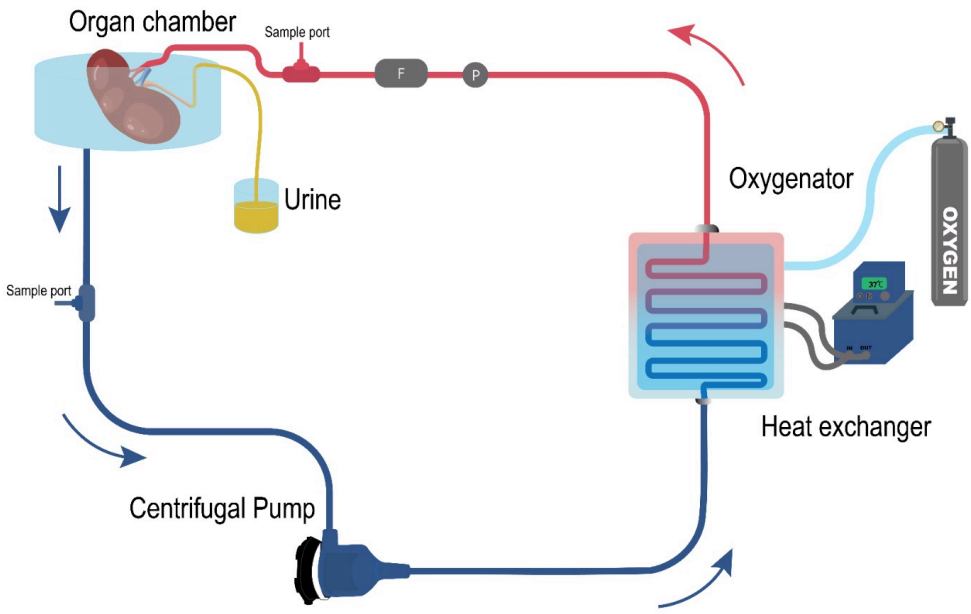


Figure 3.3. Illustration of the open normothermic machine perfusion circuit setup. In the open circuit the renal artery is cannulated and the venous outflow drains directly from the renal vein into the well of an organ chamber. Perfusate then drains from the reservoir to the centrifugal pump and is pumped through the oxygenator, where the perfusate is oxygenated and warmed at 37°C, and driven with a pressure of 70 mmHg through the renal artery in the kidney. F, flow sensor; P, pressure transducer.

After HMP, back-table preparation of the kidney and priming of the circuit were carried out in parallel. The arterial cannula (12-F) was filled with cold normal saline (0.9% sodium chloride, Baxter Healthcare) and it was closed with a clamp to prevent entry of air bubbles. The renal artery was then cannulated and secured with a 3.0 ligature. The ureter was cannulated with an 8-F cannula (Figure 3.4 A and 3.4B). The perfusion circuit was primed with either a leucocyte depleted blood solution or a version of the RBC-Albumin based perfusate described in paragraph 3.2.3.3 and 3.2.3.4. The open circuit can be primed in two different ways: (i) by adding the perfusate in the organ chamber and removing the air from the circuit with a syringe or (ii) by filling the circuit via the oxygenator valve. Perfusate temperature was kept at 37°C and oxygenated with 0.5 L/min carbogen (95% O_2 /5% CO_2, BOC, Didcot, United Kingdom). Next, the kidney was connected to the circuit and perfused for seven hours at a MAP of 70 mmHg. Perfusate and urine samples were taken hourly, centrifuged at 1,800 x g for 12 minutes at 4°C, and the supernatant stored at -80°C for further analysis. Ringer's solution (Fresenius Kabi, Cheshire, United Kingdom) was used to replace the urine output.

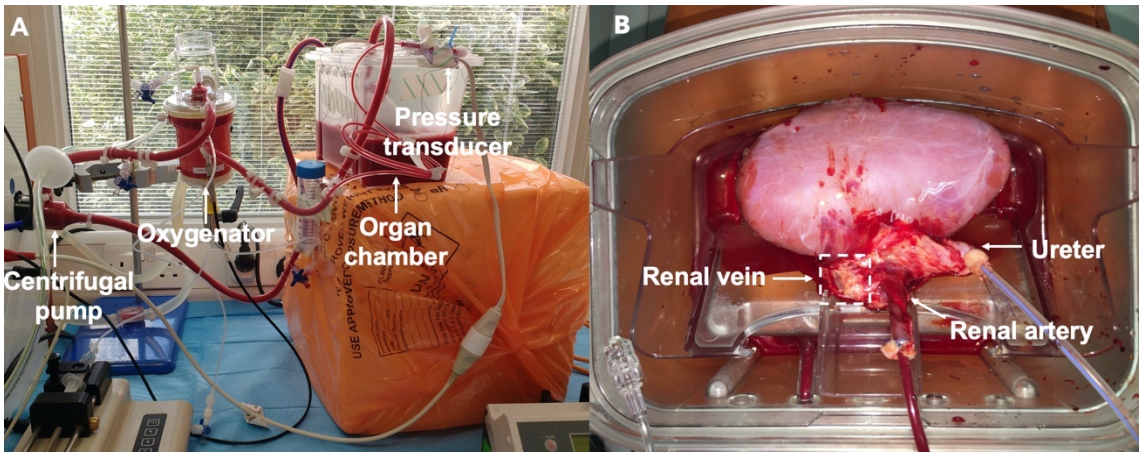


Figure 3.4. Photographic illustrations of the open normothermic machine perfusion circuit setup. In the open circuit the renal artery is cannulated and the venous outflow drains directly from the renal vein into the well of an organ chamber. Perfusate then drains from the reservoir to the centrifugal pump and is pumped through the oxygenator, where the perfusate is oxygenated and warmed at 37°C, and driven with a pressure of 70 mmHg through the renal artery in the kidney. (A) Photographic image showing the open circuit setup including the oxygenator, centrifugal pump, pressure transducer and organ chamber. (C) Photographic image of a pig kidney within an organ chamber, undergoing NMP. The renal artery and ureter are cannulated and perfusate from the renal vein is draining directly into the organ chamber.

3.2.3.2 Closed circuit set-up

In the closed circuit system the renal artery and the renal vein are both cannulated and the blood or perfusate remains within a chamber that is connected directly to both arterial and venous cannulas. In addition to the open circuit system as described in 3.2.3.1, the closed circuit contained a suspended soft shell reservoir (Capiox venous reservoir, Terumo Medical Corporation, Somerset, New Jersey, USA) that was connected directly to the venous cannula (Figure 3.5).

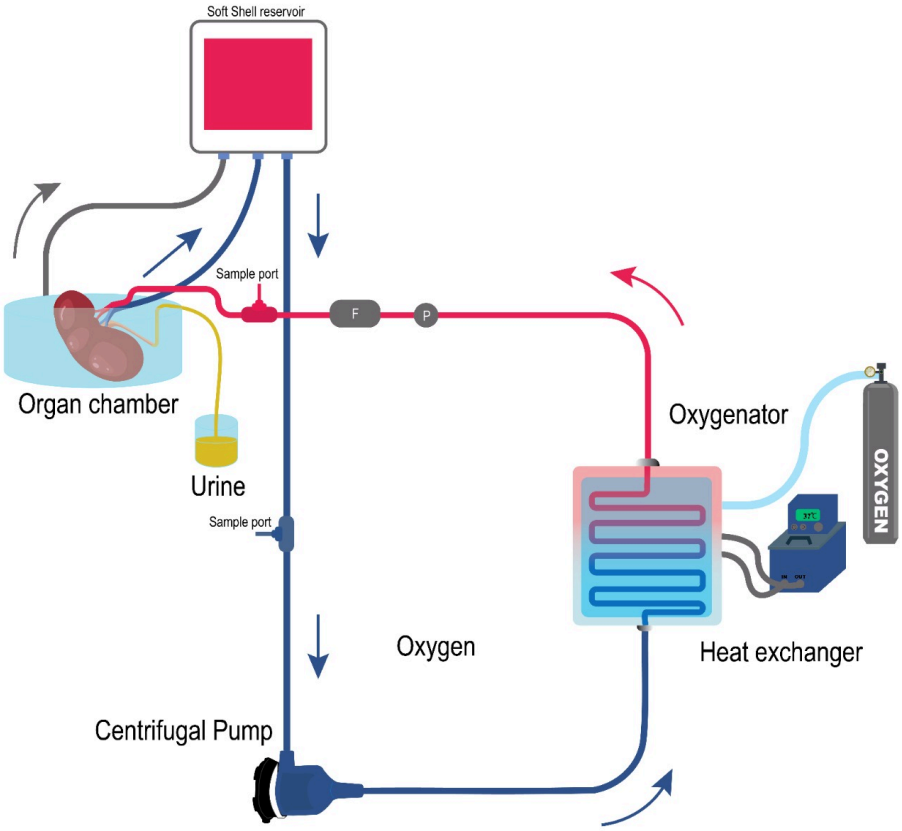


Figure 3.5. Illustration of the closed normothermic machine perfusion circuit set-up. The renal artery and vein are both cannulated in the closed circuit. A centrifugal pump pumps the perfusion solution to the oxygenator where it is oxygenated, warmed to 37°C and driven with a pressure of 70 mmHg through the renal artery of the kidney. The venous outflow drains back to the soft shell reservoir and through gravity is recirculated back in the circuit. F, flow sensor; P, pressure transducer.

During set-up, the arterial tubing and the venous input of the soft shell reservoir was connected with a y-connector to prevent air entering the system and the circuit was primed via the valve on the soft shell reservoir (Figure 3.6A). The renal artery and ureter were cannulated as described in 3.2.3.1 and the renal vein was also cannulated with a 28-F cannula (Figure 3.6B and 3.6C). The kidney was then connected to the circuit (Figure 3.6C) and perfused for seven hours at a MAP of 70 mmHg. Urine and blood samples were collected and processed as mentioned in 3.2.3.1 for further analyses.

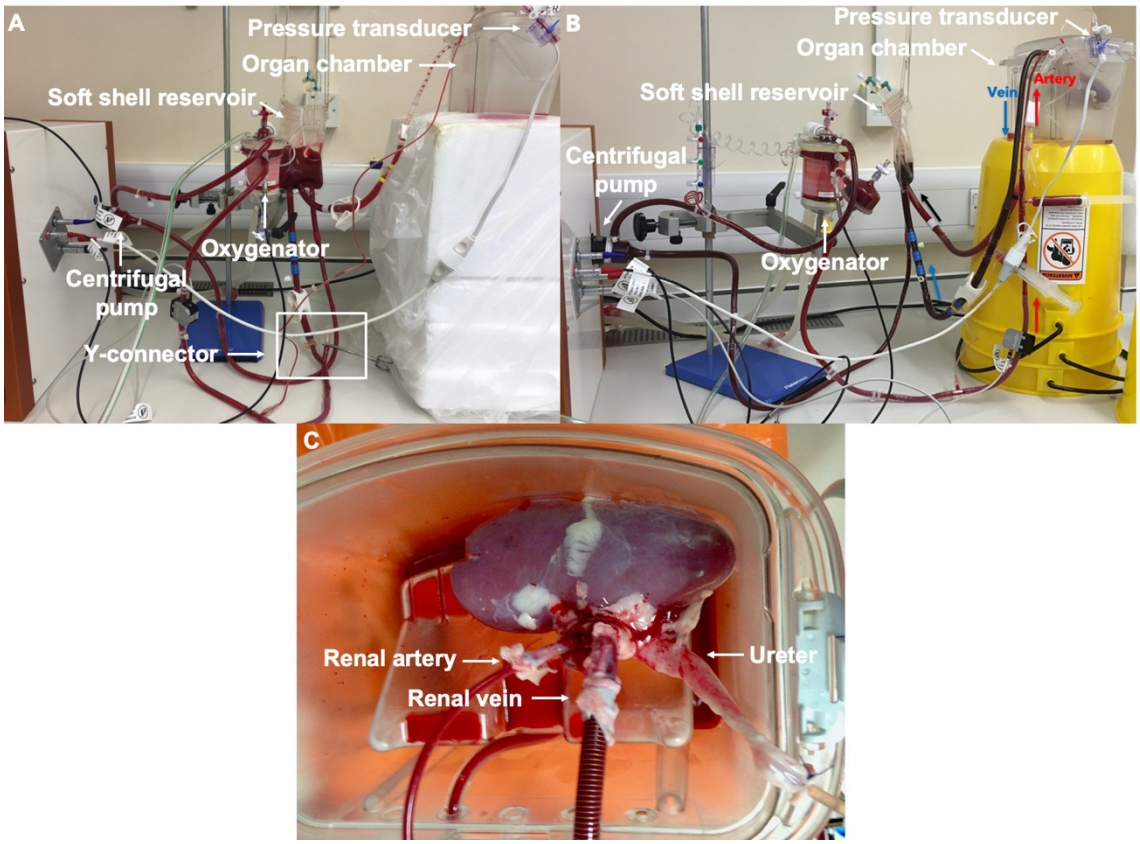


Figure 3.6. Photographic illustrations of the closed normothermic machine perfusion circuit set-up. The renal artery and vein are both cannulated in the closed circuit. A centrifugal pump pumps the perfusion solution to the oxygenator where it is oxygenated, warmed to 37°C and driven with a pressure of 70 mmHg through the renal artery (B, red arrow) of the kidney. The venous outflow drains back to the soft shell reservoir (B, blue and black arrows) and through gravity is recirculated back in the circuit. (A). Photographic image of a primed closed circuit set-up including oxygenator, centrifugal pump, pressure transducer, soft shell reservoir, organ chamber and perfusate circulating prior to connection of a kidney. A Y-connector connects the arterial and venous tubing so the system can be primed. (B) Photographic image of the closed circuit set-up connected with a kidney. (C) Photographic image of a pig kidney undergoing NMP on the closed circuit with artery, vein and ureter cannulated.

3.2.3.3 Leucocyte depleted blood perfusate

A leucocyte depleted blood-based perfusate solution was used during the comparison between open and closed circuits (n=8 kidneys in total). Perfusion in the absence of leucocytes will limit an inflammatory response and vasoconstriction. Furthermore, Harper *et al*. showed that perfusion characteristics and oxygen consumption are superior during NMP of porcine kidneys with leucocyte depleted blood compared to whole blood.[16] The perfusate consisted of 500 mL leucocyte depleted pig blood (autologous blood collected at time of donor kidney retrieval at the abattoir). Whole blood was depleted using a leucocyte filter (LeukoGuard RS filters, Haemonetics Ltd, Coventry, United Kingdom or NPT/LCR2 filters, Macopharma, Tourcoing, France). Additions to the perfusate to create a circulating volume of 750 mL included 200 mL Ringer's solution (Fresenius Kabi), 10 mL 10% (w/v) calcium gluconate (B. Braun, Meslungen, Germany), 10 mg mannitol (Sigma Aldrich, Gillingham, United Kingdom), 80 mg (1000 µmol/L) creatinine (Sigma Aldrich), 8 mg dexamethasone (Hameln Pharmaceuticals Ltd, Gloucester, United Kingdom), 1000 mg/200 mg amoxicillin-clavulanate (Bowmed, Wrexham, United Kingdom), 100 µL 20 mg/mL SNP (Sigma Aldrich), and 10 mL 8.4% (w/v) sodium bicarbonate (B. Braun). Once perfusion was started a nutrition solution of Nutriflex special (B. Braun), 8.4% (w/v) sodium bicarbonate (B. Braun) and 100 IU insulin (Novo Nordisk, Crawley, United Kingdom) was connected to the system and infused at 1.5 mL/h (Table 3.1).

Table 3.1. Components of the leucocyte depleted blood solution and supplements added during NMP.

Leucocyte depleted blood-based perfusate	
Stock solution	
Ingredient	**Amount**
Leucocyte depleted blood	500 mL
Ringer's solution	200 mL
10% calcium gluconate	10 mL
8.4% sodium bicarbonate	10 mL
Amoxicillin-clavulanate	1000 mg/200 mg
Mannitol	10 mg
Creatinine	80 mg (1000 µmol/L)
Dexamethasone	8 mg
Sodium nitroprusside	100 µL 20 mg/mL
Continuous administration during NMP	
Nutrition infusion with the following additions	1.5 mL/h
Nutriflex special (lipid free)	10.5 mL
8.4% sodium bicarbonate	2.5 mL
Insulin	100 IU

3.2.3.4 RBC-Albumin (MePEP) perfusate

To allow the use and administration of MSCs into the perfusate and reach the donor kidney in future studies (Chapters 5–6), we developed a red blood cell-albumin based perfusion solution. Three different versions of MePEP perfusates (Table 3.2) were tested to optimise perfusion conditions including variations of vasodilator and antibiotic dose. The variation of vasodilator and antibiotic are described in more detail in paragraphs 3.2.3.4.1 and 3.2.3.4.2. The MePEP perfusate was an albumin and erythrocyte based solution. The perfusion solution consisted of 250 mL 5% human serum albumin (Alburex, CSL Behring, Lyngby, Denmark) and washed autologous pig red blood cells (RBCs), to acquire a haematocrit of 25–30%. RBCs were isolated as described in paragraph 3.2.3.4.3. Additionally, 6 mL 5% (w/v) glucose (B. Braun), 3 mL 10% (w/v) calcium gluconate (B. Braun), 10 mg mannitol (Sigma Aldrich) and 1000 µmol/L creatinine (Sigma Aldrich) were added to the perfusion solution to achieve a near physiological electrolyte composition. The pH was adjusted to a near physiological value of 7.3 by adding 5–10 mL of 8.4% (w/v) sodium bicarbonate (B Braun). Once perfusion was started a nutrition solution of Nutriflex special (B. Braun), 8.4% (w/v) sodium bicarbonate (B. Braun) and either 100 IU or 5 IU insulin (Novo Nordisk) was connected and infused at 1.5 mL/h.

Table 3.2. Components of the developed RBC-Albumin (MePEP) perfusate.

	MePEP 1.0	MePEP 2.0	MePEP 3.0
Stock solution			
Ingredient	**Amount/Rate**		
RBCs	240 mL	170–190 mL	170–190 mL
5% human serum albumin	240 mL	250 mL	250 mL
Ringers solution	120 mL	15 – 50 mL	—
Sterile water	—	20 – 30 mL	20 mL
10% calcium gluconate	3 mL	3 mL	3 mL
8.4% sodium bicarbonate	10 mL	5–10 mL	5–10 mL
5% glucose	6 mL	6 mL	6 mL
Mannitol	10 mg	10 mg	10 mg
Creatinine	72 mg (1000 µmol/L)	60–67 mg (1000 µmol/L)	52–54 mg (1000 µmol/L)
Insulin	—	—	5 IU
Continuous administration during NMP			
Nutrition infusion with insulin (100 or 5 IU) and 8.4% sodium bicarbonate	1.5 mL/h (n=15)	1.5 mL/h (n=6)	—
Verapamil	—	0.25 mg/h	0.25 mg/h
Other supplements			
	—	Glucose*	Glucose*

*Continuous infusion of 5% glucose was administrated on a rate of 4 mL/h when levels dropped below 4 mmol/L.

3.2.3.4.1 The effect of different vasodilators during kidney NMP

Previous studies[7, 14, 15] had shown that verapamil and SNP were both potentially helpful options as a vasodilator during kidney NMP. Therefore, we set out to compare verapamil (Mylan, Hatfield, United Kingdom) at a continuous infusion of 0.25 mg/h (n=4) with SNP (Sigma Aldrich) at a continuous infusion of 20 mg/h (n=3) versus no vasodilator as control (n=4). After cold flush and HMP, the kidneys were perfused on an open NMP circuit at a MAP of 70 mmHg. The perfusion solution consisted of 250 mL 5% human serum albumin (Alburex, CSL Behring) and washed autologous pig RBCs to obtain a haematocrit of 25–30%. RBCs were isolated as described in paragraph 3.2.3.4.3. Additionally, 6 mL 5% (w/v) glucose (B. Braun), 3 mL 10% (w/v) calcium gluconate (B. Braun), 10 mg mannitol (Sigma Aldrich) and 1000 µmol/L creatinine (Sigma Aldrich) were added to the perfusion solution to achieve a near physiological electrolyte composition. The pH was adjusted to 7.3 with 8.4% (w/v) sodium bicarbonate (5–10 mL, B, Braun). The perfusate was supplemented with a bolus of 60 mg amoxicillin-clavulanate at baseline.

3.2.3.4.2 The effect of antibiotic doses during pig kidney NMP

Kidney NMP experimental models have included an addition of antibiotic in the perfusion circuit. [8, 9, 15, 16] In an isolated circuit without the presence of a liver and normal antibiotic breakdown and excretion, however, conventional doses may be nephrotoxic.[17] Therefore, we wanted to determine whether the antibiotic dose had a detrimental effect on pig kidney perfusion or not. Three different concentrations (n=4 per group) of amoxicillin-clavulanate were compared: 60 mg at baseline (low dose (LD)), 1200 mg at baseline (standard dose (SD)) and 300 mg at baseline with additional bolus doses of 60 mg every hour during NMP (bolus dose (BD)). The BD concentration was determined by the minimum inhibitory concentration (MIC) of amoxicillin-clavulanate measured in the LD group which equalled 256 µg/mL. This dose was selected as it was the highest MIC needed to treat the bacteria cultured in a test sample of perfusion fluid. The calculated starting concentrations of amoxicillin-clavulanate in the LD, SD and BD groups were 158

µg/mL, 3343 µg/mL and 1038 µg/mL respectively. After cold flush and HMP, kidneys were perfused in an open NMP circuit at a MAP of 70 mmHg. The perfusion solution consisted of human serum albumin (Alburex, CSL Behring) and washed autologous pig RBCs with additions described above in paragraph 3.2.3.4.1 to achieve a near physiological electrolyte composition. The perfusion was supplemented with a continuous infusion of 0.25 mg/h verapamil.

Perfusate samples (2mL) were collected throughout the perfusion and sent to Oxford University Hospitals microbiology laboratory for bacterial culture and minimum inhibitory concentration (MIC) value calculation for amoxicillin-clavulanate. MIC is the lowest concentration of the antibiotic that prevents visible growth of a bacterium or bacteria. MIC helps determine if the antibiotic dose is effective and this information can lead to an appropriate choice of an antibiotic dose. [20]

3.2.3.4.3 Red blood cell isolation

Autologous blood was collected in a heparinised container at a local abattoir. To isolate RBCs, the blood was poured into a catheter bag using a funnel and was filtered for leucocytes through a leucocyte filter (LeukoGuard RS filters, Haemonetics Ltd, United Kingdom or NPT/LCR2 filters, Macopharma, France). To remove the plasma component, the filtered blood was centrifuged at 3,000 x g for 20 minutes at room temperature (RT) and the plasma supernatant was removed and discarded. The remaining RBCs were washed with 1x phosphate-buffered saline (PBS) at 3,000 x g for 20 minutes at RT. The PBS was removed with a bladder syringe and remaining RBCs were used for the perfusion solution.

3.2.4 Development of an RBC-Plasma perfusion solution

In an attempt to improve perfusion conditions and achieve prolonged perfusions for more than seven hours, a RBC-plasma based perfusate was developed and tested. After cold flush and HMP, kidneys (n=5) were perfused on an open NMP circuit at 70 mmHg MAP. The perfusate was prepared by centrifuging 1.5 L of heparinised whole blood for 20 minutes at 3,000 x g at RT. The plasma was removed with a bladder syringe and kept separate. RBCs were isolated as described in paragraph 3.2.3.4.3. RBCs were gradually added back to 400 mL of plasma in order to achieve a haematocrit between 25 - 30%. For every 500 mL of RBC-plasma an additional 6.6 mg dexamethasone (Hameln Pharmaceuticals Ltd), 1000 mg/200 mg amoxicillin-clavulanate (Bowmed), 10 mg mannitol and 64 mg (1000 µmol/L) creatinine (Sigma Aldrich) were added. If needed, 5% (w/v) glucose (B. Braun) and 10% (w/v) calcium gluconate (B. Braun) were added to achieve a near physiological electrolyte composition.

3.2.5 Kidney assessment during NMP

Perfusion characteristics including renal blood flow, renal resistance and urine production were monitored during perfusion. Arterial and venous blood gas analysis was carried out hourly using the ABL90 FLEX blood gas analyser (Radiometer, Brønshøj, Denmark) to evaluate acid-base balance, pO_2, pCO_2, saturation, sodium, potassium, ionized calcium, glucose and lactate levels. Creatinine, sodium, albumin and haemolysis index (HI) were measured in perfusate and urine samples obtained during perfusion using routinely automated test methodology carried out by the Clinical Biochemistry laboratory in the John Radcliffe Hospital, Oxford, United Kingdom. HI of ≤ 0.4 indicated no detectable haemolysis, 0.4–2.0 haemolysis and ≥ 2.0 indicated gross haemolysis. HI is defined as plasma free haemoglobin (in grams) per 100 L of blood. In addition to the tests mentioned above, cellular injury markers lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured in perfusate samples by automated tests. The kidneys were also assessed using a published quality assessment score

(QAS) at the end of NMP.[21] The score ranges from 1 to 5, with 1 indicating the least injury, and 5 indicating severe injury. The scores were calculated using macroscopic appearance, renal blood flow and urine output. For macroscopic assessment, each kidney was categorized into one of three groups by two independent surgeons based on pictures taken at the end of NMP (Table 3.3). When the macroscopic appearance score was different, the highest score was selected to calculate the final QAS score. Human kidneys with a QAS score of 3 or less are normally considered suitable for transplantation. [21]

Table 3.3. Normothermic machine perfusion quality assessment score including the criteria for macroscopic appearance, renal blood flow and urine output.

Normothermic machine perfusion quality assessment score	
Criteria	**Score**
Excellent perfusion (global pink macroscopic appearance)	1 point
Moderate perfusion (patchy pink/purple macroscopic appearance)	2 points
Poor perfusion (global mottling and purple/black macroscopic appearance).	3 points
Mean renal blood flow < 50 mL/min/100g	1 point
Mean renal blood flow > 50 mL/min/100g	0 point
Total urine output (mL) < 43mL/hour	1 point
Total urine output (mL) > 43mL/hour	0 point
Scores for macroscopic appearance, renal blood flow and urine output will be added to yield an overall assessment score ranging from 1 (the highest quality) to 5 (the lowest quality).	

Kidney perfusions were terminated when at least two of the following four criteria were met: (i) arterial flow ≤ 50 mL/min/100g, (ii) pH < 7 or > 7.7 measured at a pCO_2 level of 5 kPa, (iii) inability to maintain physiological pCO_2 levels (4-6 kPa) and/or (iiii) patchy macroscopic appearance.

Renal metabolic activity was approximated by calculation of renal oxygen consumption using arterial and venous blood gasses. The following formula was used to calculate oxygen consumption (mL O_2/min per 100 g)[9]:

$$Oxygen\ consuption: VO_2 = \frac{(CaO_2\ -\ CvO_2)\ \text{x Q}}{g}\ \text{x } 100$$

$$Arterial\ oxygen\ content: CaO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ arterial) + (pO_2\ arterial\ x\ K))$$

$$Venous\ oxygen\ content: CvO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ venous) + (pO_2\ venous\ x\ K))$$

Where 0.024794 is the RBC oxygen binding capacity, 1.54 mL O_2 at 37°C [22], times the conversion factor of haemoglobin in g/L to mmol/L. Hb is the haemoglobin concentration in mmol/L, SO_2 is oxygen saturation in %, pO_2 is the partial oxygen pressure in kPa, K is the solubility constant of oxygen in water at 37ºC, equals to 0.0225 (ml O_2 per kPa), Q is the renal blood flow in dL/min and *g* is the weight of the kidney in grams at the beginning of NMP.

3.2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 8 (GraphPad Software Inc., USA). For all continuous longitudinally measured variables, the area under the curve (AUC) was calculated. The Shapiro–Wilk test was used to test variables for normal distribution. Normally distributed data were analysed using student's *t* test and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to compare more than two groups. Non-parametric data were analysed using Mann-Whitney U test and Kruskal-Wallis test with Dunn's multiple comparisons test to compare more than two groups. Categorical data were compared using Chi-square test. Data are presented as mean ± standard deviation. Two-sided p-values of 0.05 or less were considered to indicate statistical significance. To indicate the level of significance in the graphs, asterisks were plotted in the graphs. One asterisk (*) indicates a level of significance of

$p \leq 0.05$. Two asterisks (**) indicate a p value of ≤ 0.01 and three asterisks (***) a p value of $p \leq 0.001$.

3.3 RESULTS

3.3.1 HMP perfusion characteristics

During HMP on either the self-assembled or portable HMP device all kidneys (n=40) showed similar flow patterns with starting values ranging between 1 and 40 mL/min/100g and an increase over time to a maximum of 85 mL/min/100g at the end of HMP. Temperature during HMP on the self-assembled HMP device was 14 ± 2°C (n=10). Perfusions on the portable HMP had a mean temperature of 5 ± 2°C (n=30).

3.3.2 Comparison of open and closed circuit on NMP of pig kidneys

Previous studies have shown that both open and closed circuits can be used for kidney NMP. It was unclear which type of circuit provided optimal conditions for kidney perfusion and as a result we compared open (n=4) and closed circuits (n=4).

3.3.2.1 Perfusion characteristics

During the first hour of NMP, renal blood flow increased in both the open and closed NMP circuits and then remained stable until the end of perfusion. There was no difference in mean flow per 100 grams (74 ± 17 vs 74 ± 22 mL/min/100g, open circuit vs closed circuit, respectively, p=0.783, student's *t* test) or mean renal resistance (0.49 ± 0.11 vs 0.51 ± 0.15 mmHg/mL/min, open circuit vs closed circuit, respectively, p=0.952, student's *t* test) between the open and closed NMP circuits (Figure 3.7A and 3.7B). Kidneys allocated to the closed circuit had a higher cumulative urine output than the kidneys in the open circuit, however variability was quite high with only two of the four kidneys in the open circuit producing any urine, and this difference was not statistically significant (60 ± 42 mL vs 7 ± 10, closed circuit vs open circuit, respectively, p=0.081, Welch's test; Figure 3.7C).

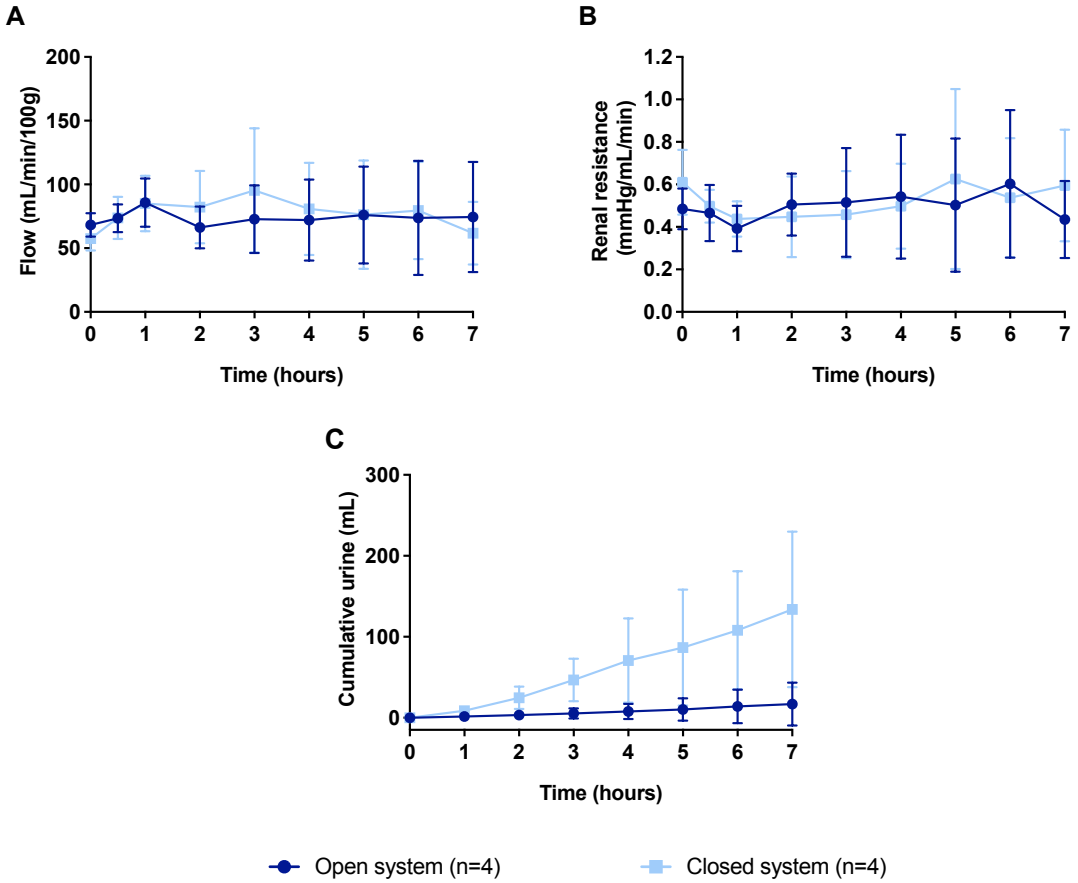


Figure 3.7. NMP perfusion characteristics of pig kidneys perfused in open and closed circuits. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were allocated to closed (n=4, renal vein cannulated) or open (n=4, no renal vein cannula) circuit NMP for seven hours. Perfusion remained stable for (A) mean renal blood flow per 100 grams and (B) mean renal resistance and there were no differences between the groups. (C) There were no differences between cumulative urine outputs between the groups. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

3.3.2.2 Acid-base balance and lactate levels

Partial pressure of carbon dioxide and pH were in physiological range and were similar between the groups, p=0.269 and p=0.439 (student's *t* test), respectively (Figure 3.8A and 3.8B). Perfusate lactate increased throughout perfusion in both groups and at the end of perfusion there were no differences between the groups (p=0.507, student's *t* test; Figure 3.8C).

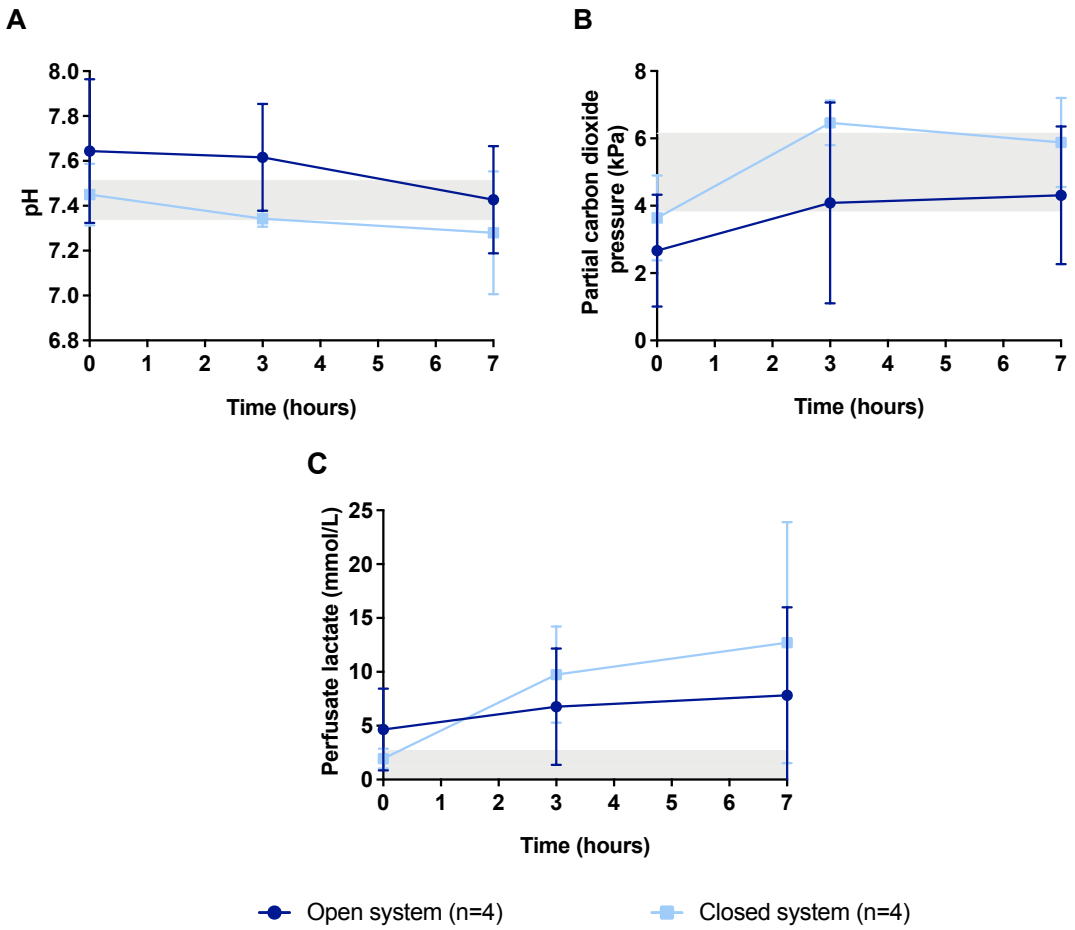


Figure 3.8. Acid-base balance and perfusate lactate levels during pig kidney NMP in open and closed circuits. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were allocated to closed (n=4, renal vein cannulated) or open (n=4, no renal vein cannula) circuit NMP for seven hours. (A) pH and (B) partial carbon dioxide pressure remained stable during NMP and there were no differences between the groups. (C) Perfusate lactate increased throughout perfusion and there were no differences between the groups. Data are presented as mean ± standard deviation. The grey shaded area represents physiological range in pigs.[23, 24] HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

3.3.2.3 Cellular injury markers, haemolysis and LDH

HI and LDH levels were measured as markers of cellular damage (Figure 3.9). Both groups showed detectable haemolysis (0.4<HI<2.0), however this was not significantly different between the groups (p=0.325, student *t* test; Figure 3.9A). There were no differences in LDH between the groups (p=0.110, student *t* test; Figure 3.9B). Given these results and the simpler configuration, the open circuit was selected for use in subsequent experiments.

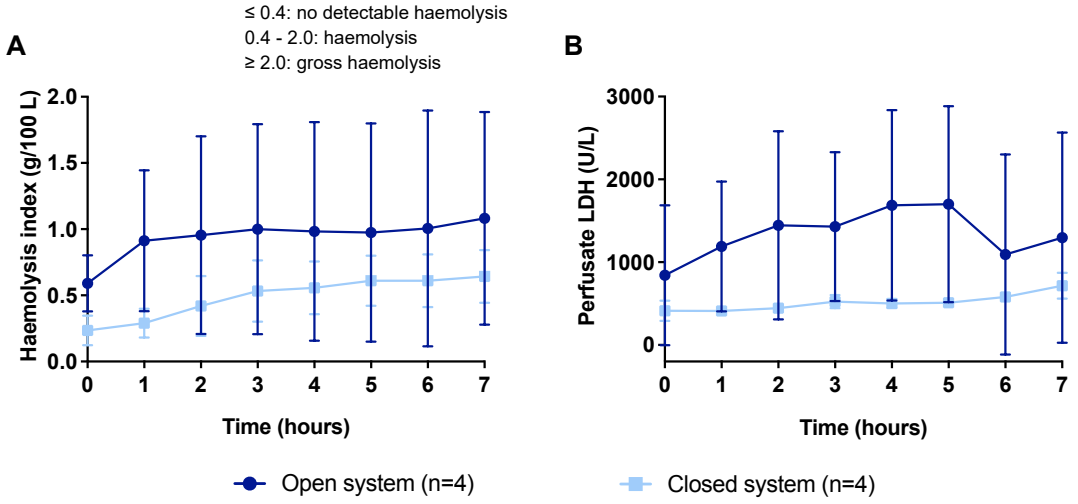


Figure 3.9. Measure of cellular damage during NMP in open and closed circuit. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were allocated to closed (n=4, renal vein cannulated) or open (n=4, no renal vein cannula) circuit NMP for seven hours. (A) Haemolysis index and (B) perfusate lactate dehydrogenase (LDH) during NMP. There were no differences between the groups. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

3.3.3 Optimisation of an RBC-based perfusion solution in a porcine slaughterhouse model of kidney NMP

During development of the NMP circuit in paragraph 3.3.2 a whole blood (leucocyte depleted) perfusate was used as it was less complex and cheap to produce. However, for use in the MSC perfusion experiments (Chapters 5–6) we decided upon a human albumin based perfusate as whole blood would not be translatable into clinical practice and the removal of plasma would make the detection of MSC-secreted proteins more straightforward. Table 3.4 shows the evolution of the perfusate composition.

Table 3.4. Evolution of the optimisation of the RBC-Albumin based perfusate (MePEP perfusate)

Slaughterhouse kidneys																			
Protocol variation	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8 & 9**	**10 to 13**	**14**	**15**	**16**	**17 & 18**	**19**	**20**	**21**	**22**	**23**	**24 & 25**	**26 & 27**
Cold perfusion	*sub*-HMP (at the lab)		HMP on Kidney Assist device, immediately after cold flush																	
Perfusion solution	MePEP 1.0										MePEP 2.0									MePEP 3.0
Insulin in nutrition solution	100 IU		N/A	5 IU												N/A				
Vasodilator	20 mg/h SNP		bolus 0.25 mg/h verapamil			0.25 mg verapamil		20 mg/h SNP continuous infusion	N/A	20 mg/h SNP continuous infusion	0.25 mg/h verapamil continuous infusion	N/A	0.25 mg/h verapamil continuous infusion							
Amoxicillin-clavulanate	1x bolus 180 mg		1x bolus 60 mg								1x bolus 1200 mg			1x bolus 60 mg	1x bolus 1200 mg	bolus 120 mg/h	1x bolus 1200 mg	300 mg at baseline and bolus 120 mg/h	300 mg at baseline and bolus 60 mg/h	

HMP, hypothermic machine perfusion; SNP, sodium nitroprusside.

In our initial experiments (Kidneys 1–15) we added Ringer's solution to the perfusate (MePEP 1.0, Table 3.4) but this was later omitted from the protocol, as when sterile water was added (MePEP 2.0 and 3.0, Table 3.4) the electrolyte composition remained within physiological range at baseline (Table 3.5).

Table 3.5. Baseline electrolytes and pH values in the three different perfusion solutions: MePEP 1.0, MePEP 2.0 and MePEP 3.0. Perfusate baseline blood gas analysis, perfusate phosphate and perfusate albumin concentrations. Data are presented as mean ± standard deviation. pCO_2, partial carbon dioxide pressure; pO_2, partial oxygen pressure; Hb, haemoglobin; Hct, haematocrit; Na+, sodium; K+, potassium; iCa^{2+}, ionized calcium.

	MePEP 1.0 (n=15)	MePEP 2.0 (n=11)	MePEP 3.0 (n=2)	p-value
pH	7.43 ± 0.12	7.39 ± 0.08	7.29 ± 0.02	0.141
pCO_2 (kPa)	3.42 ± 0.10	4.23 ± 0.80	4.61 ± 0.52	0.060
pO_2 (kPa)	75.7 ± 5.3	80.3 ± 6.1	86.3 ± 2.6	**0.015***
Hb (g/L)	100 ± 21	94 ± 4	109 ± 3	0.116
Hct (%)	30 ± 6	29 ± 1	33 ± 1	0.132
Na^+ (mmol/L)	139 ± 10	138 ± 4	141 ± 2	0.622
K^+ (mmol/L)	2.2 ± 1.5	3.0 ± 1.2	1.5 ± 0.2	0.141
iCa^{2+} (mmol/L)	1.31 ± 0.16	1.25 ± 0.29	1.31 ± 0.06	0.608
PO_4^{3-} (mmol/L)	1.16 ± 1.06 (n=14)	1.27 ± 1.37 (n=10)	1.35 ± 0.95	0.831
Glucose (mmol/L)	4.2 ± 0.7	4.7 ± 0.8	5.7 ± 0.2	**0.043***
Lactate (mmol/L)	0.3 ± 0.3	0.2 ± 0.2	0.2 ± 0	0.999
Albumin (g/L)	31 ± 3 (n=14)	34 ± 2 (n=10)	42 ± 0	**0.009***

* MePEP 1.0 significantly lower than MePEP 3.0

We also administered a nutrition infusion with insulin concentrations of 100 IU and later 5 IU of insulin (MePEP 1.0 and 2.0, Table 3.4), but neither had an effect on the perfusate glucose concentration during NMP (Figure 3.10). Therefore, the continuous infusion of nutrition was omitted from the protocol. Instead, we decided to start glucose infusion only when glucose levels dropped ≤ 4 mmol/L. In all three perfusate protocols, glucose levels increased and peaked during the first two hours of NMP and decreased gradually thereafter (Figure 3.10). Albumin concentrations during NMP were also monitored to determine if they were adequate to support perfusion and stable oncotic pressure.

Starting albumin concentrations were 31 ± 3 mmol/L for MePEP perfusate 1.0 (n=14), 34 ± 2 mmol/L for MePEP perfusate 2.0 (n=10) and 42 ± 0 mmol/L for MePEP perfusate 3.0 (n=2).

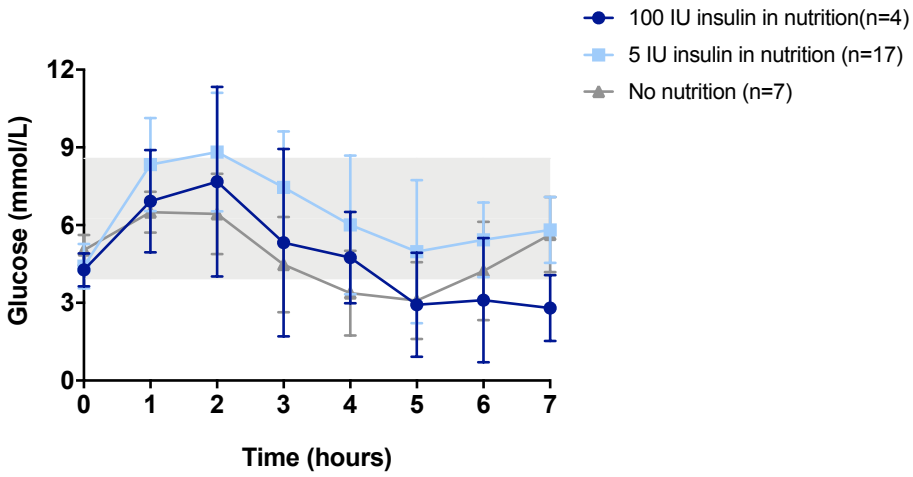


Figure 3.10. Glucose concentration in the different RBC-Albumin based perfusates (MePEP perfusate). Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with an RBC-albumin based perfusate with different insulin concentrations. The different insulin concentrations did not cause any difference in the perfusate glucose levels. Data are presented as mean ± standard deviation. Grey area represents physiological range in pigs.[23, 24] HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; RBC, red blood cell.

During the development of the MePEP perfusate, different types of vasodilators and antibiotic concentrations were also tested (Table 3.4). The effect of different vasodilators during pig kidney NMP (described in more details in paragraph 3.3.4) was investigated with MePEP perfusate 1.0 and the effect of different antibiotic doses (described in more details in paragraph 3.3.5) was investigated with MePEP perfusates 2.0 and 3.0. Perfusate electrolyte and blood gas baseline values from both studies are displayed in Table S3.1 and S3.2 in the appendix.

3.3.4 The effect of different vasodilators during pig kidney NMP

Previous work had shown that SNP and verapamil were both vasodilator options during kidney NMP.[7, 14, 15] Therefore, we set out to compare verapamil (n=4), SNP (n=3), and no vasodilator (n=4) during kidney NMP.

3.3.4.1 Perfusion characteristics

There was no significant difference in mean blood flow between the groups (86 ± 30 mL/min/100g, 94 ± 28 mL/min/100g and 98 ± 53 mL/min/100g, verapamil vs SNP vs no vasodilator respectively, p=0.668, ANOVA; Figure 3.11A). Not all kidneys perfused up to seven hours. Renal resistance initially reduced and then slowly increased throughout perfusion and there was no difference between the groups in the overall mean values (0.58 ± 0.18 mmHg/mL/min, 0.63 ± 0.13 mmHg/mL/min and 0.81 ± 0.71 mmHg/mL/min, verapamil vs SNP vs no vasodilator, respectively p=0.668, Kruskal-Wallis test; Figure 3.11B). One of the four kidneys in the no vasodilator group had a high resistance at the end of perfusion (renal resistance=6.45 mmHg/mL/min). There was no difference between the groups in overall urine output (data not shown) (48 ± 16 mL, 23 ± 22 mL and 10 ± 10 mL, verapamil vs SNP vs no vasodilator, respectively, p=0.447, ANOVA).

3.3.4.2 pH and perfusate lactate levels

The SNP group had a significantly lower pH (7.27 ± 0.06 vs 7.45 ± 0.04 and 7.44 ± 0.07, p=0.006, ANOVA) and increased lactate levels (10.3 ± 3.4 mmol/L vs 1.3 ± 0.6 and 4.8 ± 3.9 mmol/L, p=0.001, ANOVA) compared with the verapamil and no vasodilator groups, respectively (Figure 3.12).

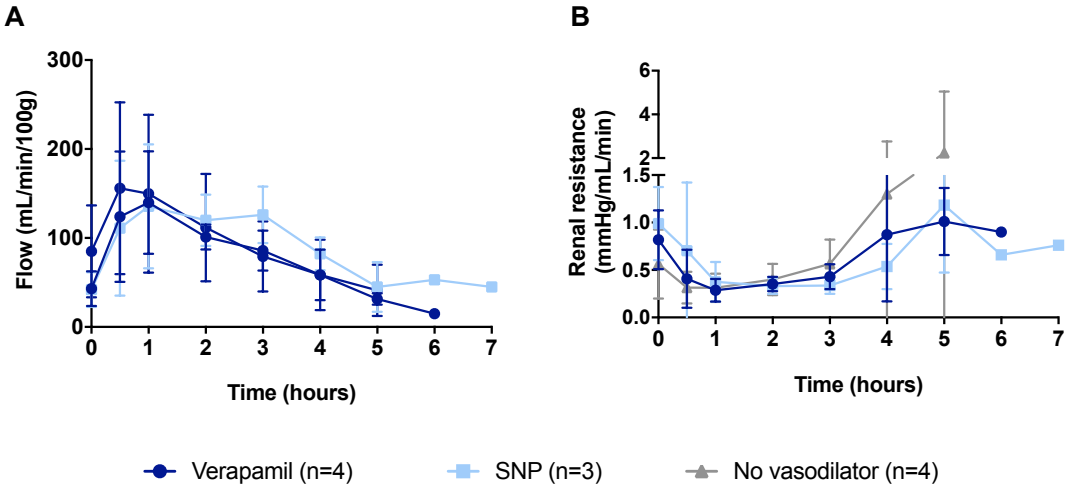


Figure 3.11. Perfusion characteristics during NMP with different vasodilators. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with no vasodilator (n=4), verapamil (n=4) and sodium nitroprusside (n=3, SNP). There were no differences in (A) mean renal blood flow per 100 grams and (B) mean renal resistance between the groups. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

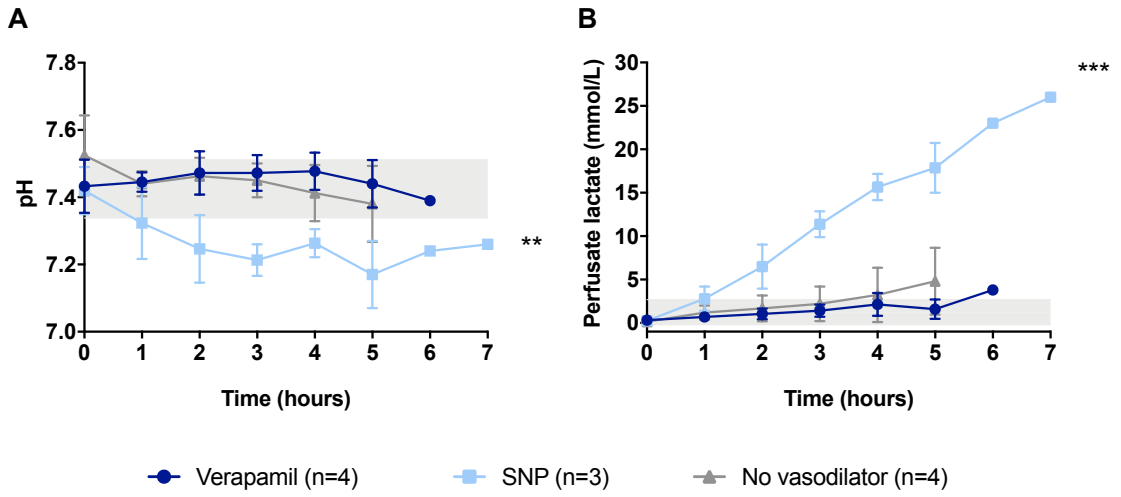


Figure 3.12. pH and perfusate lactate levels during NMP with three different vasodilators. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with no vasodilator (n=4), verapamil (n=4) and SNP (n=3). There was a significant reduction in (A) pH and an increase in (B) perfusate lactate during NMP in the SNP group compared to the verapamil and no vasodilator groups. Grey area represents physiological range in pigs.[23, 24] Data are presented as mean ± standard deviation. ** and *** $p \leq 0.05$ SNP versus verapamil and no vasodilator groups. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; SNP, sodium nitroprusside.

3.3.4.3 Perfusate albumin, proteinuria and urinary albumin:creatinine ratio

In all groups, there was minimal loss of perfusate albumin compared to baseline levels, with overall mean loss values of 3 ± 5 g/L for the verapamil group, 5 ± 4 g/L for the SNP group and 3 ± 2 g/L for the no vasodilator group (Figure 3.13A). Proteinuria was measured as marker of glomerular function. Proteinuria increased in the SNP group, while in the verapamil and no vasodilator groups decreased over time (9.0 ± 7.0 g/L vs. 3.0 ± 0.8 g/L and 2.0 ± 1.4 g/L). There was however no difference in proteinuria (AUC) between the groups, p=0.114 (ANOVA; Figure 3.13B). There were no differences in urinary albumin:creatinine ratio between the groups (verapamil group: 2879 ± 3954 mg/mmol, SNP group: 10660 ± 10940 mg/mmol and no vasodilator group: 1384 ± 1007 mg/mmol, p=0.165; Kruskal-Wallis test; Figure 3.13C). Overall, the macroscopic appearance of all kidneys in all groups was patchy at the end of perfusion and scored 5 according to the QAS score (data not shown).

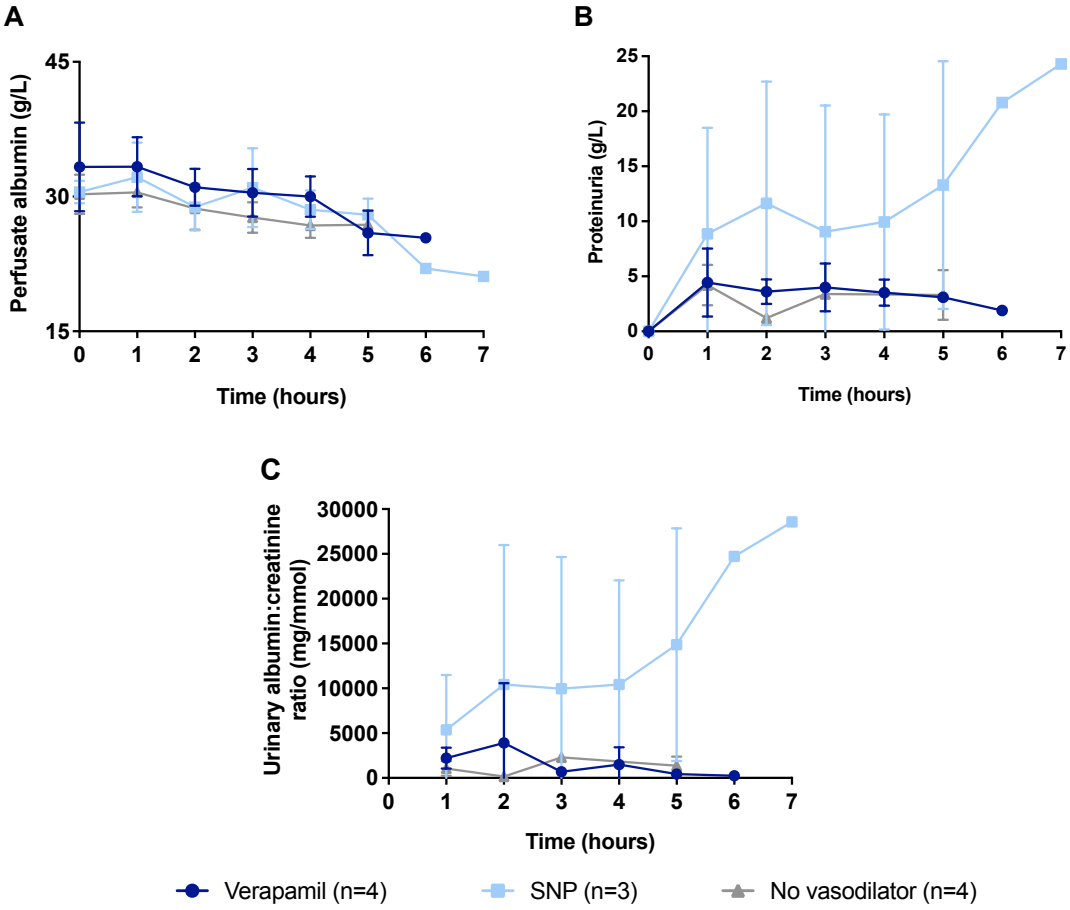


Figure 3.13. Perfusate albumin, proteinuria and urinary albumin:creatinine ratio during NMP with different vasodilators. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with no vasodilator (n=4), verapamil (n=4) and SNP (n=3). There were minimal losses in (A) perfusate albumin compared to baseline values. (B) Proteinuria increased in the SNP group over time, while in the verapamil and no vasodilator groups it decreased overtime with no statistical differences between the groups. (C) There were no differences between the groups in urinary albumin:creatinine ratio. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; SNP, sodium nitroprusside.

3.3.5 The effect of antibiotic dosing on pig kidneys during NMP

Previous kidney NMP experimental models have included the addition of an antibiotic to the perfusion circuit.[8, 9, 15, 16] A series of *in vitro* experiments performed by Sierra-Parraga *et al.* (personal communication) have shown that 1000 mg/200 mg amoxicillin-clavulanate in blood-based perfusate, used by other groups during NMP, reduced MSC viability (unpublished data). As a result, we wanted to determine whether different antibiotic doses had an effect on pig kidney perfusion and we compared low dose (LD, n=4), standard dose antibiotic (SD, n=4) and bolus dose (BD, n=4).

3.3.5.1 Perfusion characteristics

During NMP, flow rates peaked in the first hour of NMP for all groups with overall mean flow values of 86 ± 30 mL/min/100g in the LD group, 126 ± 48 mL/min/100g in the SD group and 104 ± 8 mL/min/100g in the BD group (Figure 3.14A). Renal blood flow for kidneys perfused in the LD group was significantly lower than in the kidneys perfused in the SD group, p=0.024 (ANOVA). Kidneys in the LD group had a significantly lower value at the end of NMP than kidneys in the SD and BD groups (30 ± 26 vs 111 ± 33 and 78 ± 16 mL/min, p=0.003, ANOVA). Mean renal resistance was 0.58 ± 0.19 mmHg/mL/min/100g in the LD group, 0.41 ± 0.09 mmHg/mL/min/100g in the SD group and 0.42 ± 0.07 mmHg/mL/min/100g in the BD group and there were no significant differences between the groups (p=0.452, Kruskal-Wallis test; Figure 3.14B). At the end of perfusion kidneys in the LD group had significantly higher resistance values than kidneys in the SD and BD groups (1.21 ± 0.50 vs 0.41 ± 0.09, p=0.009 and 0.42 ± 0.06 mmHg/mL/min, p=0.011, ANOVA). There was no difference in urine output between the groups (LD group: 23 ± 22 mL, SD group: 4.5 ± 2.6 mL and BD group: 5.5 ± 5 mL, p=0.452, Kruskal-Wallis test; Figure 3.14C).

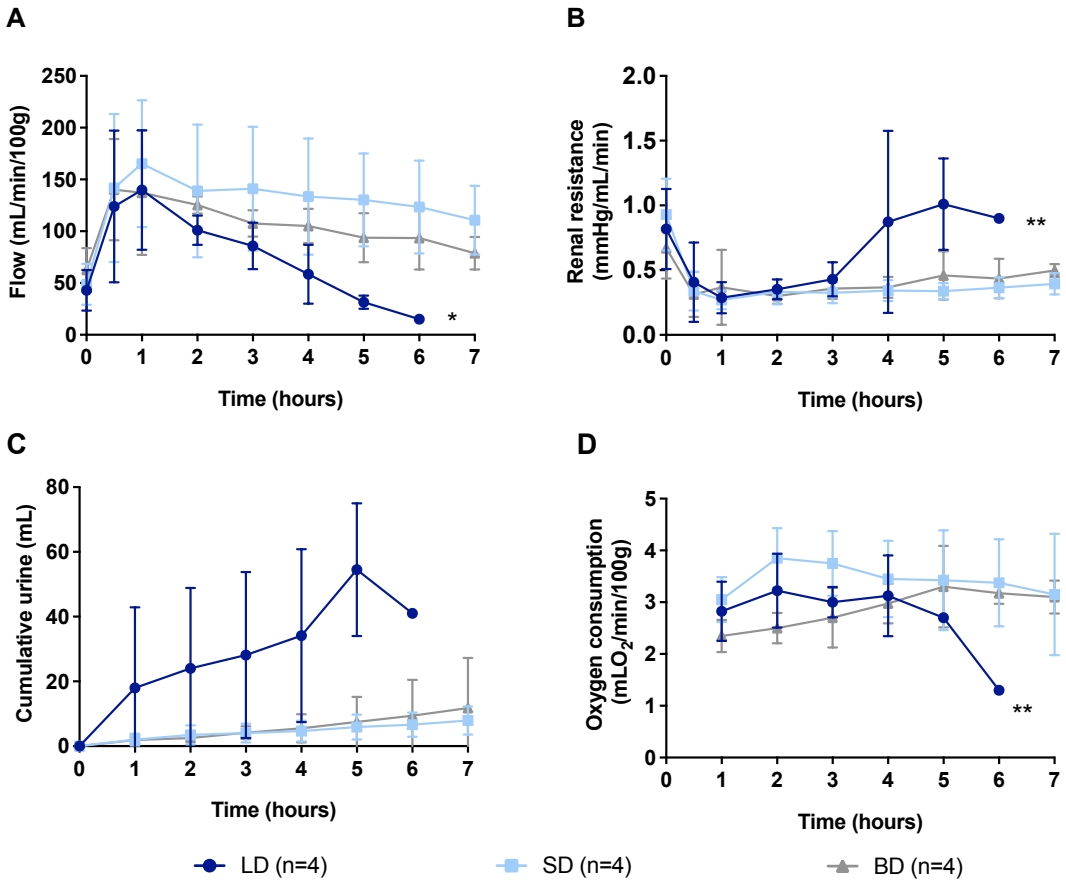


Figure 3.14. Perfusion characteristics, urine production and oxygen consumption during NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP for seven hours with low dose antibiotic (LD, n=4), standard dose antibiotic (SD, n=4) and bolus dose antibiotic (BD, n=4). There was a significant reduction in (A) mean renal blood flow per 100 grams and an increase in (B) mean renal resistance during NMP in the LD group compared to the SD and BD groups. There were no differences in (C) cumulative urine output between the groups. (D) Oxygen consumption in the LD group was significantly reduced in comparison with the SD and BD groups. Data are presented as mean ± standard deviation. * flow and ** oxygen consumption $p \leq 0.05$ AUC LD versus AUC SD and BD groups. ** $p \leq 0.05$ renal resistance end NMP LD versus SD and BD groups. AUC, area under the curve; HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

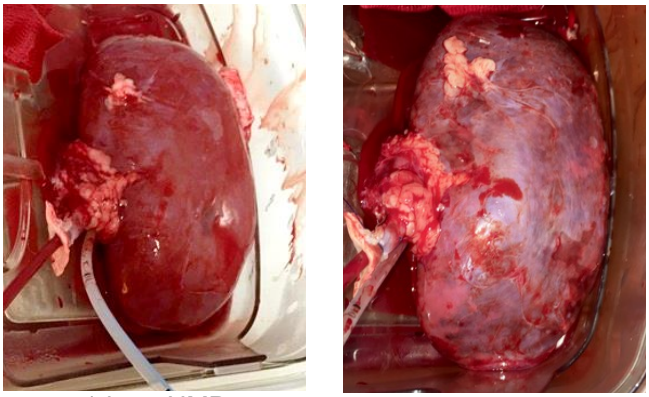
3.3.5.2 Kidney viability, proteinuria and urinary albumin:creatinine ratio

Kidney quality and metabolic activity were assessed by the QAS score and oxygen consumption, respectively. Oxygen consumption in the SD and BD group showed a similar trend, while oxygen consumption in the LD group was significantly reduced in comparison to the BD and SD group (p=0.002, ANOVA; Figure 3.14D). At the end of NMP mean oxygen consumption levels were 2.35 ± 0.90 mLO_2/min/100g in the LD group, 3.16 ± 1.12 mLO_2/min/100g in the BD group and 3.10 ± 0.32 mLO_2/min/100g in the SD group, p=0.388 (ANOVA). A patchy, ischemic macroscopic appearance of the kidneys was observed during NMP in the LD group compared to the SD and BD groups (Figure 3.15). Kidneys in the SD and BD groups had lower QAS scores compared to kidneys in the LD group (p=0.003, Chi-square test; Table 3.6).

Table 3.6. Quality assessment score (QAS) for kidneys perfused with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate during NMP. The QAS is based on urine production, renal blood flow and macroscopic appearance at the end of NMP.

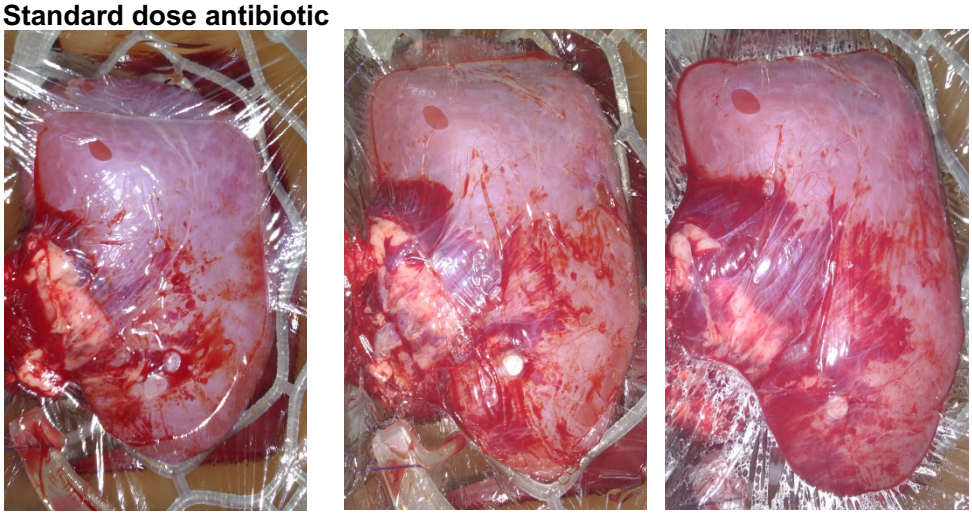
Kidney	Urine	Flow	Macroscopic appearance	QAS score
LD 1	1	1	3	4
LD 2	1	1	3	5
LD 3	1	0	3	4
LD 4	1	1	3	5
SD 1	1	0	1	2
SD 2	1	0	2	3
SD 3	1	0	1	2
SD 4	1	0	2	3
BD 1	1	0	1	2
BD 2	1	0	2	3
BD 3	1	0	2	3
BD 4	1	0	2	3

A. Low dose antibiotic



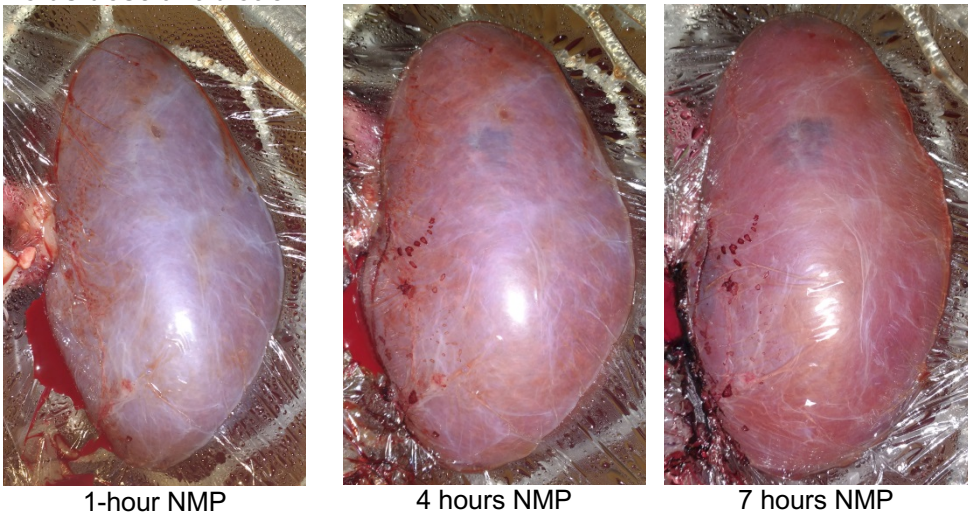
1-hour NMP 4 hours NMP

B. Standard dose antibiotic



1-hour NMP 4 hours NMP 7 hours NMP

C. Bolus dose antibiotic



1-hour NMP 4 hours NMP 7 hours NMP

Figure 3.15. Kidney macroscopic appearance during NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP for seven hours with low dose antibiotic (LD, n=4), standard dose antibiotic (SD, n=4) and bolus dose antibiotic (BD, n=4). (A) Low dose antibiotic kidney showing severe patchy macroscopic appearance after 4 hours of NMP. (B) Standard dose antibiotic kidney showing homogenous pink appearance during seven hours of NMP. (C) Bolus dose antibiotic kidney with mild patchy appearance.

There were no differences in urinary protein levels between LD (3.9 ± 1.1 g/L), SD (15 ± 9.4 g/L) and BD (6.2 ± 3.6 g/L, p=0.071, Kruskal-Wallis test; Figure 3.16A). There were no differences in urinary albumin:creatinine ratio between the groups (LD: 7777 ± 13611 mg/mmol, SD: 5844 ± 3548 mg/mmol and BD group: 10471 ± 9737 mg/mmol, p=0.284; Kruskal-Wallis test; Figure 3.16B).

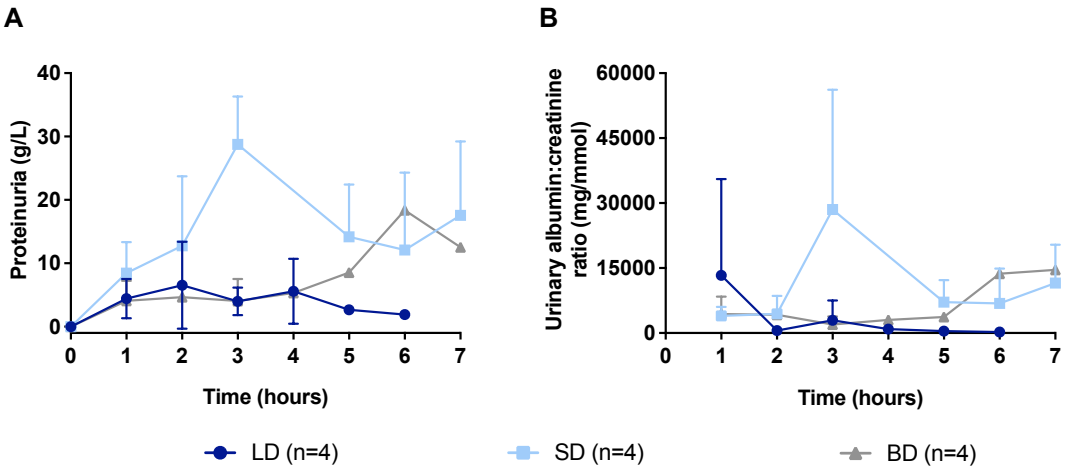


Figure 3.16. Proteinuria and urinary albumin:creatine ratio in kidneys perfused on NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP for seven hours with low dose antibiotic (LD, n=4), standard dose antibiotic (SD, n=4) and bolus dose antibiotic (BD, n=4). There were no differences in (A) proteinuria and (B) urinary albumin:creatinine ratio between the groups. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

3.3.5.3 Injury markers lactate, LDH and AST

Perfusate lactate increased over time during NMP and there were no differences between the groups (LD group: 1.3 ± 0.61 mmol/L, SD group: 1.8 ± 1.2 mmol/L and BD group: 2.4 ± 0.9 mmol/L, p=0.269, Kruskal-Wallis test; Figure 3.17A). Similarly, cellular injury marker LDH increased over time during NMP and was similar at the end of NMP (p=0.073, ANOVA; Figure 3.17B). There were no differences in perfusate AST levels between the groups (p=0.227, ANOVA; Figure 3.17C).

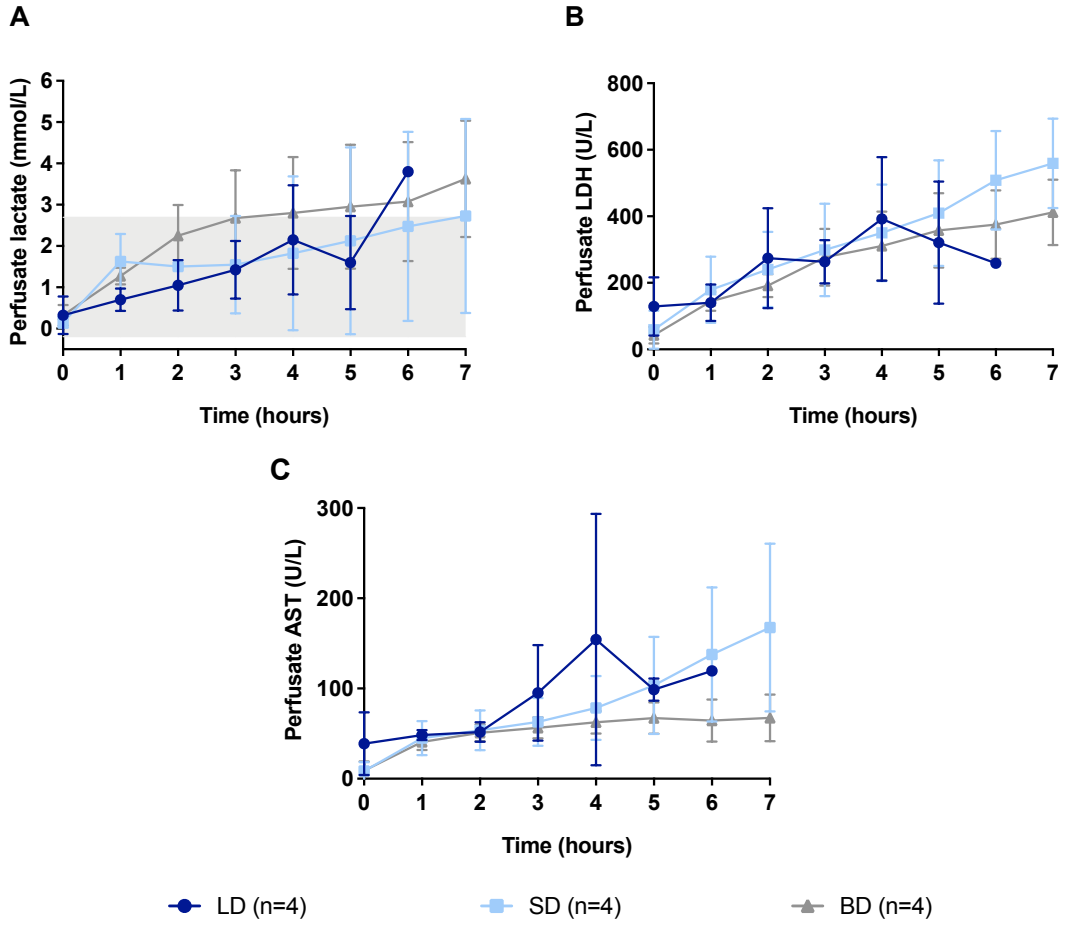


Figure 3.17. Injury markers LDH and AST as well as lactate during NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP for seven hours with low dose antibiotic (LD, n=4), standard dose antibiotic (SD, n=4) and bolus dose antibiotic (BD, n=4). Perfusate (A) lactate, (B) LDH and (C) AST increased in all groups during NMP and there were no significant differences between the groups. Grey area represents physiological range in pigs [23, 24] Data are presented as mean ± standard deviation. AST, aspartate aminotransferase; HMP, hypothermic machine perfusion; LDH, lactate dehydrogenase; NMP, normothermic machine perfusion.

3.3.5.4 Acid-base balance and electrolytes

All kidneys had a physiological pH during NMP with overall mean values of 7.45 ± 0.04 in the LD group, 7.38 ± 0.04 in the BD group and 7.41 ± 0.04 in the SD group (Figure 3.18A). Partial pressure of carbon dioxide remained stable during NMP for the LD and BD group. In the SD group, it slowly increased. Mean pCO_2 levels were 5.36 ± 0.86 kPa in the LD group, 6.60 ± 1.33 in the SD group and 5.22 ± 0.86 kPa in the BD group (Figure 3.18B). Perfusate bicarbonate remained physiological in all the groups with mean values of 29 ± 3 mmol/L in the LD group, 27 ± 4 mmol/L in the SD group and 26 ± 2 mmol/L in the BD group (Figure 3.18C).

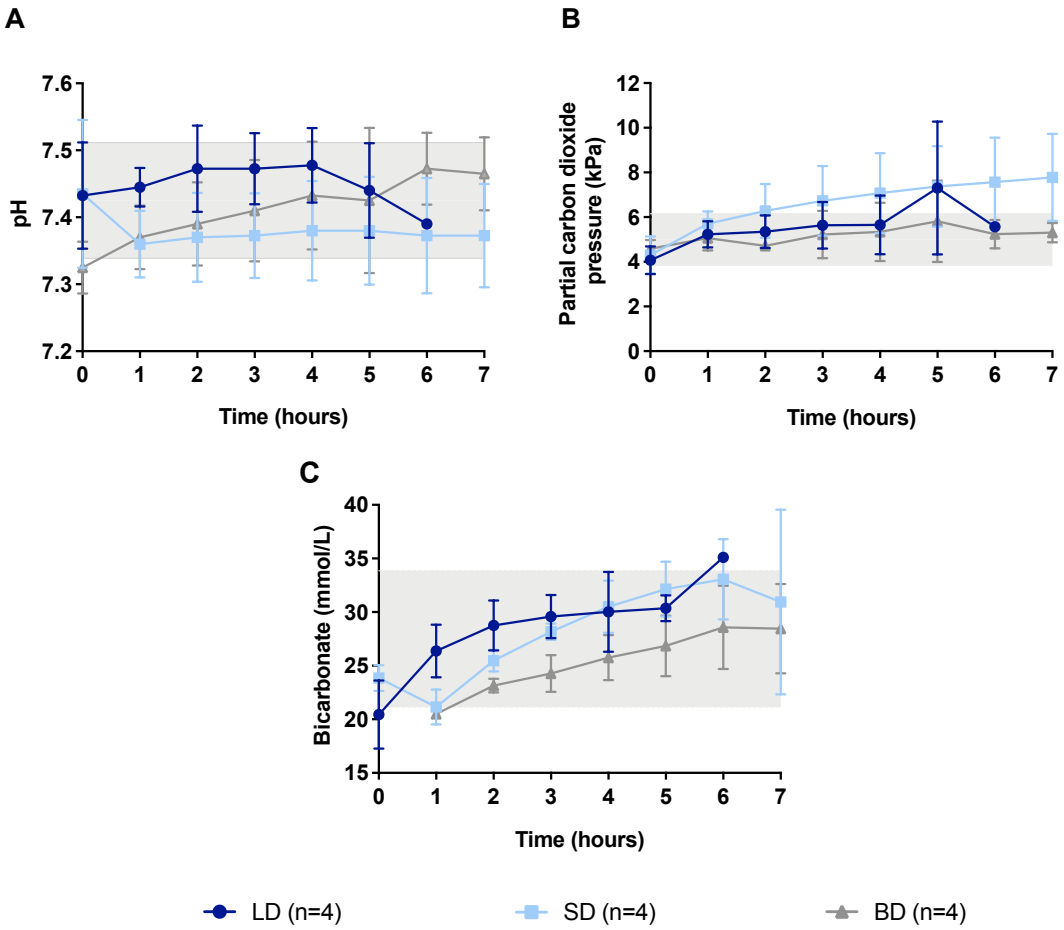


Figure 3.18. Acid-base balance during NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP for seven hours with low dose (LD, n=4), standard dose (SD, n=4) and bolus dose antibiotic (BD, n=4). (A) pH remained within physiological range throughout perfusion in all groups. (B) Partial carbon dioxide pressure slowly increased in the SD group compared to the LD and BD groups. (C) Perfusate bicarbonate levels remained within physiological values in all groups. Data are presented as mean ± standard deviation. Grey area represents physiological range in pigs.[24,25] HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

Perfusate sodium levels were within physiological range in all groups throughout perfusion with overall mean levels of 138 ± 2 mmol/L in the LD group, 143 ± 3 mmol/L in the SD group and 139 ± 2 mmol/L in the BD group (Figure 3.19A). Initial perfusate potassium levels were lower than normal physiological values and thereafter increased slowly during NMP with end values of 6.1 ± 0.0 mmol/L for the LD group, 5.4 ± 1.1 mmol/L for the SD group and 3.5 ± 0.3 mmol/L for the BD group (Figure 3.19B). Ionized calcium levels in the perfusate decreased during the first hour of perfusion in all groups and remained within physiological levels throughout perfusion. Overall mean ionized calcium values were 0.9 ± 0.0 mmol/L for the LD group, 1.0 ± 0.0 mmol/L for the SD group and 1.1 ± 0.1 mmol/L for the BD group (Figure 3.19C). There was an increase in phosphate levels during the first hour of NMP in all groups and they remained stable throughout perfusion (Figure 3.19D).

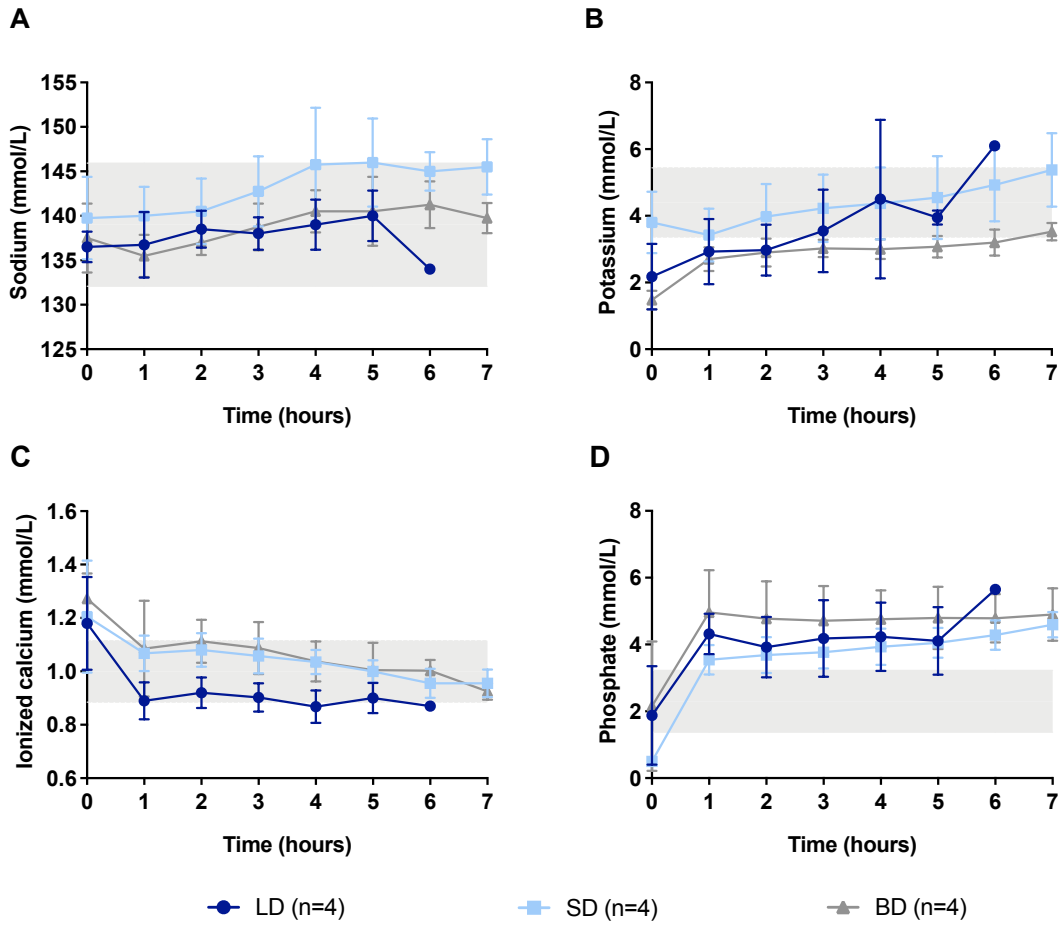


Figure 3.19. Perfusate electrolyte levels during NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP for seven hours with low dose (LD, n=4), standard dose (SD, n=4) and bolus dose antibiotic (BD, n=4). (A) Perfusate sodium levels remained within physiological values in all groups and (B) potassium increased from baseline during the first hour and remained in physiological range in all groups. (C) Ionized calcium remained stable during NMP after the first hour of perfusion and (D) phosphate increased during the first hour of perfusion and remained stable afterwards in all groups. Data are presented as mean ± standard deviation. Grey area represents physiological range in pigs.[23, 24] HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

3.3.5.5 Micro-organism culture and minimum inhibitory concentration

The concentration of amoxicillin-clavulanate in the perfusate at the beginning of perfusion was 160 μg/mL, 1038 μg/mL and 3343 μg/mL in the LD, BD and SD groups respectively. The MICs for amoxicillin-clavulanate for a selection of frequently occurring bacteria isolated in the perfusate are displayed in Table 3.7. Numerous environmental bacteria were isolated from the perfusate prior to the initiation of perfusion and the full list is available in Table S3.3 in the Appendix.

Table 3.7. Frequently isolated bacteria in the perfusate during NMP and relative amoxicillin-clavulanate minimum inhibitory concentration.

Isolated bacteria	Minimum inhibitory concentration (μg/mL)
Klebsiella oxytoca	4
Stenotrophomas species	> 256
Pseudomonas fluorescens	64
Agromyces mediolanus	16
Sphingobacterium multivorum	16
Pseudonomas chloraphis	> 256
Acinetobacter bereziniae	32
Brevundimonas dimunta	> 256
Acinetobacter pitti	16

In two experiments using the bolus dose antibiotic, at the start of perfusion there was moderate growth of a number of the bacteria listed in Table S3.3 in the Appendix, including *Pseudomonas spp*. and *Stenotrophomas spp*. At the end of perfusion, during hour 7, *Pseudomonas spp.* remained with profuse growth in one experiment and moderate in the other but overall the number of bacteria had reduced.

3.3.6 RBC-plasma perfusion solution for prolonged NMP

Previous studies have focussed predominantly on a leucocyte depleted RBC-containing perfusate including a colloid and electrolytes. We have also tested a perfusate with RBCs and plasma on NMP (n=5) with the idea of combining advantages from both the leucocyte depleted blood with its remaining plasma compartment and the RBC-albumin based compositions, as we hypothesised that a RBC-plasma perfusate being more *physiological* would support prolonged NMP.

3.3.6.1 Perfusion characteristics and kidney quality

In all kidneys (n=5) the flow increased during the first hours of NMP with kidneys being perfused for a minimum of eight hours. Kidneys 1 and 3 were perfused for the longest duration and reached 16 and 19 hours, respectively. Renal blood flow for all kidneys is displayed in Figure 3.20A. At the beginning of NMP flow values ranged between 37-103 mL/min/100g and peaked at one hour for kidney 1, 2 and 5 (181, 116 and 126 mL/min/100g respectively), at four hours for kidney 3 (202 mL/min/100g) and at five hours for kidney 4 (127 mL/min/100g). Renal resistance decreased during the first hour of perfusion and remained stable for kidneys 1, 2 and 4 and ranged from 0.19–0.40 mmHg/mL/min. The highest renal resistance was observed in kidney 5 where the resistance rapidly increased in the final five hours of NMP (Figure 3.20B). There was a relatively large difference in cumulative urine production between the kidneys. Two of the five kidneys did not produce any urine during NMP (Figure 3.20C). All kidneys consumed oxygen with values ranging from 1.26 to 4.51 mL O_2/min/100g. Kidney 2 appeared to consume less oxygen than the rest of the kidneys and in kidney 3 oxygen consumption rapidly decreased in the last three hours of NMP (figure 3.20D).

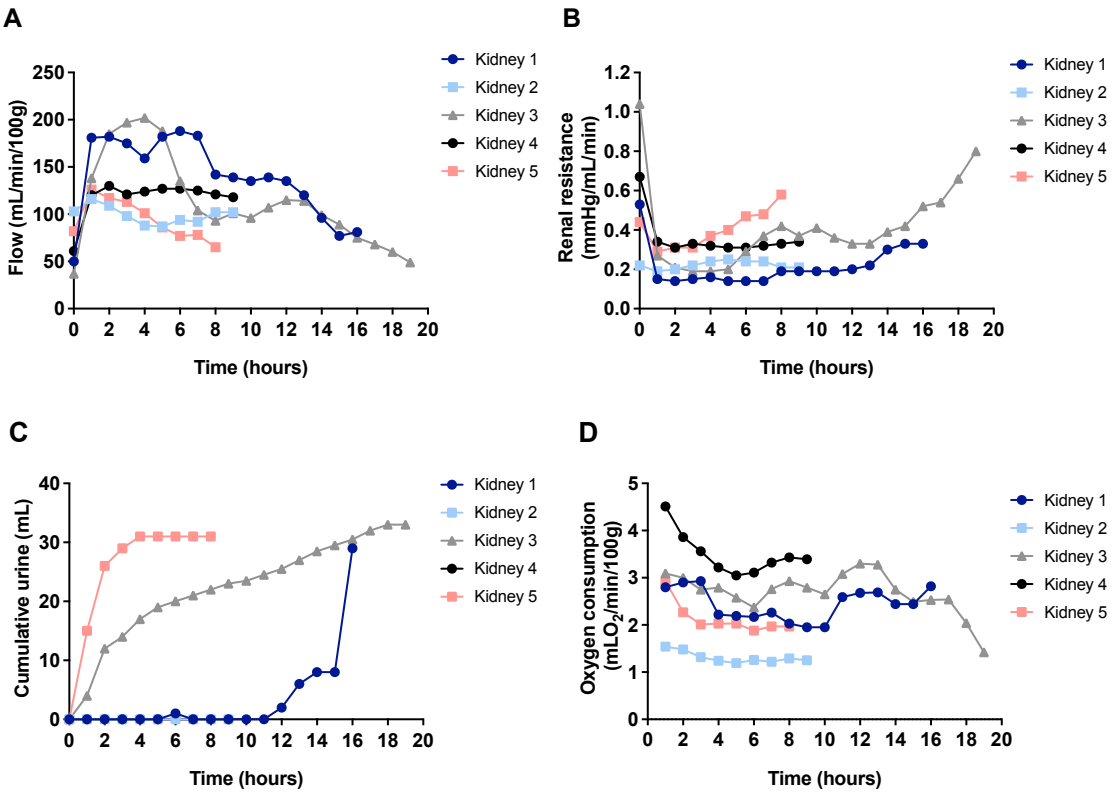


Figure 3.20. Perfusion characteristics and oxygen consumption during NMP for kidneys perfused with RBC-plasma perfusion solution. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with an RBC-plasma based perfusate. (A) Renal blood flow per 100 grams increased in all kidneys during the first hour of NMP and kidneys were perfused a minimum of eight hours. (B) Renal resistance remained stable for kidneys 1, 2 and 4 during NMP. There was a relatively large difference in (C) cumulative urine output. (D) Kidneys 1, 3 and 5 consumed oxygen in a similar manner during NMP whereas kidney 2 consumed less oxygen than the rest of the kidneys. HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; RBC, red blood cell.

An example of the macroscopic appearance is illustrated in Figure 3.21, where a homogenous pink macroscopic appearance was observed in all kidneys during and at the end of NMP. All kidneys scored 3 or lower according to the QAS (Table 3.8).

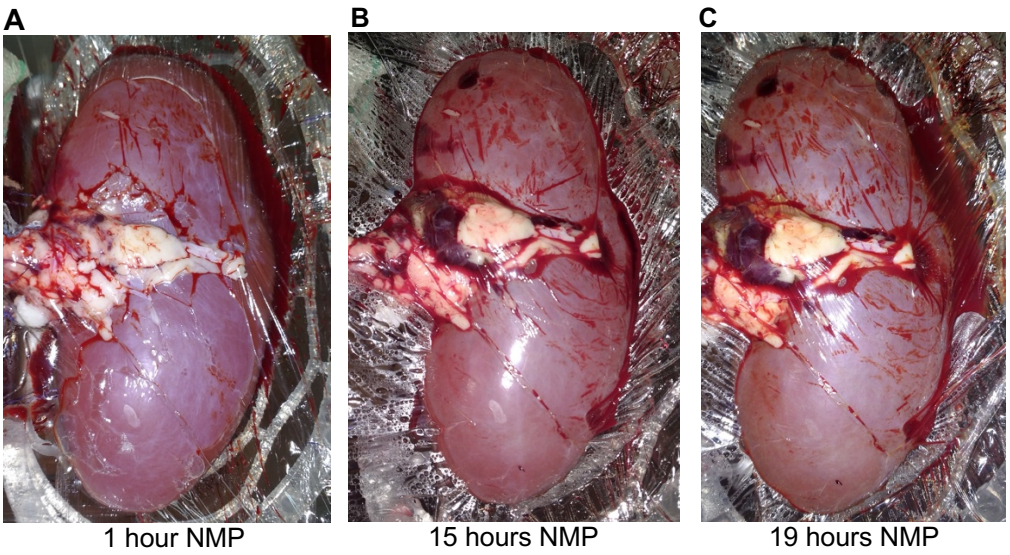


Figure 3.21. Kidney macroscopic appearance during NMP with RBC-plasma perfusion solution. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with an RBC-plasma based perfusate. A homogenous pink macroscopic appearance is observed after (A) 1 hour, (B) 15 hours and (C) 19 hours of NMP.

Table 3.8. Quality assessment score (QAS) for kidneys perfused with the RBC-plasma perfusion solution. The QAS is based on the urine production, renal blood flow and macroscopic appearance at the end of NMP.

Kidney	Urine	Flow	Macroscopic appearance	QAS score
1	1	0	2	3
2	1	0	1	2
3	1	0	2	3
4	1	0	1	2
5	1	0	1	2

3.3.6.2 Injury markers lactate, LDH and AST

In this cohort of kidneys, perfusate lactate levels decreased from the beginning of perfusion (2.7–10.5 mmol/L) until nine hours (0.3–1.5 mmol/L) of NMP for kidneys 1–4 and slowly increased afterwards until the end of NMP. Lactate in kidney 5 remained stable for six hours and then rapidly increased (Figure 3.22A). At baseline, injury marker LDH ranged from 454–690 U/L and increased until the end of perfusion for all kidneys, ranging from 616–1570 U/L (Figure 3.22B). Similarly, AST increased until the end of NMP and at the end of perfusion ranged from 86–461 U/L (Figure 3.22C).

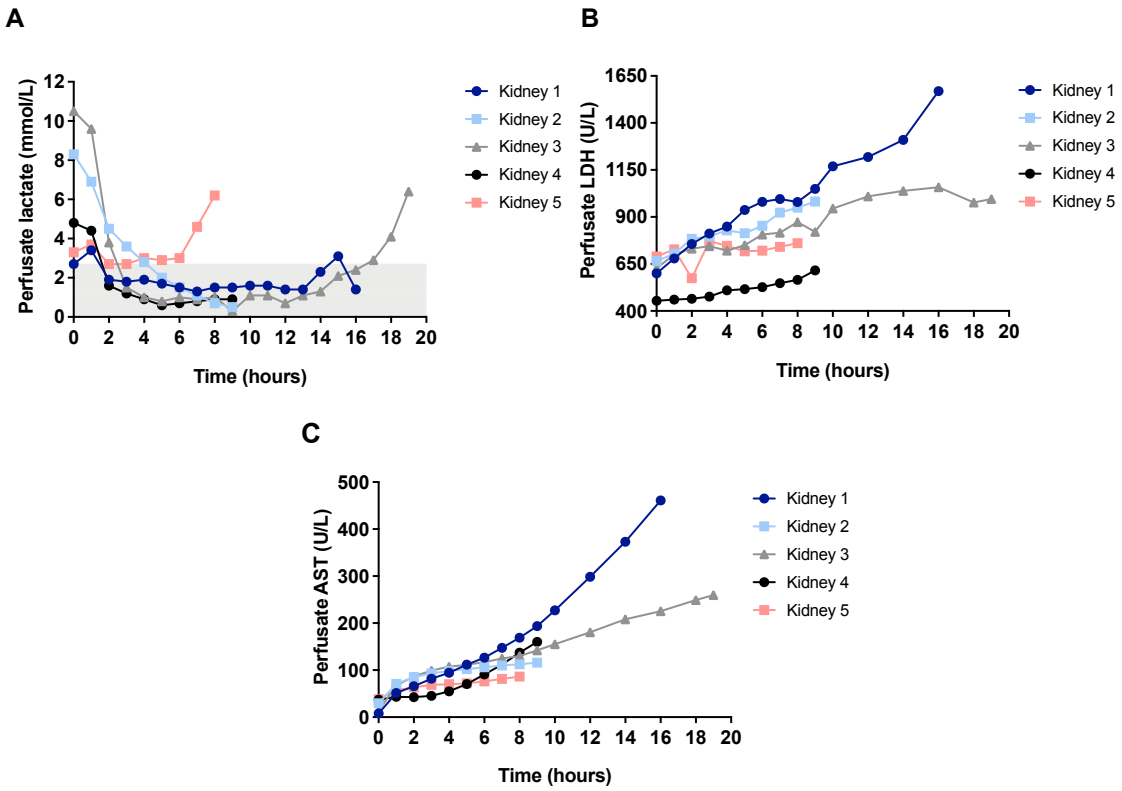


Figure 3.22. Injury markers for kidneys perfused with RBC-plasma perfusion solution during NMP. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with an RBC-plasma based perfusate. **(**A) Lactate decreased from baseline until nine hours NMP for kidneys 1 – 4. Lactate in kidney 5 remained stable for six hours and then rapidly increased. (B) LDH and (C) AST increased in all kidneys during NMP. Grey area represents physiological range in pigs.[23, 24] AST, aspartate aminotransferase; HMP, hypothermic machine perfusion; LDH, lactate dehydrogenase; NMP, normothermic machine perfusion; RBC, red blood cell.

3.3.6.3 Acid-base balance and electrolytes levels

Perfusate electrolyte and blood gas baseline values are displayed in Table S3.4 in the appendix. The pH ranged between 7.25 and 7.70 and was predominantly alkalotic throughout perfusion for kidneys 1, 3 and 5. In kidney 2, pH rapidly decreased after five hours and in kidney 4 the pH was in physiological range. Interestingly, kidneys with physiological pH during NMP (kidney 2 and 4) had higher partial pressure of carbon dioxide levels during NMP and were therefore terminated (Figure 3.23A and 3.23B). For all kidneys perfusate bicarbonate levels were higher than physiological levels and increased in kidneys 1, 2 and 4 overtime during perfusion. For kidney 3 perfusate bicarbonate increased until 12 hours and then rapidly decreased (Figure 3.23C).

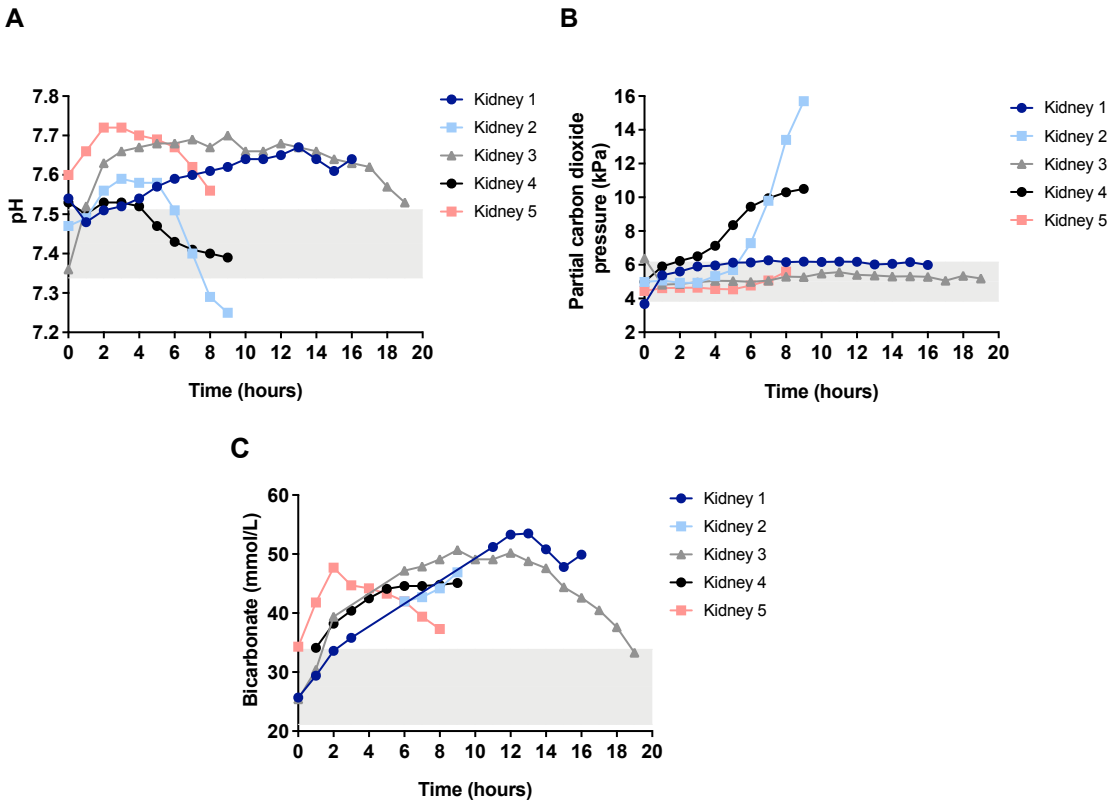


Figure 3.23. Acid-base balance for kidneys perfused with RBC-plasma perfusion solution during NMP. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with an RBC-plasma based perfusate. (A) pH varied between kidneys and was predominantly alkalotic. (B) Partial carbon dioxide pressure increased in kidney 2 and 4. All kidneys had higher (C) perfusate bicarbonate levels than physiological values. Grey area represents physiological range in pigs.[24,25] HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; RBC, red blood cell.

All five kidneys had higher perfusate sodium than physiological values throughout perfusion with mean values ranging from 147mmol/L–151mmol/L (Figure 3.24A). Initial perfusate potassium levels were higher than the physiological range (8.5 ± 0.5mmol/L), and decreased during the first hour of perfusion and remained in physiological range for kidneys 1 and 5. This was similar for kidney 3 except for the last three hours of NMP (Figure 3.24B). Ionized calcium was in physiological range for all five kidneys throughout perfusion (Figure 3.24C). Perfusate phosphate increased during the first hour of perfusion and remained stable afterwards during NMP (Figure 3.24D).

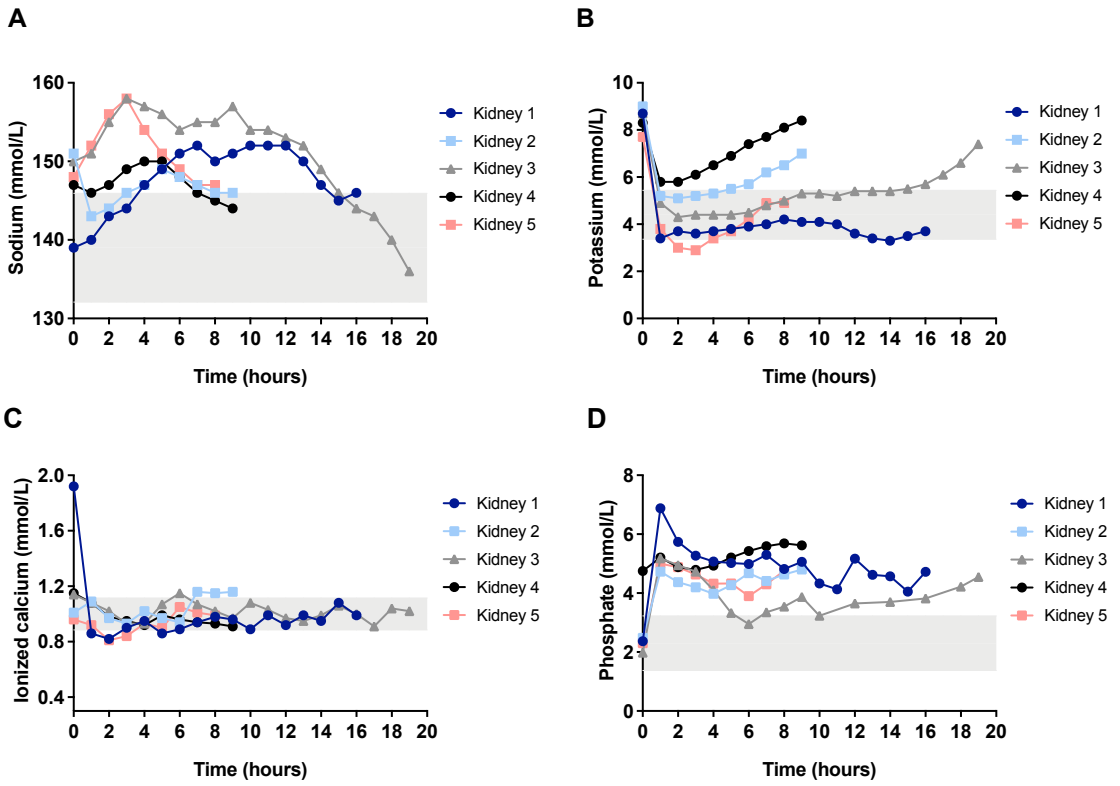


Figure 3.24. Perfusate electrolyte levels for kidneys perfused with RBC-plasma perfusion solution during NMP. Pig kidneys retrieved from an abattoir underwent two hours of HMP and were perfused on NMP with an RBC-plasma based perfusate. (A) Perfusate sodium levels were higher than physiological values. (B) Potassium decreased from baseline during the first hour and remained in physiological range for kidneys 1, 3 and 5. (C) Ionized calcium remained stable in all kidneys during NMP and (D) phosphate increased during the first hour of perfusion and remained stable afterwards. Grey area represents physiological range in pigs.[23, 24] HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; RBC, red blood cell.

3.4 DISCUSSION

This chapter describes the development and subsequent evolution of the NMP protocol adopted in this thesis to achieve prolonged kidney perfusion in a porcine slaughterhouse model. The experiments described involved comparisons between different NMP circuits, comparison of different vasodilators and comparison between different antibiotic doses. In addition, the optimisation of an RBC-albumin based perfusion solution with stepwise adjustments and improvements is described.

At the beginning of the establishment of the NMP model we first focused on the comparison between the use of an open or closed perfusion circuit. Studies have found that both systems can be used during NMP.[6-9] In the open circuit, blood returning from the kidney via the renal vein drains directly into a reservoir without resistance. In the closed circuit the renal vein is cannulated and the blood remains in the circuit under constant positive pressure, which is maintained by height regulation of the venous reservoir.[25] Flow values and renal resistance did not differ significantly between kidneys perfused in the open and closed circuits, and this was also the case in other studies using slaughterhouse kidneys with a similar protocol.[9, 15, 16, 26] There was a notable, albeit not statistically significant, higher cumulative urine output in the closed circuit in comparison to the open circuit. The closed circuit increases pressure across the glomerulus which may help filtration and may therefore result in a higher urine output. There was no difference in cellular damage markers such as LDH or haemolysis between groups. Given that there was no significant difference between the two circuit types, an open circuit was selected for further research use as it was more straightforward to assemble and less problematic during NMP compared to the closed circuit.

In the second part of this study we focused on the optimisation of a mixture of human albumin and washed autologous RBC-based perfusate. Obtaining a good perfusate

solution is essential for optimal perfusion. Pig renal arteries have the tendency to vasospasm[27] and the addition of a vasodilator can relax the blood vessels and increase the blood flow in the kidney. Work by Hosgood *et al.* and Kaths *et al.* has shown that SNP and verapamil are both options as vasodilators during pig kidney NMP. [7, 14, 15] SNP is a fast-acting vasodilator with a half-life of around two minutes.[28] It dilates arterioles and venous capacitance vessels by interacting with sulfhydryl groups in erythrocytes where it generates cyanide ions and nitric oxide (NO).[28] NO, in turn, reduces peripheral resistance and relaxes vascular smooth muscle cells. Verapamil is a voltage-dependent calcium channel blocker with a plasma half-life of three to six hours.[29] Calcium channels are present in the smooth muscle cells of blood vessels and are responsible for regulating the influx of calcium into muscle cells. Calcium plays a central role in contraction of vascular smooth muscle cells and regulation of blood flow and blocking of calcium channels dilates the blood vessels.[29-31] Intravenous administration of verapamil hydrochloride is primarily metabolised in the liver and approximately 70% of the administered dose is excreted in the urine.[29, 32] We were also aware that the Groningen group did not use any vasodilator during pig kidney NMP[33] and so we set out to establish whether, in our model, a vasodilator was necessary and if so which one was the best choice. We found that the addition of a vasodilator during NMP did not have a significant effect on the renal blood flow or renal resistance. This is in line with the Groningen model[33] mentioned above, but is in contrast with results of NMP of DCD livers, where a vasodilator appears to be vital for optimal microcirculatory support.[13] Kidneys perfused in the SNP group had significantly higher lactate and lower pH than kidneys in the verapamil and no vasodilator groups. In normal physiology, the cyanide produced by SNP is converted to thiocyanate by rhodanese in the liver, and then excreted by the kidney. However, with prolonged administration of SNP, or in patients with hepatic impairment or renal insufficiency, free cyanide may accumulate and bind to ferric ions on cytochrome C. This can result in uncoupling of mitochondrial oxidative phosphorylation and inhibition of cellular respiration, resulting in metabolic acidosis.[34, 35] Given that there

was no liver present on the NMP circuit the lactic acidosis we observed in kidneys perfused with SNP was likely due to the cumulative effect of cyanide on mitochondrial respiration. A similar pattern was also observed in perfusions of open and closed circuits where SNP was used as vasodilator. Several studies have added SNP as a vasodilator during pig kidney NMP without observing acid-base imbalance nor significant increase in lactate, but the majority were perfusions of only short duration.[9, 15, 26] Even though we acknowledge that the sample size in each group is small and randomisation of the groups was not possible, as this was an observational study where the perfusion protocol evolved over time as experience accrued, we did observe similar lactic acidosis in an unpublished series of 10 kidneys undergoing NMP with SNP as vasodilator. We also acknowledge that the kidneys perfused in the vasodilator comparisons were conducted in the initial phase of the optimisation of the perfusion protocol and achieving prolonged perfusion at this stage was still not feasible.

The majority of kidney NMP experimental models have included the addition of an antibiotic to the perfusion circuit.[8, 9, 15, 16] Bacterial contamination in the organ donor's perfusion fluid is common and contamination during the procurement seemed to be the primary cause. [36-38] However, the only currently ongoing randomised controlled trial of human kidney NMP for one hour does not include the addition of antibiotics to the perfusion circuit.[6] The conditions in human kidney NMP are sterile, the perfusion is performed in the operating theatre and the patient receives antibiotics at induction of anaesthesia. However, a recent clinical study showed 56% (n=9) of positive bacterial growth in perfusion fluid of kidneys undergoing NMP. [39] In experimental research using slaughterhouse animals the conditions are not sterile and contamination during the retrieval process is inevitable. Therefore, for prolonged perfusion of slaughterhouse pig kidneys the use of antibiotics during NMP, to minimise bacterial proliferation, is logical. However, the pharmacology of antibiotics during NMP is currently unknown. In an isolated circuit without the presence of a liver and normal antibiotic breakdown and

excretion, conventional doses may be nephrotoxic.[17] Furthermore, given the intent to use this system as a delivery platform for MSC, it was important to also consider the effect such antibiotic doses have on MSC. *In vitro* studies by Sierra-Parraga *et al.* (personal communication) showed that standard dose antibiotic concentration of 1000 mg/200 mg amoxicillin-clavulanate, used by other groups during NMP, lowered the viability of MSC by 50% after 30 minutes when in suspension in the perfusion solution. MSC viability increased to approximately 90% when the concentration of amoxicillin-clavulanate was diluted 100x. Thus, as part of this study, we wanted to establish whether reducing the dose of antibiotic would lead to excessive microbial contamination and compromise the perfusion. Kidneys perfused in the low antibiotic dose group performed worse during NMP than kidneys in the bolus and standard dose groups, presenting reduced renal blood flow and higher QAS scores. There was no difference between cellular injury markers LDH and AST in the perfusate. The measured concentration of antibiotic in the low dose group of 160 μg/mL was significantly lower than the minimum inhibitory concentration of > 256 μg/mL for a number of the micro-organisms cultured from the perfusate. Data on the extent of bacterial growth was not available for all antibiotic doses, but results from the bolus dose experiments showed that the MIC was reached for all of the cultured micro-organisms, however one species of *pseudomonas* continued to proliferate, which is likely to be due to resistance to amoxicillin-clavulanate.[38] Other broad-spectrum antibiotics can be added during NMP instead of amoxicillin-clavulanate. There was no clear difference in perfusion characteristics between a standard dose of antibiotic given at the beginning of perfusion and bolus doses given throughout perfusion, although the concentration of 3343 μg/mL in the SD was unnecessarily high.

In addition to our standard perfusate solution, we also developed a RBC-plasma based perfusate, with the idea to combine the advantages of both the leucocyte depleted blood whilst retaining its plasma component and the RBC-albumin based compositions as this

might be more *physiological*. The difference between the leucocyte depleted blood and RBC-plasma perfusate is that the use of Ringer's solution can be avoided and the RBCs can be re-suspended in plasma to achieve a haematocrit of 25%. The use of plasma might be beneficial in maintaining a more physiological oncotic pressure than albumin on its own. Plasma contains albumin and globulins to maintain osmotic pressure, electrolytes to help maintain blood pH and immunoglobulins to fight infection and therefore beneficial for NMP conditions.[40] All kidneys perfused with the RBC-plasma perfusate perfused for a minimum of 8 hours without too much difficulty and the longest duration of NMP was even 19 hours. Albumin is one of the proteins that determines oncotic pressure and has the ability to bind and transport a wide range of substances, scavenge free radicals and protect the endothelial surface layer.[41] The RBC-plasma perfusate had a similar composition of albumin to the perfusate used by Weissenbacher *et al.* who demonstrated a series of 24 hour perfusions of human discarded kidneys.[8] Kidney 2 in our series appeared to consume less oxygen than the rest of the kidneys and in kidney 3 oxygen consumption rapidly decreased in the last three hours of NMP. Injury markers LDH and AST were similar in all kidneys and both increased with perfusion time and this was in line with studies that used leucocyte depleted blood during NMP of slaughterhouse pig kidneys.[9, 42] Lactate was stable and in physiological range in four kidneys until the end of perfusion, regardless of the duration of NMP. Electrolytes were stable during perfusion and in physiological range for kidneys 1, 3 and 5, except for perfusate sodium with values higher than physiological range for all five kidneys. The pH was predominantly alkalotic throughout perfusion for kidneys 1, 3 and 5 whereas pH for kidneys 2 and 4 rapidly decreased overtime with an increasing partial pressure of carbon dioxide. In normal circumstances the body regulates pH via the respiratory system, by alternating the partial pressure of carbon dioxide, and via the kidneys by reabsorption, secretion, and in some circumstances excretion of bicarbonate.[43] NMP is a pseudo-physiological *ex situ* system without lungs, and the regulation of the acid-base balance depends solely on the metabolic activity of the kidney. As perfusate bicarbonate

levels are higher than physiological values, contributing to alkalosis, and despite having a constant gas mixture of oxygen and carbon dioxide, an increase in partial carbon dioxide pressure was observed which resulted in a more acidic pH.

A current issue in kidney NMP is what parameters and/or criteria to use to assess kidney quality during perfusion. In this study organ viability was assessed according to a previously published QAS.[21] This is a score based on the renal blood flow, macroscopic appearance of the kidney, and the urine output, however, it has only been validated for use after one hour of NMP and only in human kidneys. Kidneys perfused with SD and BD of amoxicillin-clavulanate as well as the kidneys perfused with the RBC-plasma perfusate had a score of 3 or less which, if taken in a clinical context, means they could be considered for transplantation. None of the kidneys were transplanted and the score was used as a means of comparison, rather than to demonstrate they were suitable for transplant. Urine production has also been considered, by some, as an indicator of kidney quality. In general, across all experiments, there was only minimal urine production and a relatively large difference in cumulative urine production between the kidneys. Urine production may be influenced by the composition of the colloid in the perfusion solution, but the variable nature of slaughterhouse kidneys makes it difficult to be conclusive. Urine production in itself should not be considered synonymous with kidney function, as no creatinine clearance nor fractional excretion of sodium can be observed when measured in subsequent experiments (data not shown). Functional parameters used in the literature such as creatinine clearance, fractional excretion of sodium, and total filtered load all depend on urine production.[9, 16, 26, 44] Previous studies have also concluded that slaughterhouse models are better to allow investigation of early cellular and molecular changes and that renal function during *ex vivo* NMP is not a reliable marker for viability.[9, 45] The latter was demonstrated by Kaths *et al.* who showed in a porcine DCD auto-transplant model, that there was no correlation between the amount of urine produced during NMP and graft function after transplantation.[46]

Proteinuria is a consequence of two mechanisms: abnormal trans-glomerular passage of proteins, due to increased permeability of the glomerular capillary wall (glomerular proteinuria) and subsequent impaired reabsorption by the epithelial cells of the proximal tubuli (tubular proteinuria).[47, 48] Under physiological circumstances more than 99% of filtered proteins are reabsorbed in the tubules.[49] One of the main causes of tubular proteinuria after transplantation is ischaemia-reperfusion injury.[50] Currently, there is only limited data on urinary protein loss during kidney NMP. Hamar *et al.* mentioned minimal protein urinary loss in a DCD porcine auto-transplant model but no values were reported.[51] Venema *et al.* reported reduced proteinuria in slaughterhouse porcine kidneys perfused with HMP in comparison to SCS prior to NMP. Urinary protein levels ranged from 6–10 g/L and decreased to 4 g/L after four hours of NMP with the Hosgood *et al.* leucocyte depleted blood perfusate protocol.[9] In our study proteinuria was quite high in comparison with normal physiological values and the urinary protein levels by Venema *et al*., especially in kidneys perfused with the SD of amoxicillin-clavulanate.[9] The MePEP perfusate contains albumin to maintain oncotic pressure. Losing high amounts of protein in the urine, high proteinuria, and replacing urine volume with Ringers solution might further decrease the albumin concentration in the perfusate, lowering oncotic pressure which can further result in oedema and unstable perfusion of the donor kidney. Additionally, Weissenbacher *et al.* demonstrated that urine recirculation can help, maintaining a physiological pH and electrolyte balance.[8] For the reasons above and to avoid loss of albumin in the urine, we decided to recirculate urine in future experiments.

A limitation of using a slaughterhouse model is that the conditions of the kidneys and the injury sustained are less controlled than in the animal house setting which contributes to variability between animals and within groups.[45] The definition of warm ischaemia time is also different in a commercial slaughterhouse setting to that of laboratory animals, as there is no definite end of blood flow as exsanguination continues with decreasing blood volume and a beating heart. In addition, exsanguinated pigs undergo a hot wash in water

of around 60°C for about five minutes, which compounds the warm ischaemia duration and its severity. Nevertheless, as we have demonstrated, the slaughterhouse model will provide viable kidneys as well as autologous blood to be successfully and reproducibly used in normothermic perfusion experiments, establishing a basis for translational research.[4, 14] The use of the slaughterhouse model is advantageous as the organs are easily accessible, it avoids the use of laboratory animals, which is costly, and more importantly takes ethical considerations and animal welfare into account.

In summary, in this study we have demonstrated that NMP of pig kidneys can be performed both on an open or closed circuit and without the addition of a vasodilator. The use of SNP as vasodilator caused worsening lactic acidosis, potentially by accumulation of its toxic metabolite cyanide and it should probably be avoided. Furthermore, stable kidney NMP with an erythrocyte-albumin based solution can be performed for a minimal of seven hours using a porcine slaughterhouse model, maintaining an appropriate acid-base balance, concentration of electrolytes as well as metabolic parameters. On the basis of these results MePEP perfusate 3.0 with BD antibiotic administration and urine recirculation was used in all subsequent MSC related experiments.

REFERENCES

1. Brat A, Pol RA, Leuvenink HG. Novel preservation methods to increase the quality of older kidneys. *Curr Opin Organ Transplant*. 2015;20:438-443.

2. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? *Transpl Int*. 2015;28:657-664.

3. Yong C, Hosgood SA, Nicholson ML. Ex-vivo normothermic perfusion in renal transplantation: past, present and future. *Curr Opin Organ Transplant*. 2016;21:301-307.

4. Grosse-Siestrup C, Unger V, Fehrenberg C, et al. A model of isolated autologously hemoperfused porcine slaughterhouse kidneys. *Nephron*. 2002;92:414-421.

5. Golriz M, Fonouni H, Nickkholgh A, Hafezi M, Garoussi C, Mehrabi A. Pig kidney transplantation: an up-to-date guideline. *Eur Surg Res*. 2012;49:121-129.

6. Hosgood SA, Saeb-Parsy K, Wilson C, Callaghan C, Collett D, Nicholson ML. Protocol of a randomised controlled, open-label trial of ex vivo normothermic perfusion versus static cold storage in donation after circulatory death renal transplantation. *BMJ Open*. 2017;7:e012237-012237.

7. Kaths JM, Spetzler VN, Goldaracena N, et al. Normothermic Ex Vivo Kidney Perfusion for the Preservation of Kidney Grafts prior to Transplantation. *J Vis Exp*. 2015;(101):e52909. doi:e52909.

8. Weissenbacher A, Lo Faro L, Boubriak O, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. *Am J Transplant*. 2019;19:178-192.

9. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation*. 2019;103:2057-2064.

10. Scallan J, Huxley VH, Korthuis RJ. Chapter 4. pathophysiology of edema formation. In: *Capillary Fluid Exchange: Regulation, Functions, and Pathology. .* Morgan & Claypool Life Sciences; 2010.

11. Arykbaeva AS, De Vries DK, Doppenberg JB, Huurman VAL, Minnee RC, Moers C, Pol RA, Ploeg RJ, Alwayn IPJ. Prolonged Normothermic Machine Perfusion of Human Discarded Donor Kidneys: First Results of the PROPER Study. *Transplant International.* 2019:25.

12. Pool M. *Towards ex vivo repair of damaged donor kidneys.* University of Groningen; 2020.

13. Nassar A, Liu Q, Farias K, et al. Role of vasodilation during normothermic machine perfusion of DCD porcine livers. *Int J Artif Organs*. 2014;37:165-172.

14. Hosgood S, Harper S, Kay M, Bagul A, Waller H, Nicholson ML. Effects of arterial pressure in an experimental isolated haemoperfused porcine kidney preservation system. *Br J Surg*. 2006;93:879-884.

15. Hosgood SA, Barlow AD, Yates PJ, Snoeijs MG, van Heurn EL, Nicholson ML. A pilot study assessing the feasibility of a short period of normothermic preservation in an experimental model of non heart beating donor kidneys. *J Surg Res*. 2011;171:283-290.

16. Harper S, Hosgood S, Kay M, Nicholson M. Leucocyte depletion improves renal function during reperfusion using an experimental isolated haemoperfused organ preservation system. *Br J Surg*. 2006;93:623-629.

17. Phillips BL, Chandak P, Uwechue R, van Nispen Tot Pannerden, C., Hemsley C, Callaghan CJ. Microbial Contamination During Kidney Ex Vivo Normothermic Perfusion. *Transplantation*. 2018;102:e186-e188.

18. Belzer FO. Renal preservation. *N Engl J Med*. 1974;291:402-404.

19. Belzer FO, Hoffman RM, Southard JH. Kidney preservation. *Surg Clin North Am*. 1978;58:261-271.

20. Idexx Laboratories. Microbiology guide to interpreting minimum inhibitory concentration (MIC). 2020.

21. Hosgood SA, Barlow AD, Hunter JP, Nicholson ML. Ex vivo normothermic perfusion for quality assessment of marginal donor kidney transplants. *Br J Surg*. 2015;102:1433-1440.

22. Ronco C, Bellomo R, Kellum JA. Chapter 116. arterial and venous blood gases - oxygen content. In: *Critical Care in Nephrology.* 2nd ed. Saunders; 2008:608.

23. Hannon JP, Bossone CA, Wade CE. Normal physiological values for conscious pigs used in biomedical research. *Lab Anim Sci*. 1990;40:293-298.

24. Laber KE, Whary MT, Bingel SA, Goodrich JA, Smith AC, Swindle MM. Chapter 15 - biology and diseases of swine. In: *Laboratory Animal Medicine.* Academic Press:615-673.

25. Kaths JM, Echeverri J, Goldaracena N, et al. Eight-Hour Continuous Normothermic Ex Vivo Kidney Perfusion Is a Safe Preservation Technique for Kidney Transplantation: A New Opportunity for the Storage,

Assessment, and Repair of Kidney Grafts. *Transplantation*. 2016;100:1862-1870.

26. Hosgood SA, Shah K, Patel M, Nicholson ML. The effect of prolonged of warm ischaemic injury on renal function in an experimental ex vivo normothermic perfusion system. *J Transl Med*. 2015;13:207-4.

27. Kaths JM, Echeverri J, Goldaracena N, et al. Heterotopic Renal Autotransplantation in a Porcine Model: A Step-by-Step Protocol. *J Vis Exp*. 2016;(108):53765. doi:53765.

28. Friederich JA, Butterworth JF,4th. Sodium nitroprusside: twenty years and counting. *Anesth Analg*. 1995;81:152-162.

29. Infomed Ltd. Verapamil Pharmacology. 2020.

30. Hamann SR, Blouin RA, McAllister RG,Jr. Clinical pharmacokinetics of verapamil. *Clin Pharmacokinet*. 1984;9:26-41.

31. Katz AM. Pharmacology and mechanisms of action of calcium-channel blockers. *J Clin Hypertens*. 1986;2:28S-37S.

32. Mylan. Securon 2.5 mg/ml IV Intravenous Injection. 2020.

33. Pool MBF, Hartveld L, Leuvenink HGD, Moers C. Normothermic machine perfusion of ischaemically damaged porcine kidneys with autologous, allogeneic porcine and human red blood cells. *PLoS One*. 2020;15:e0229566.

34. Schulz V. Clinical pharmacokinetics of nitroprusside, cyanide, thiosulphate and thiocyanate. *Clin Pharmacokinet*. 1984;9:239-251.

35. Baud FJ, Haidar MK, Jouffroy R, Raphalen JH, Lamhaut L, Carli P. Determinants of Lactic Acidosis in Acute Cyanide Poisonings. *Crit Care Med*. 2018;46:e523-e529.

36. Yahav D, Manuel O. Clinical relevance of preservation-fluid contamination in solid-organ transplantation: a call for mounting the evidence. *Clin Microbiol Infect*. 2019;25:536-537.

37. Corbel A, Ladrière M, Le Berre N, et al. Microbiological epidemiology of preservation fluids in transplanted kidney: a nationwide retrospective observational study. *Clin Microbiol Infect*. 2020;26:475-484.

38. Oriol I, Sabé N, Tebé C, Veroux M, Boin, I. F. S. F., Carratalà J. Clinical impact of culture-positive preservation fluid on solid organ transplantation: A systematic review and meta-analysis. *Transplant Rev (Orlando)*. 2018;32:85-91.

39. Phillips BL, Chandak P, Uwechue R, van Nispen Tot Pannerden, C., Hemsley C, Callaghan CJ. Microbial Contamination During Kidney Ex Vivo Normothermic

Perfusion. *Transplantation*. 2018;102:e186-e188.

40. Mathew J, Sankar P, Varacallo M. Physiology, blood plasma. In: *StatPearls.* Treasure Island (FL): StatPearls Publishing LLC; 2020.

41. Martensson J, Bellomo R. Are all fluids bad for the kidney? *Curr Opin Crit Care*. 2015;21:292-301.

42. Bagul A, Hosgood SA, Kaushik M, Kay MD, Waller HL, Nicholson ML. Experimental renal preservation by normothermic resuscitation perfusion with autologous blood. *Br J Surg*. 2008;95:111-118.

43. Hamm LL, Nakhoul N, Hering-Smith KS. Acid-Base Homeostasis. *Clin J Am Soc Nephrol*. 2015;10:2232-2242.

44. Mancina E, Kalenski J, Paschenda P, et al. Determination of the preferred conditions for the isolated perfusion of porcine kidneys. *Eur Surg Res*. 2015;54:44-54.

45. Hosgood SA, Nicholson ML. Oxygen Supplementation Supports Energy Production During Hypothermic Machine Perfusion in a Model of Donation After Circulatory Death Donors. *Transplantation*. 2019;103:1980-1981.

46. Kaths JM, Hamar M, Echeverri J, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. *Am J Transplant*. 2018;18:580-589.

47. D'Amico G, Bazzi C. Pathophysiology of proteinuria. *Kidney Int*. 2003;63:809-825.

48. Haraldsson B, Nystrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. *Physiol Rev*. 2008;88:451-487.

49. Bockenhauer D, Kleta R. Approach to the patient with renal fanconi syndrome, glycosuria, or aminoaciduria. In: Turner N, Lameire N, Goldsmith D, et al, eds. *Oxford Textbook of Clinical Nephrology.* 4th edition ed. Oxford: Oxford University Press; 2015.

50. Ponticelli C, Graziani G. Proteinuria after kidney transplantation. *Transpl Int*. 2012;25:909-917.

51. Hamar M, Urbanellis P, Kaths MJ, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. *Transplantation*. 2018;102:1262-1270.

APPENDIX: SUPPLEMENTARY INFORMATION

Table S3.1. Perfusate electrolytes and blood gases baseline values in the vasodilator study groups comparing verapamil, sodium nitroprusside (SNP) and no vasodilator. Perfusate baseline blood gas analysis and perfusate albumin concentration. Data are presented as mean ± standard deviation. pCO_2, partial carbon dioxide pressure; pO_2, partial oxygen pressure; Hb, haemoglobin; Hct, haematocrit; Na+, sodium; K+, potassium; Ca^{2+}, calcium.

	Verapamil (n=4)	SNP (n=3)	No vasodilator (n=4)	p-value
pH	7.43 ± 0.08	7.43 ± 0.11	7.53 ± 0.12	0.305
pCO_2 (kPa)	4.07 ± 0.62	4.17 ± 0.64	3.26 ± 0.72	0.182
pO_2 (kPa)	74.1 ± 6.3	58.8 ± 37.1	50.0 ± 35.4	0.916
Hb (g/L)	111 ± 36	91 ± 14	98 ± 16	0.331
Hct (%)	33 ± 11	28 ± 4	29 ± 5	0.970
O_2 saturation (%)	101.6 ± 0.5	102.0 ± 0	101.7 ± 0.6	0.709
Na^+ (mmol/L)	137 ± 2	152 ± 5	144 ± 6	**0.005***
K^+ (mmol/L)	2.2 ± 1.0	1.7 ± 0.2	2.1 ± 1.0	0.943
ionized Ca^{2+} (mmol/L)	1.18 ± 0.17	1.40 ± 0.22	1.32 ± 0.09	0.252
Glucose (mmol/L)	3.9 ± 0.5	4.4 ± 0.7	4.8 ± 0.7	0.161
Lactate (mmol/L)	0.3 ± 0.5	0.3 ± 0.2	0.2 ± 0.1	0.801
Albumin (g/L)	32 ± 5	31 ± 1	30 ± 2	0.867

*SNP group significant higher than the verapamil group

Table S3.2. Perfusate electrolytes and blood gases baseline values in the low dose (LD), standard dose (SD) and bolus dose (BD) antibiotic groups. Perfusate baseline blood gas analysis and perfusate albumin concentration. Data are presented as mean ± standard deviation (n=4/group). pCO_2, partial carbon dioxide pressure; pO_2, partial oxygen pressure; Hb, haemoglobin; Hct, haematocrit; Na+, sodium; K+, potassium; Ca^{2+}, calcium.

	Low dose (n=4)	Standard dose (n=4)	Bolus dose (n=4)	p-value
pH	7.43 ± 0.08	7.43 ± 0.11	7.32 ± 0.04	0.149
pCO_2 (kPa)	4.07 ± 0.62	4.31 ± 0.83	4.59 ± 0.39	0.536
pO_2 (kPa)	74.1 ± 6.3	84.1 ± 1.2	83.9 ± 3.4	**0.013***
Hb (g/L)	111 ± 36	95 ± 4	100 ± 11	0.949
Hct (%)	33 ± 11	29 ± 1	31 ± 3	0.861
O_2 saturation (%)	101.6 ± 0.5	101.6 ± 0.5	102.0 ± 0	0.418
Na^+ (mmol/L)	137 ± 2	140 ± 5	138 ± 4	0.840
K^+ (mmol/L)	2.2 ± 1.0	3.8 ± 0.9	1.5 ± 0.3	**0.007****
ionized Ca^{2+} (mmol/L)	1.18 ± 0.17	1.21 ± 0.21	1.27 ± 0.09	0.727
Glucose (mmol/L)	3.9 ± 0.5	5.0 ± 1.2	5.0 ± 0.7	0.159
Lactate (mmol/L)	0.3 ± 0.5	0.2 ± 0.1	0.3 ± 0.3	0.594
Albumin (g/L)	32 ± 5	34 ± 1	38 ± 4	0.075

*Low dose group significant lower than the standard dose and bolus dose groups,**standard dose significant higher than the low dose and bolus dose groups.

Table S3.3. Isolated bacteria in the perfusate and minimum inhibitory concentration (MIC) values for amoxicillin-clavulanate during NMP with low dose (LD), standard dose (SD) and bolus dose (BD) amoxicillin-clavulanate administration.

Experimental group	Kidney	Timepoint (hours)	Bacteria isolated	Growth	Amoxicillin-clavulanate MIC
Low dose 60 mg amoxicillin-clavulanate	LD1	T0	*Stenotrophomonas Maltophilia*	N/A	N/A
			Pseudonomas Aeruginosa	N/A	N/A
			Acinetobacter Pitti	N/A	N/A
			Pseudomonas Synxantha	N/A	N/A
		T1	*Stenotrophomonas Maltophilia*	N/A	N/A
			Pseudomonas Synxantha	N/A	N/A
		T4	*Stenotrophomonas Maltophilia*	N/A	N/A
			Pseudomonas Synxantha	N/A	N/A
Standard dose 1200 mg amoxicillin-clavulanate	SD 1	T0	*Klebsiella Oxytoca*	N/A	N/A
			Stenotrophomonas Maltophilia	N/A	N/A
		T1	*Klebsiella Oxytoca*	N/A	4
			Acinetobacter Pitti	N/A	N/A
		T7	*Acinetobacter Pitti*	N/A	16
Bolus dose 300 mg amoxicillin-clavulanate at baseline and additional 60 mg/h	BD 1	T0	*Burkholderia Cepacia complex*	moderate	4
			Delftia Lacustris	moderate	2
			Brevundimonas Diminuta	moderate	0.5
			Stenotrophomas Species	moderate	256
			Pseudomonas Fluorescens	moderate	64
		T1	*Burkholderia Cepacia complex*	moderate	N/A
			Delftia Lacustris	moderate	N/A
			Agromyces Mediolanus	moderate	16
			Sphingobacterium Multivorum	moderate	16
			Pseudonomas Chloraphis	moderate	256
		T7	*Acinetobacter Bereziniae*	scanty	32
			Stenotrophomas Maltophilia	N/A	N/A
			Brevundimonas Dimunta	moderate	256
			Pseudonomas Tolaasii	moderate	N/A
	BD 2	T0	*Stenotrophomonas Maltophilia*	moderate	N/A

(Continues)

Table S3.3. (Continued)

Experimental group	Kidney	Timepoint (hours)	Bacteria isolated	Growth	Amoxicillin-clavulanate MIC
			Microbacterium Oxydans	moderate	N/A
			Pseudomonas Synxantha	moderate	N/A
			Stenotrophomonas Acidaminiphilla	moderate	N/A
			Sphingobacterium Multivorum	moderate	N/A
		T1	*Pseudomonas Synxantha*	profuse	N/A
			Delftia Lacustris	scanty	N/A
		T5	*Pseudomonas Poae*	profuse	N/A
			Stenotrophomonas Maltophilia	moderate	N/A
		T7	*Pseudonomas Cerdina*	profuse	N/A
			Stenotrophomonas Maltophilia	scanty	N/A

Table S3.4. Perfusate baseline electrolyte and blood gases values in the RBC-plasma perfusion solution. Perfusate baseline blood gas analysis. Data are presented as mean ± standard deviation. pCO_2, partial carbon dioxide pressure; pO_2, partial oxygen pressure; Hb, haemoglobin; Hct, haematocrit Na+, sodium; K+, potassium; Ca^{2+}, calcium.

	RBC-plasma perfusion solution (n=5)
pH	7.50 ± 0.09
pCO_2 (kPa)	4.90 ± 0.99
pO_2 (kPa)	74.3 ± 28.6
Hb (g/L)	85 ± 3.4
Hct (%)	26.0 ± 1.0
O_2 saturation (%)	101.2 ± 0.5
Na^+ (mmol/L)	147 ± 5
K^+ (mmol/L)	8.5 ± 0.5
ionized Ca^{2+} (mmol/L)	1.24 ± 0.39
Glucose (mmol/L)	6.4 ± 1.2
Lactate (mmol/L)	5.9 ± 3.4

Chapter 4

Comparison of *ex vivo* kidney perfusion using red blood cells, a synthetic haemoglobin-based oxygen carrier and without an oxygen carrier

ABBREVIATIONS

AST	aspartate aminotransferase
AUC	area under the curve
ELISA	enzyme linked immunosorbent assay
FENa	fractional excretion of sodium
HBOC	haemoglobin based oxygen carriers
HI	haemolysis index
LDH	lactate dehydrogenase
metHb	methaemoglobin
MDA	malondialdehyde
PAS	periodic acid-Schiff
NADH	nicotinamide adenine dinucleotide hydrogen
NGAL	neutrophil gelatinase-associated lipocalin
NMP	normothermic machine perfusion
RBC	red blood cell
RT	room temperature
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
UW-CSS	Belzer UW® Cold Storage Solution

4.1 INTRODUCTION

With a growing number of studies focussing on the use of normothermic machine perfusion (NMP) as a method to preserve, assess and resuscitate donor kidneys, there is a need for developing a medical grade perfusion solution with a standardised composition.[1-5] In Chapter 3, we established and optimised an erythrocyte and albumin based perfusion solution for NMP of porcine kidneys. However, its focus was on the optimisation of a red blood cell (RBC)-based perfusion solution and not so much on the oxygen carrier capacity of the perfusate. Most NMP studies have relied on a RBC-based perfusion solution, with RBCs being utilised as the oxygen carriers to deliver oxygen to the tissues.[6-10] Although the use of RBCs resembles normal physiology, it has several limitations. Firstly, RBCs need to be retrieved from human donors and they have a relatively short shelf life as their capacity to release oxygen will diminish after processing and storage at low temperatures within days. Secondly, there is a risk of infection and transmission of blood-borne infections.[11-13] In addition, prolonged *ex vivo* perfusion either by the use of centrifugal or roller pump devices can damage RBCs, leading to significant haemolysis.[14-16]

Acellular synthetic oxygen carriers were initially developed and tested as an alternative to packed RBCs transfusions[17, 18] and these haemoglobin-based oxygen carriers (HBOC) could be a promising alternative to RBCs during NMP. The polymerized bovine derived HBOC-201 also known as Hemopure® (HBO2 Therapeutics) is smaller than a human erythrocyte, is less viscous than RBCs, and has the ability to release oxygen more easily than human haemoglobin.[19] HBOC-201 has previously been studied on liver NMP in pre-clinical and clinical studies achieving promising results.[20-23] In a study with discarded human livers, NMP with either an HBOC-201-based perfusion fluid or RBCs enabled similar tissue oxygenation and yielded in similar perfusion outcomes, indicating HBOC-201 was a suitable alternative for RBCs.[20] HBOC-201 is universally compatible

and requires no tissue or blood group compatibility testing, it is readily accessible and is stable at room temperature (RT) for at least three years.[17, 18]

The aim of this chapter was to determine if an oxygen carrier is necessary for renal NMP and if HBOC-201 can act as an RBC substitute to carry and deliver oxygen to the kidney.

4.2 MATERIALS & METHODS

4.2.1 Study design

Pig kidneys were retrieved from anaesthetised animals and randomly allocated to one of three experimental groups (n=5 per group). Healthy kidneys were retrieved and cold flushed with 500 mL of Belzer UW® Cold Storage Solution (UW-CSS, Bridge to Life Ltd, Columbia, SC, USA). To mimic living kidney donation, warm ischaemia time was kept as short as possible. Subsequently, kidneys were cold stored in UW-CSS for two hours to allow for preparation of the perfusion solution. Next, the kidneys in the three groups were subjected to six hours of pulsatile NMP with a mean arterial pressure of 85 mmHg. The experimental groups are detailed below:

Group 1: Perfusion solution without an oxygen carrier (No Oxygen Carrier group);

Group 2: Standard perfusion solution, including allogeneic concentrated cross-matched porcine RBCs (RBC group);

Group 3: Perfusion solution (without RBCs) supplemented with HBOC-201 (Haemoglobin glutamer – 250 bovine, HbO2 Therapeutics, Souderton, PA, USA) (HBOC-201 group).

4.2.2 Ethics and animals

This study utilised right kidneys from 50 kg female laboratory pigs (Danish Landrace and Yorkshire crossbreed) that were under anaesthesia for another series of auto-transplantation experiments described in Chapter 6. All animal care and animal procedures followed local regulations and guidelines set by the European Union (directive 2010/63/EU). The study which led to the availability of these kidneys was approved by The Animal Experimentation Council of the University of Aarhus, Denmark (reference-number 2016-15-0201-01145).

4.2.3 Surgical procedure

To allow vein cannulation, the fasting pigs were sedated with intramuscular injection of midazolam (0.5 mg/kg). After induction of anaesthesia with intravenous administration of ketamine (6 mg/kg) and midazolam (0.5 mg/kg), intubation and ventilation followed, using a mixture of atmospheric air and oxygen (2:1) to maintain expiratory partial pressure of carbon dioxide between 4.5 and 5.5 kPa. Anaesthesia was maintained by sevoflurane (gas, maintaining minimum alveolar concentration between 1.1 and 1.3) and intravenously administrated remifentanyl (0.03 and 0.06 mg/kg/h) for analgesia. Monitoring included heart rate, respiratory rate, oxygen saturation, and rectal temperature, whereas continuous blood pressure monitoring (non-invasive blood pressure) was done by placing a child's cuff (DURA-CUF 2751, GE Healthcare, Brøndby, Denmark) on the right foreleg. Hourly venous blood gas levels of lactate, glucose, electrolytes and pH were monitored. One litre of Ringer's acetate (Fresenius Kabi, Bad Homburg, Germany) was intravenously administrated within the first hour and after the surgical procedure with an infusion rate of 500 mL/h to maintain hydration. In a sterile setting, a permanent semi-central venous catheter (5 Fr, 20 cm, Careflow, BD, NJ, USA) was placed through an ear vein into the jugular vein. After midline incision, a left retroperitoneal nephrectomy was performed for other experimental purposes. After closure of the midline incision the pig was returned to the stables. The next day the pig was anaesthetised again following the same procedures mentioned above and the right contralateral kidney, which was used in this study, was removed prior to auto-transplantation of the left kidney.

4.2.4 Normothermic machine perfusion

4.2.4.1 Perfusion circuit

Kidneys preserved during NMP were perfused with a pressure controlled perfusion system without venous cannulation. This setup was controlled by a custom-made electronic interface and software (LabVIEW Software, National Instruments Netherlands BV, Woerden, the Netherlands). The perfusion circuit consisted of an oxygenator with built-in heat exchanger (Terumo Denmark, Herlev, Denmark), a magnetic pump head (Medos Deltastream Pumpdrive DP2, Medos Medizintechnik AG, Stolberg, Germany) connected to a centrifugal pump (Deltastream DP2, Medos Medizintechnik) and a LifePort® organ chamber (Organ Recovery Systems, Illinois, USA) with a LifePort® Disposable Cannula 3mm Straight (Organ Recovery Systems). Flow was monitored using an ultrasonic clamp-on flow probe (Transonic Systems Europe BV, Elsloo, the Netherlands). Pressure was measured directly after the straight cannula using a pressure transducer (TruWave disposable pressure transducer, Edwards Lifesciences, Irvine, California, USA). Perfusate temperature was kept at 37°C and oxygenated with 0.5 L/min carbogen (95% O_2 / 5% CO_2, Air Liquide supplies, Aarhus University hospital, Aarhus, Denmark).

4.2.4.2 Collection of allogenic red blood cells

All experimental animals and blood donor pigs were blood typed prior to the start of this series of experiments (Ortho BioVue^{TM} Card, Ortho Clinical Diagnostics, Raritan, USA), and only blood type A pigs were used. Under sterile conditions, a 12 Fr central sheet (Radifocus® Introducer II, Terumo Europe, Leuven, Belgium) was inserted into the external jugular vein of porcine blood donors weighing between 60 to 80 kg. Blood was collected in quadruple-blood bags (Macopharma, Mouvaux, France), containing 63 mL of anticoagulant citrate-phosphate-dextrose solution. Subsequently, the blood was fractionated (Baxter Optipress II Blood Component Separator, Baxter, Chicago, USA).

Next, the RBCs were leucocyte filtered, transferred into bags containing the storage solution saline-adenine-glucose-mannitol and stored for a maximum of 14 days at 4°C. RBCs were washed twice in phosphate-buffered Saline (PBS, Dulbecco's, Thermo Fisher) one hour prior to the start of NMP.

4.2.4.3 Perfusion solutions

The components of the three different perfusion solutions are displayed in table 4.1. Each perfusate was supplemented with a bolus of 300 mg amoxicillin-clavulanate (Bowmed, Wrexham, United Kingdom) at baseline, with additional doses of 60 mg amoxicillin-clavulanate every hour during NMP. A bolus dose of 1.25 mg verapamil (Orion Pharma, Copenhagen, Denmark) was given at baseline and continuous infusion of 0.25 mg/h verapamil was administered once the kidney was connected. The total volume of each solution was 450 mL. The plasma (non-cellular) volume in the RBC group was 269 mL. To obtain a plasma volume of 450 mL in group 3 (No Oxygen Carrier), all components, apart from the RBCs, were multiplied by 1.67. To allow for comparison between the RBC group and HBOC-201 group we prepared an HBOC-201 based perfusate that had a similar electrolyte concentration, oncotic pressure and haemoglobin concentration as the RBC group. The calculated oncotic pressure[24] in the RBC group was 1.872 J/L and in the HBOC-201 group 1.868 J/L. Haemoglobin concentration was 89 g/L in the RBC group and 86 g/L in the HBOC-201 group.

Table 4.1. The composition of the perfusion solutions.

No Oxygen Carrier group	RBC group	HBOC-201 group
	181 mL of cross-matched allogeneic RBCs	277 mL HBOC-201 (HBO2 Therapeutics)
417.5 mL Albumen 50 g/L (Alburex, CSL Behring, Lyngby,Denmark)	250 mL Albumen 50 g/L (CSL Behring)	125 mL Albumen 50 g/L (CSL Behring)
		23.5 mL Albumen 200 g/L (CSL Behring)
5 mL 10% calcium gluconate (B. Braun, Melsungen, Germany)	3 mL 10% calcium gluconate (B. Braun)	1.3 mL 10% calcium gluconate (B. Braun)
8.35 mL 8.4% $NaHCO_3$ (B. Braun)	5 mL 8.4% $NaHCO_3$ (B. Braun)	9 mL 8.4% $NaHCO_3$ (B. Braun)
10 mL 5% glucose (B. Braun)	6 mL 5% glucose (B. Braun)	10 mL 5% glucose (B. Braun)
8 IU insulin (Novo Nordisk A/S, Bagsværd, Denmark)	5 IU insulin (Novo Nordisk A/S)	8 IU insulin (Novo Nordisk A/S)
16.7 mg mannitol (Sigma Aldrich)	10 mg mannitol (Sigma Aldrich)	16.7 mg mannitol (Sigma Aldrich)
1000 µmol/L creatinine (Sigma Aldrich)	1000 µmol/L creatinine (Sigma Aldrich)	1000 µmol/L creatinine (Sigma Aldrich)
Total plasma = 450 mL	Total plasma = 269 mL	Total plasma = 450 mL
Total volume = 450 mL	**Total volume = 450 mL**	**Total volume = 450 mL**

4.2.5 Perfusate and urine collection and analysis

4.2.5.1 Perfusion parameters, perfusate, urine and blood gas collection

During NMP, arterial and venous blood gas samples were analysed every 30 minutes on the ABL90 FLEX blood gas analyser (Radiometer Medical ApS, Brønshøj, Denmark) for pH, sodium, potassium, lactate, partial oxygen and carbon dioxide pressure, oxygen saturation and methaemoglobin (metHb) levels. Perfusate and urine samples were taken hourly, centrifuged at 1,800 x g for 12 minutes at 4°C and stored at -80°C for further analysis. Perfusion parameters such as renal perfusate flow, renal vascular resistance and urine production were monitored regularly.

4.2.5.2 Biochemistry perfusate and urine analysis

The concentration of creatinine and sodium were measured in the perfusate and urine with routinely automated test methodology carried out by the clinical biochemistry laboratory at University Medical Center Groningen in the Netherlands. In addition to the tests mentioned above, kidney injury markers lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured in perfusate samples. HBOC-201 samples required a tenfold dilution, as the intense red colour of these samples (caused by dissolved HBOC-201) would otherwise interfere with colorimetric-based routine analyses. Due to this issue, it proved impossible to measure several other standard markers such as LDH in the HBOC-201 samples. Creatinine clearance, fractional excretion of sodium (FENa) and total sodium transport were calculated to determine renal function during NMP.

4.2.5.3 Thiobarbituric acid reactive substances (TBARS) analysis

To quantify oxidative stress, thiobarbituric acid reactive substances (TBARS) (Lipid Peroxidation (MDA) Assay Kit, Sigma-Aldrich B.V., Zwijndrecht, the Netherlands) were measured according to the manufacturer's protocol. TBARS including malondialdehyde (MDA) are an end-product of lipid peroxidation. Lipid peroxidation has been used as an indirect measurement of oxidative damage induced by reactive oxygen species.[25] In this assay MDA reacts with thiobarbituric acid (TBA) to produce a colour (532 nm). 600 µL TBA was added to each standard and sample and incubated for one hour at 95°C followed by cooling to RT in an ice bath for 10 minutes. Each reaction mix was mixed with 300 µL of 1-butanol and 100 µL of 5 M NaCl to enhance the sensitivity of the assay. The samples were vortexed and then centrifuged for three minutes at 16,000 x g at RT. The 1-butanol was removed by heating on a hot block at 55°C. The pellet was resuspended in 200 µL of ultrapure water and added to a 96 well plate. The output was immediately measured at OD 532 nm on a microplate reader. A standard curve was obtained and the MDA concentration was determined by interpolation from the standard curve.

4.2.5.4 Neutrophil gelatinase-associated lipocalin (NGAL) analysis

Levels of neutrophil gelatinase-associated lipocalin (NGAL), one of the earliest induced proteins that indicate acute kidney injury[26], were determined in urine using an enzyme linked immunosorbent assay (NGAL pig ELISA kit, Enzo Life Sciences, Zandhoven, Belgium) according to the manufacturer's protocol. In brief, samples and a series of standards were added to a pre-coated 96-well plate with a monoclonal antibody specific for pig NGAL and incubated for one hour at RT. Wells were washed and afterwards incubated with biotinylated Pig-NGAL antibody for one hour at RT. Horseradish peroxidase (HRP)-Streptavidin was added after washing and again incubated for one hour at RT. After washing, hydrogen peroxide and tetramethylbenzidine (TMB) substrate

were added to the wells and the plate was incubated for 10 minutes at RT in the dark. The reaction was stopped with a stop solution and the optical density was measured at 450 nm using a spectrophotometer. A standard curve was also obtained and the NGAL concentration was determined by interpolation from the standard curve. We calculated the absolute amount of NGAL in the urine every hour by correcting it with the volume of urine produced.

4.2.5.5 Oxygen consumption calculation

Oxygen consumption (mL O_2/min per 100 g) was calculated with the following formulas[27,28]:

$$Oxygen\ consuption\ in\ the\ no\ oxygen\ carrier\ group: VO_2 = \frac{(pO_2\ arterial\ -\ pO_2\ \text{venous}) \text{ x Q}}{g} \text{ x } 100$$

$$Oxygen\ consuption\ in\ the\ RBC\ and\ HBOC-201\ group: VO_2 = \frac{(CaO_2\ -\ CvO_2) \text{ x Q}}{g} \text{ x } 100$$

$$RBC\ arterial\ oxygen\ content: CaO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ arterial) + (pO_2\ arterial\ x\ K))$$
$$RBC\ venous\ oxygen\ content: CvO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ venous) + (pO_2\ venous\ x\ K))$$

$$HBOC-201\ arterial\ oxygen\ content: CaO_2 = ((0.020286\ x\ Hb\ x\ SO_2\ arterial) + (pO_2\ arterial\ x\ K))$$
$$HBOC-201\ venous\ oxygen\ content: CvO_2 = ((0.020286\ x\ Hb\ x\ SO_2\ venous) + (pO_2\ venous\ x\ K))$$

The number 0.024794 is the equation of the oxygen binding capacity of RBCs, 1.54 mL O_2 at 37°C[28], times the conversion factor of haemoglobin in g/L to mmol/L. The number 0.020286 is the oxygen binding capacity of HBOC-201, 1.26 mL O_2[29], times the conversion factor of haemoglobin in g/L to mmol/L. Hb is the haemoglobin concentration in mmol/L, SO_2 is oxygen saturation in %, pO_2 is the partial oxygen pressure in mmHg, K is the solubility constant of oxygen in water at 37ºC and equals 0.0225 (mL O_2 per kPa), Q is the renal blood flow in (dL/min) and *g* is the weight of the kidney in grams prior to the start of NMP.

4.2.6 Histology

Cortical needle biopsies (3mm x 20mm) were taken from the upper pole of the kidney prior to the start of NMP. At the end of each experiment a wedge biopsy of the upper pole cortex was collected. Biopsies were formalin fixed, paraffin embedded and stained with Periodic Acid-Schiff (PAS) to assess changes in renal morphology. A semi-quantitative scoring method, based on existing histological scoring systems[31], was developed. Biopsies were evaluated using light microscopy by two independent examiners, in random order. If the examiners did not report the same scores, they discussed their observations until a consensus was reached. All biopsies were scored on a scale of 0-3 (0 = none; 1 = mild; 2 = moderate; 3 = severe) for glomerular dilatation, tubular dilatation and tubular necrosis.

4.2.7 Statistical analysis

Data analysis was performed using GraphPad Prism Version 8.3.1 (GraphPad software Inc., La Jolla, CA, USA). For all continuous longitudinally measured variables, the area under the curve (AUC) was calculated. A one-way analysis of variance (ANOVA) with multiple comparisons was used to compare AUC values between groups if the data were normally distributed (Shapiro Wilk test) and had homogeneity of variances (tested by means of a Bartlett test). If data failed these assumptions the Kruskal-Wallis test with Dunn's multiple comparisons test was used. Two-sided p-values of 0.05 or less were considered to indicate statistical significance. To indicate the level of significance, asterisks were plotted in the graphs. One asterisk (*) indicates a level of significance of $p \leq 0.05$. Two asterisks (**) indicate a p value of ≤ 0.01 and three asterisks (***) a p value of $p \leq 0.001$.

4.3 RESULTS

4.3.1 Warm and cold ischaemia time and kidney weight

Warm ischaemia time was kept as short as possible and the mean duration was 5 ± 3 minutes in the No Oxygen Carrier group, 4 ± 3 minutes in the RBC group and 3 ± 1 min in the HBOC-201 group. There were no significant differences between the groups (p=0.126, Kruskal-Wallis test). Static cold storage times did not differ significantly between the groups (p=0.669, Kruskal-Wallis test) and was 2.0 ± 0.00 hours in the No Oxygen Carrier group, 2.01 ± 0.03 hours in the RBC group and 1.96 ± 0.07 hours in the HBOC-201 group. Kidney weight did not differ significantly between the groups with a mean weight of 189 ± 13 g in the No Oxygen Carrier group, 204 ± 30 g in the RBC group and 211 ± 12 g in the HBOC-201 group (p=0.243, ANOVA) before the start of NMP.

4.3.2 NMP perfusion characteristics

Flow values were stable throughout perfusion in all groups with mean values of 145 ± 42 mL/min/100g in the No Oxygen Carrier group, 126 ± 26 mL/min/100g in the RBC group and 123 ± 9 mL/min/100g in the HBOC-201 group (Figure 4.1A and 4.1B) and there were no differences between the groups (p=0.514, ANOVA). Mean renal resistance was 0.35 ± 0.10 mmHg/mL/min in the No Oxygen Carrier group, 0.34 ± 0.07 mmHg/mL/min in the RBC group and 0.34 ± 0.04 mmHg/mL/min in the HBOC-201 group and there were no significant differences between groups (p=0.971, ANOVA; Figure 4.1C and 4.1D).

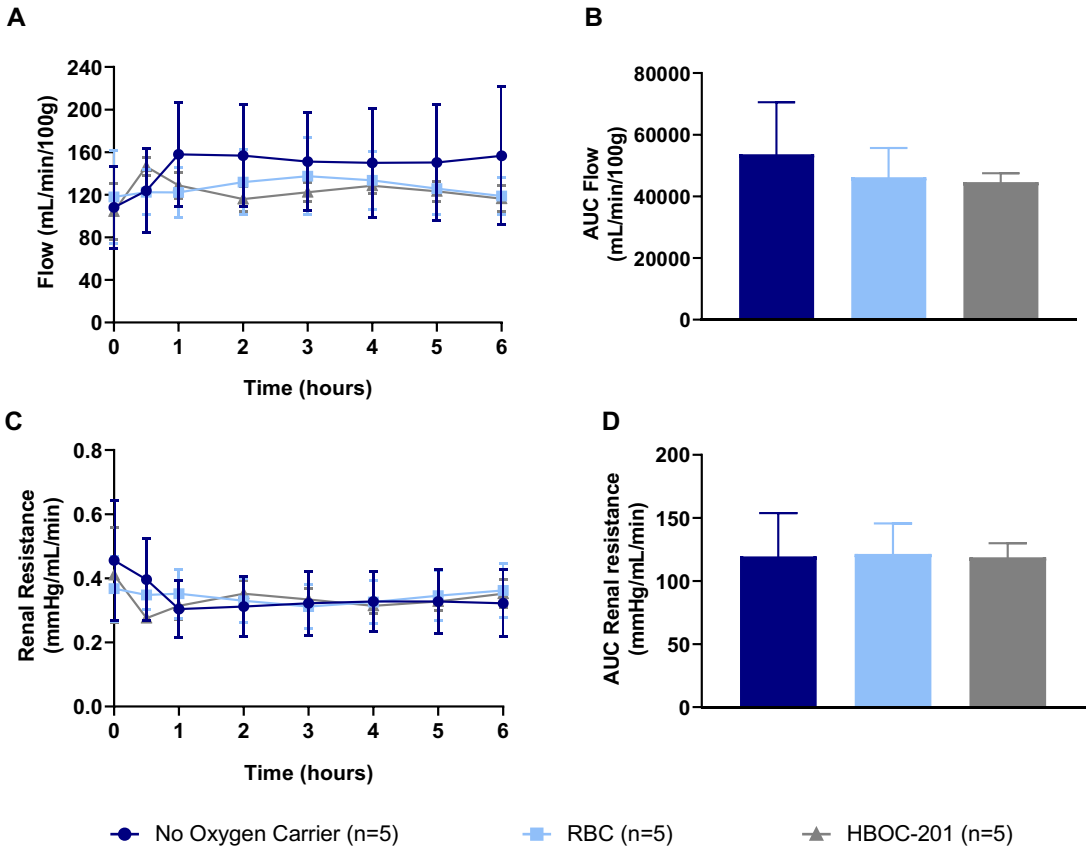


Figure 4.1. **Flow and renal resistance during NMP with different oxygen carriers.** Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier (n=5), RBC (n=5) or HBOC-201 (n=5) at 37°C. (A, B) Mean renal blood flow per 100 grams remained stable during perfusion and there were no differences between the groups. (C, D) Mean renal resistance remained stable during NMP and there were no differences between the groups. Data are presented as mean ± standard deviation. AUC, area under the curve; HBOC, haemoglobin based oxygen carrier; NMP, normothermic machine perfusion; RBC, red blood cell.

4.3.3 Renal function and metabolic activity during NMP

There was a relatively large difference in cumulative urine production between the groups. Total urine production after six hours of NMP was 42 ± 21 mL in the No Oxygen Carrier group, 98 ± 92 mL in the RBC group and 5 ± 2 mL in the HBOC-201 group (Figure 4.2A and 4.2B). This difference was only statistically significant when comparing the RBC group to the HBOC-201 group (p=0.049, ANOVA). After six hours of perfusion, mean creatinine clearance levels were 0.79 ± 0.70 mL/min in the No Oxygen Carrier group, 6.11 ± 7.08 mL/min in the RBC group and 0.23 ± 0.20 mL/min in the HBOC-201 group. The creatinine clearance of the RBC group was significantly higher in comparison with the No Oxygen Carrier group (p=0.014, ANOVA) and the HBOC-201 group (p=0.008, ANOVA; Figure 4.2C and 4.2D). Fractional excretion of Sodium (FENa) throughout perfusion was significantly lower in the RBC group in comparison with the No Oxygen Carrier group (p=0.006, Kruskal-Wallis test). At the end of perfusion, mean FENa was 6 ± 7% in the RBC group compared to 79 ± 38% in the No Oxygen Carrier group (p=0.018, Kruskal-Wallis test) and 62 ± 44% in the HBOC-201 group (p=0.169, Kruskal-Wallis test; Figure 4.2E and 4.2F).

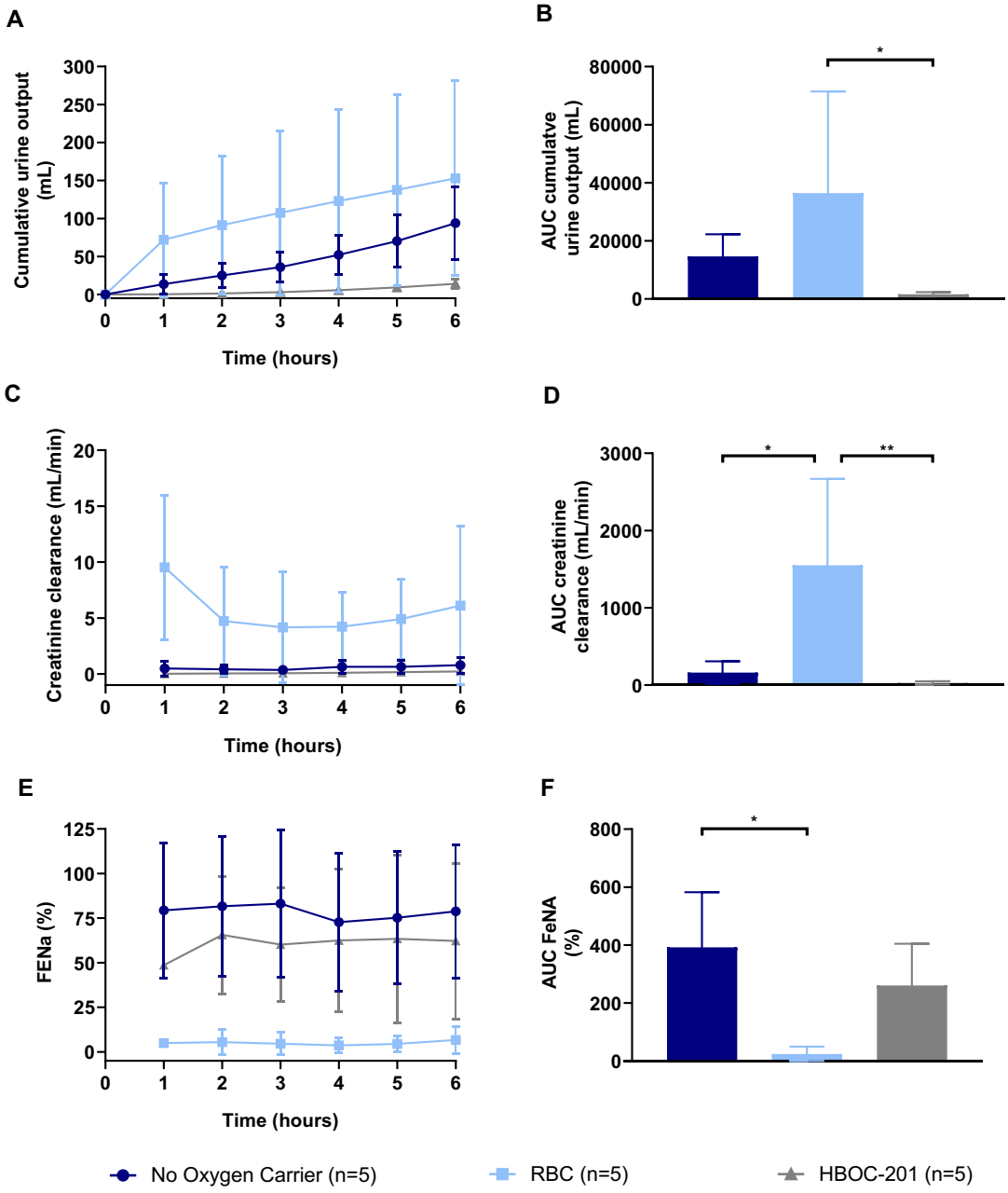


Figure 4.2. Renal function during NMP with different oxygen carriers. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier, RBC or HBOC-201 at 37°C. (A) There was a relatively large difference in cumulative urine production between the groups. (B) Kidneys in the RBC group had a significantly higher cumulative urine output than the HBOC-201 group *p<0.05. (C) There was no creatinine clearance in the No Oxygen Carrier and HBOC-201 groups. (D) Creatinine clearance was higher in the RBC group compared to the other two groups *p<0.05 vs No Oxygen Carrier and **p<0.01 vs HBOC-201. (E, F) Fractional excretion of sodium (FENa) was higher in the No Oxygen Carrier and HBOC-201 groups throughout perfusion and it was significantly lower in the RBC group in comparison with the No Oxygen Carrier group *p<0.05. Data are presented as mean ± standard deviation. AUC, area under the curve; HBOC, haemoglobin based oxygen carrier; NMP, normothermic machine perfusion; RBC, red blood cell.

Total transport of sodium was significantly higher in the RBC group in comparison with the No Oxygen Carrier group (p=0.027, Kruskal-Wallis test) and the HBOC-201 group (p=0.022, Kruskal-Wallis test; Figure 4.3A and 4.3B). The group with No Oxygen Carrier had a significantly lower renal oxygen consumption than the HBOC-201 group during the entire period of NMP (p=0.009, ANOVA). The difference between the No Oxygen Carrier group and the RBC group almost reached statistical significance (p=0.059, ANOVA). At the end of perfusion, mean oxygen consumption levels were 1.12 ± 0.17 mLO_2/min/100g in the No Oxygen Carrier group, and this was significantly lower than values of 1.53 ± 0.13 mLO_2/min/100g in the RBC group (p=0.008, ANOVA) and 1.62 ± 0.22 mL O_2/min/100g in the HBOC-201 group (p=0.003, ANOVA; Figure 4.3C and 4.3D).

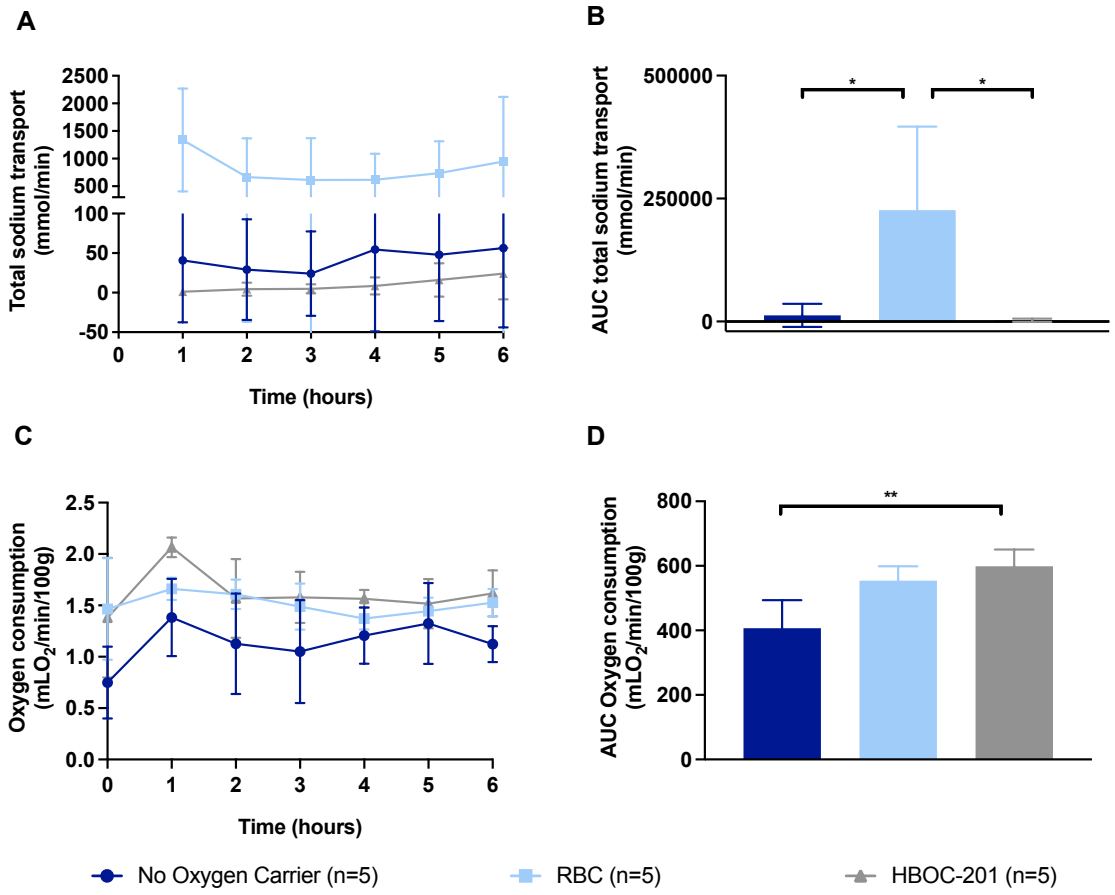


Figure 4.3. Total sodium transport and oxygen consumption during NMP with different oxygen carriers. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier, RBC or HBOC-201 at 37°C. (A, B) Total transport of sodium was significantly higher in the RBC group in comparison with the No Oxygen Carrier and HBOC-201 group. (C) Mean oxygen consumption was similar in the RBC and HBOC-201 groups. (D). Kidneys in the No Oxygen Carrier group consumed less oxygen than kidneys in the HBOC-201 group. Data are presented as mean ± standard deviation. AUC, area under the curve; HBOC, haemoglobin based oxygen carrier; NMP, normothermic machine perfusion; RBC, red blood cell.

4.3.4 Injury markers during NMP

In contrast with the RBC and HBOC-201 groups, in the No Oxygen Carrier group lactate increased throughout the six hours of perfusion. The differences in lactate levels at the end of perfusion in comparison with the start of NMP (delta lactate) were 8.8 ± 3.0 mmol/L in the No Oxygen Carrier, 1.1 ± 1.5 mmol/L in the RBC group and -1.3 ± 2.1 mmol/L in the HBOC-201 group. The increase in lactate during perfusion was significantly higher in the No Oxygen Carrier group in comparison with the RBC group ($p=0.001$, ANOVA) and in comparison with the HBOC-201 group ($p<0.0001$, ANOVA; Figure 4.4A and 4.4B). Along with the lactate levels, AST values also significantly increased in the No Oxygen Carrier group during NMP in comparison with the RBC group ($p=0.017$, Kruskal-Wallis test). End perfusion levels were also significantly higher in the No Oxygen Carrier group at 929 ± 858 U/L in comparison with 134 ± 77 U/L in the RBC group ($p=0.006$, Kruskal-Wallis test), but not in comparison with 200 ± 61 U/L in the HBOC-201 group ($p=0.105$, Kruskal-Wallis test; Figure 4.4C and 4.4D).

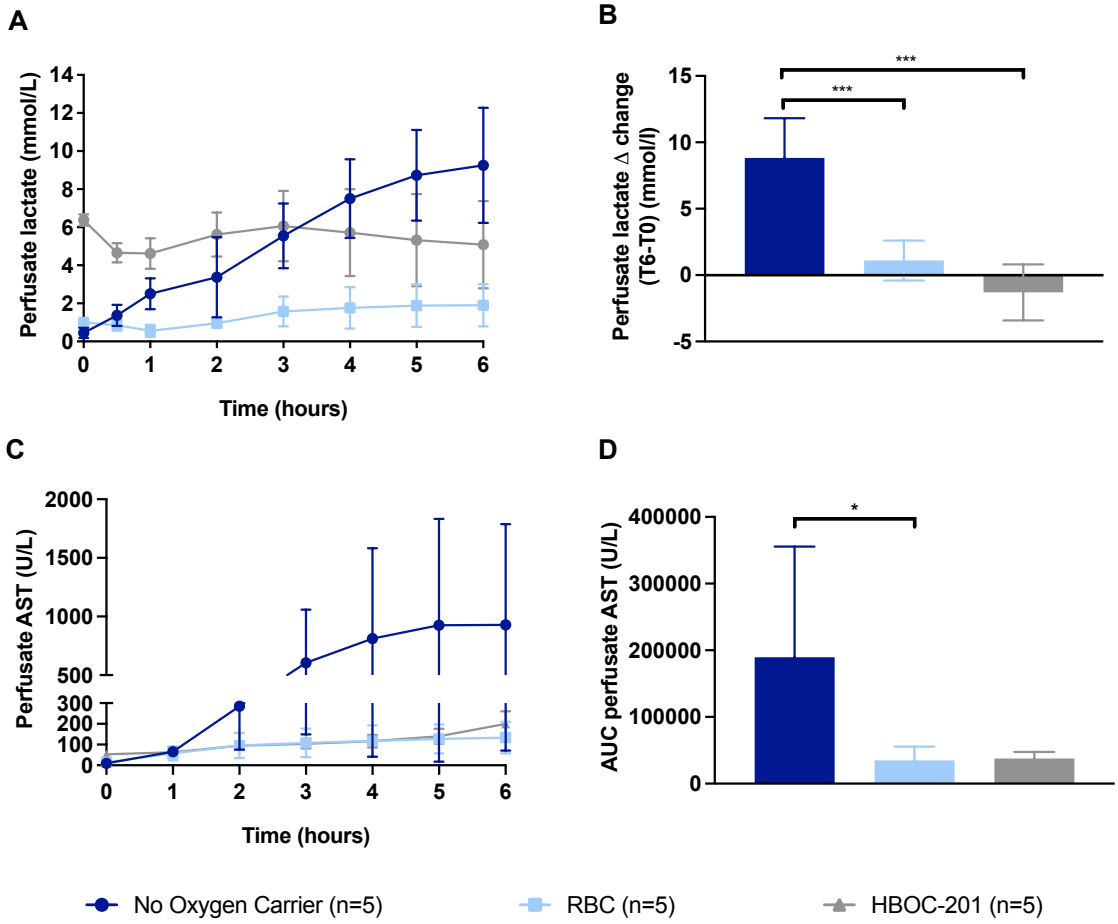


Figure 4.4. Injury markers lactate and AST during NMP with different oxygen carriers. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier, RBC or HBOC-201 at 37°C. (A) Mean lactate remained similar during perfusion in the RBC and HBOC-201 groups. (B) Delta change in lactate (t = 6 hours minus t= 0 hour) was significantly higher in the No Oxygen Carrier group compared to the other two groups. (C) Mean AST remained stable during perfusion in the RBC and HBOC-201 groups. (D) Kidneys in the No Oxygen Carrier group had significantly higher AST than the other two groups. Data are presented as mean ± standard deviation. AST, aspartate aminotransferase; AUC, area under the curve; HBOC, haemoglobin based oxygen carrier; NMP, normothermic machine perfusion; RBC, red blood cell.

TBARS were quantified by measuring the MDA concentration in the perfusate as a marker of oxidative stress. The starting levels of MDA were highest in the HBOC-201 group and these levels increased during perfusion. In the RBC group levels started close to zero and increased during perfusion to levels just below end values of the HBOC-201 group (Figure 4.5A and 4.5B). The differences in MDA levels at the end of perfusion in comparison with the start of NMP (delta MDA) were 1.28 ± 0.54 μM in the No Oxygen Carrier, 11.34 ± 5.72 μM in the RBC group and 4 ± 0 μM in the HBOC-201 group. The increase in MDA during perfusion was significantly higher in the RBC group in comparison with the No Oxygen Carrier group ($p=0.007$, ANOVA) and almost significantly higher than the HBOC-201 group ($p=0.072$, ANOVA). NGAL concentration in the urine remained stable throughout perfusion in the RBC group and the No Oxygen Carrier group and decreased in the HBOC-201 group (Figure 4.5C). As a result of the higher cumulative urine production in the RBC group, absolute NGAL levels were calculated by correcting for the urine production and were highest in this group. Calculated absolute NGAL levels in the last hour of NMP were significantly higher in the RBC group compared to the HBOC-201 group ($p=0.040$, Kruskal-Wallis test; Figure 4.5D). The AUC of absolute NGAL was also significantly higher in the RBC group in comparison with the HBOC-201 group ($p=0.008$, ANOVA) and the No Oxygen Carrier group but it did not reach significance in this case ($p=0.072$; ANOVA). The differences in absolute urinary NGAL levels at the end of perfusion in comparison with the first hour of NMP (delta absolute urinary NGAL) showed a significant decrease over time in the RBC group in comparison with the No Oxygen Carrier group ($p=0.011$, ANOVA) and the RBC group ($p=0.049$, ANOVA; Figure 4.5E).

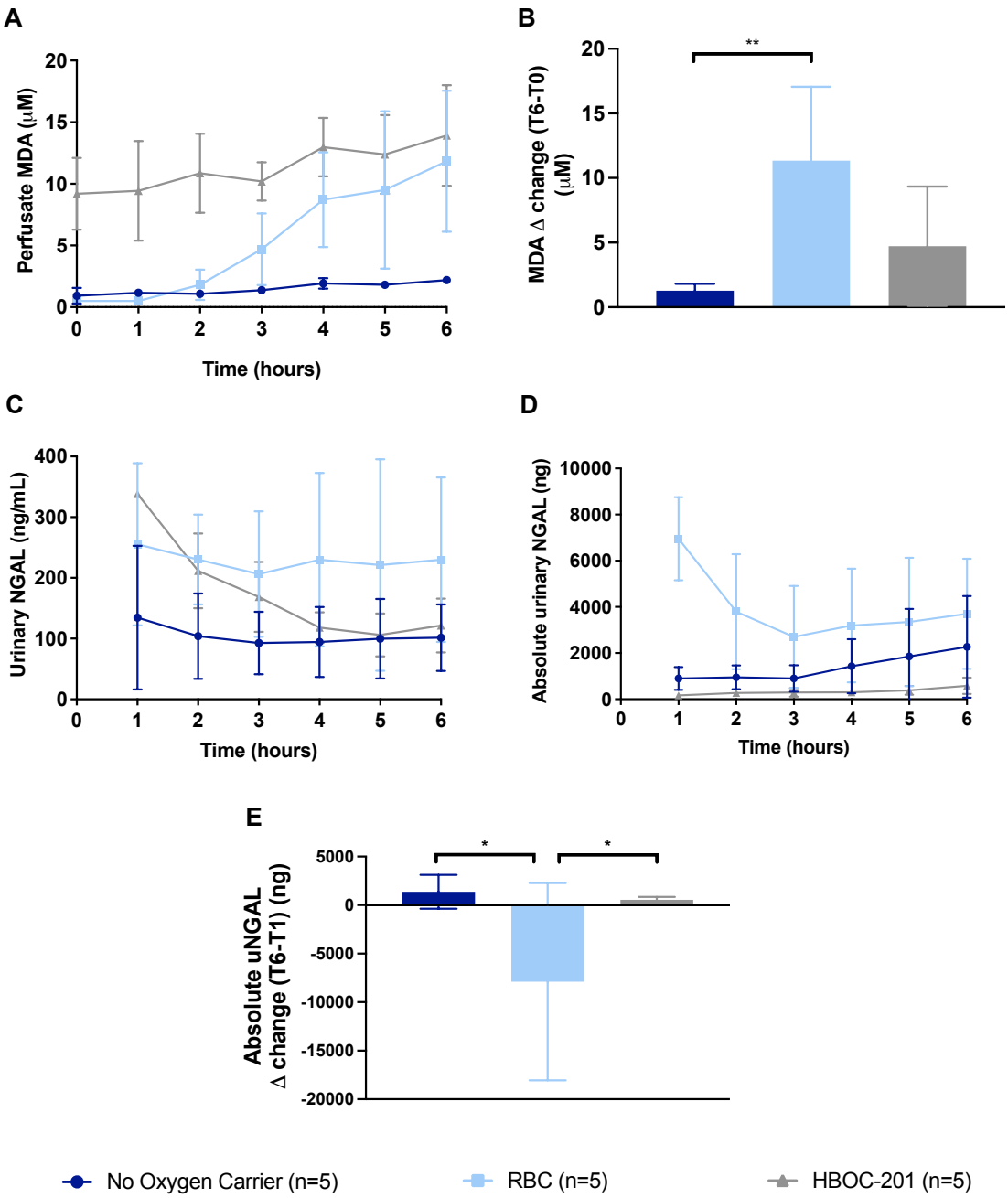


Figure 4.5. Oxidative stress (MDA) and injury (NGAL) markers during NMP with different oxygen carriers. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier, RBC or HBOC-201 at 37°C. (A) MDA concentration in the perfusate remained similar in the No Oxygen Carrier group and increased from baseline to end of perfusion in the RBC and HBOC-201 groups. (B) Delta change in MDA (t = 6 hours minus t = 0 hour) was significantly higher in RBC group compared to the No Oxygen Carrier group. (C) Urinary NGAL concentration in ng/mL during NMP. (D) Absolute NGAL in the urine remained stable in No Oxygen Carrier and HBOC-201 groups and decreased in the RBC group. (E) Delta change in urinary NGAL (t = 6 hours minus t = 1 hour) was significantly decreased in the RBC group compared to the No Oxygen Carrier and HBOC-201 groups. Data are presented as mean ± standard deviation. AUC, area under the curve; HBOC, haemoglobin based oxygen carrier; MDA, malondialdehyde; NGAL, neutrophil gelatinase-associated lipocalin; NMP, normothermic machine perfusion; RBC, red blood cell.

4.3.5 Haemolysis in the RBC group

For this series of experiments, RBCs of a maximum 14 days old were used. In some of the experiments, the haemolysis index was significantly higher than in others (Figure 4.6), which might be related to the age and stability of the RBCs.

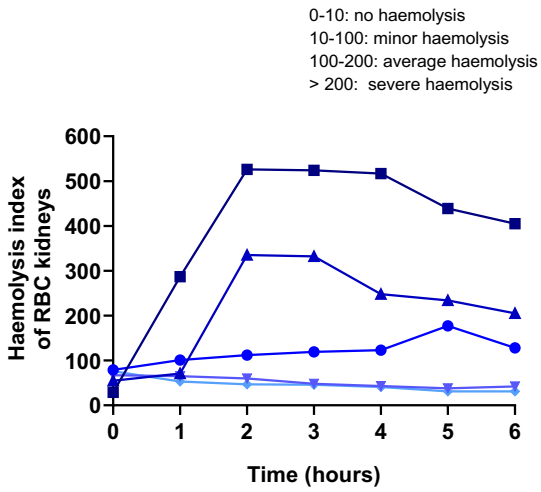


Figure 4.6. Haemolysis index per kidney perfused with red blood cells. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing RBCs at 37°C. Maximum of 14 days old stored RBCs were used. Some kidneys had no haemolysis while other kidneys presented severe haemolysis. NMP, normothermic machine perfusion; RBC, red blood cell.

4.3.6 Formation of methaemoglobin

Methaemoglobin is a form of haemoglobin in which one or more of the iron atoms are in ferric (Fe^{3+}) rather than ferrous (Fe^{2+}) state, leading to impaired oxygen binding in the affected haemoglobin monomer and higher oxygen affinity in the remaining monomers of the tetrameric haemoglobin molecule. Thus, metHb is incapable of effectively delivering oxygen to tissues. The blood gas analyses that were performed every 30 minutes revealed that metHb levels increased in each kidney perfused with HBOC-201. At the start of perfusion the percentage of metHb was 8.66 ± 1.40%, while after six hours of perfusion levels had increased to 44.48 ± 1.45%. Individual values per kidney of the HBOC-201 and the RBC groups are shown in Figure 4.7A and 4.7B. The delta metHb at the end of perfusion was significantly higher in the HBOC-201 group (35.82 ± 2.03 %) in

comparison with -0.50 ± 0.87 % in the RBC group (p=0.008, Mann-Whitney U test; Figure 4.7C).

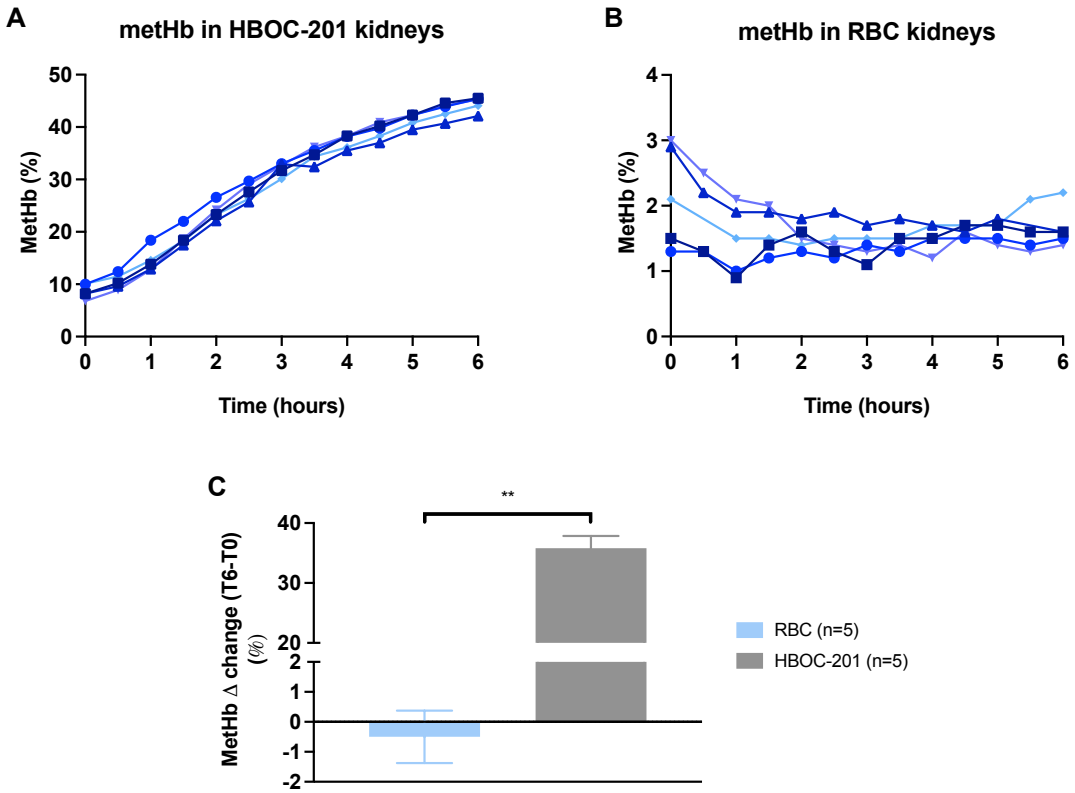


Figure 4.7. Individual methaemoglobin levels of kidneys perfused with HBOC-201 and red blood cells. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier, RBC or HBOC-201 at 37°C. (A) HBOC-201 kidneys showed an increase in methaemoglobin levels during perfusion. (B) Methaemoglobin in the RBC kidneys remained stable during perfusion. (C) Accumulation of methaemoglobin was higher in kidneys perfused with HBOC-201 compared to kidneys perfused with RBCs. Data are presented as mean ± standard deviation. AUC, area under the curve; HBOC, haemoglobin based oxygen carrier; metHb, methaemoglobin; NMP, normothermic machine perfusion; RBC, red blood cell.

During perfusion it was also noticeable that the HBOC-201 kidneys became darker in colour. Flushing out of the HBOC-201 after NMP resulted in the kidneys returning to their original pale colour (Figure 4.8A - D).

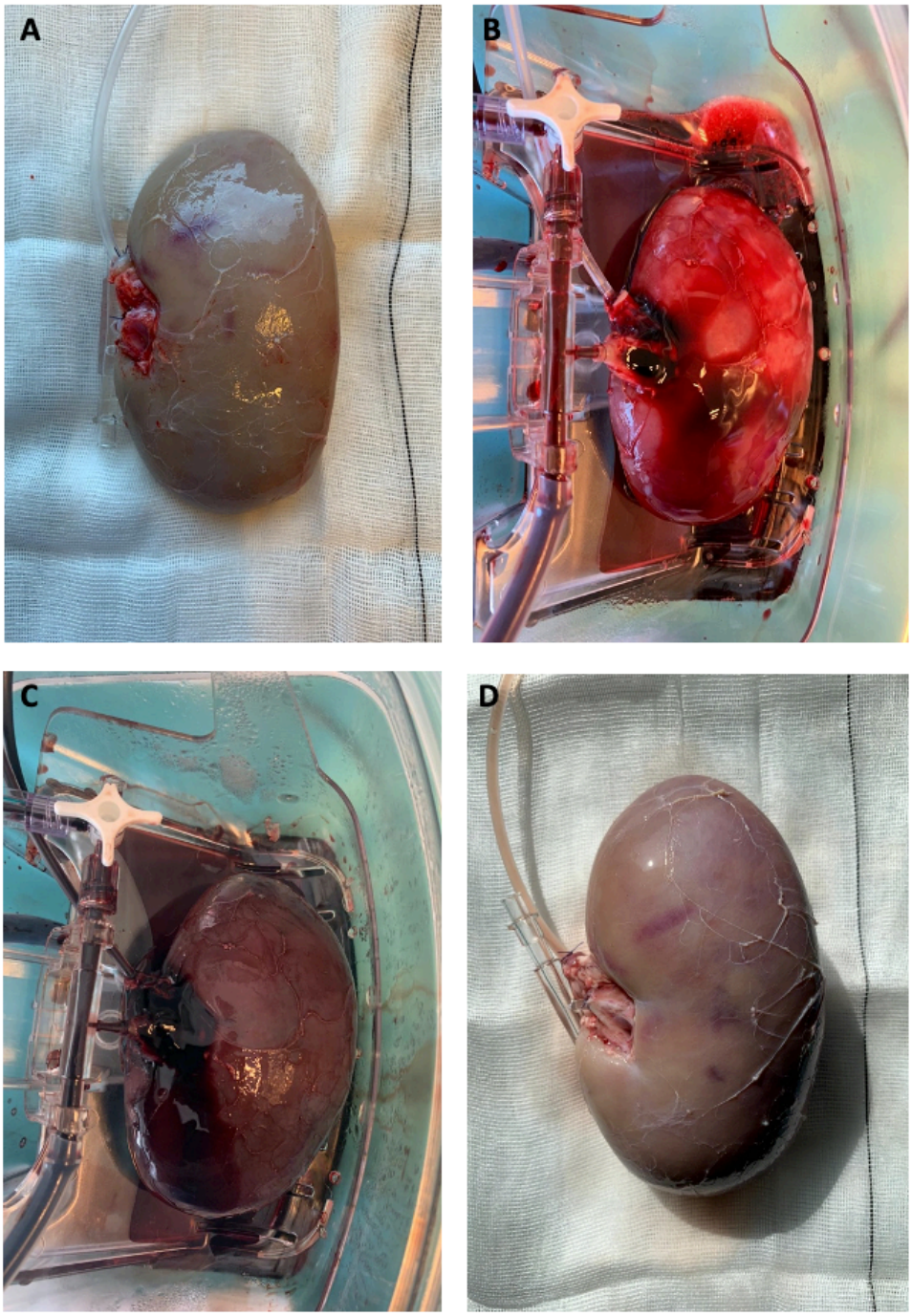


Figure 4.8. Macroscopic appearance of kidneys perfused with HBOC-201. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing HBOC-201 at 37°C. (A) Kidney after cold flush showing a pale colour, (B) kidney after one hour of perfusion with HBOC-201 showing a red/pink homogenous colour, (C) kidney after six hours of perfusion with HBOC-201 showing darker colour and (D) kidney after flush with 250 mL of saline at the end of perfusion, pale in colour.

4.3.7 Macroscopic appearance and histology

As described in section 4.2.2, a left retroperitoneal nephrectomy was performed as part of an auto-transplantation study described in Chapter 6, one day before nephrectomy of the contralateral right kidney. On the day of the left nephrectomy, inspection revealed well perfused, pink, left kidneys without patches or abnormalities. The kidney was not inspected or touched whilst *in situ*. Interestingly, after anaesthetising the pig and reopening the midline incision the next day for nephrectomy, the right kidneys showed evidence of several erythematous patches. These areas remained evident after nephrectomy and cold flush (Figure 4.8A). During NMP all kidneys were all globally perfused (pale in the No Oxygen Carrier group, pink in the RBC group and globally dark purple in the HBOC-201 group as a result of the metHb formation). None of these organs had a patchy appearance.

Histological analysis revealed that all kidneys had already sustained some injury despite very short warm ischaemic times, which was evident with the presence of tubular necrosis before the start of NMP. Table 4.2 shows tubular necrosis and dilatation as well as glomerular dilatation scores before the start of NMP (t = 0 hour) and after six hours of NMP (t = 6 hours). There was no difference in injury scores between the groups at either time point.

Table 4.2. Histological damage scores at baseline and after six hours of NMP. Pig kidneys were retrieved from anaesthetised animals, statically cold stored for two hours and perfused on NMP for six hours with a perfusion solution containing either No Oxygen Carrier, RBC or HBOC-201 at 37°C. Biopsies at baseline showed that all kidney sustained some degree of injury despite having short warm ischaemic times. After NMP, tubular necrosis, tubular dilation and glomerular dilation were present, but did not show any difference between the groups (ANOVA).

	Baseline (t= 0 hour)				**After NMP (t = 6 hours)**			
	No oxygen carrier	**RBCs**	**HBOC-201**	**p-value**	**No oxygen carrier**	**RBCs**	**HBOC-201**	**p-value**
Tubular necrosis	1 (1-1)	2 (1-2)	1.5 (1-2)	0.42	2 (1-3)	2 (1-3)	2 (1-3)	0.40
Tubular dilatation	0 (0-0)	0 (0-1)	0 (0-0)	0.09	1 (0-1)	0 (0-1)	0 (0-1)	0.56
Glomerular dilatation	0 (0-0)	0 (0-1)	0 (0-1)	0.72	0.5 (0-1)	0 (0-1)	1 (0-1)	0.85

HBOC, haemoglobin-based oxygen carrier, NMP: normothermic machine perfusion, RBC: red blood cell.

4.4 DISCUSSION

With a growing number of studies focussing on the use of NMP to better preserve, assess and resuscitate donor kidneys prior to transplantation, there is a need to develop a medical grade perfusion solution with a standardised composition. To date, most NMP studies have used either pure RBCs or whole blood as the oxygen carrier in the perfusate. The aim of this chapter was to establish whether a cellular oxygen carrier was necessary at all and if HBOC-201 could be an acceptable alternative to RBCs during of *ex vivo* NMP of six hours.

Vascular resistance and renal blood flow values did not differ significantly between groups, despite the fact that HBOC-201 was less viscous and with a diameter approximately one thousandth of the diameter of a red blood cell, which should theoretically result in a net lower resistance and higher flow values, when perfusion pressure is kept constant.[20] The group of kidneys perfused without an oxygen carrier and with perfusate of the lowest viscosity, showed higher flow values than the other two groups although this did not reach statistical significance. Urine production in an NMP setting may be influenced by the perfusate oncotic pressure and has also been considered, by some, as an indicator of kidney quality. Despite comparable calculated oncotic pressure values in the RBC and HBOC-201 group, the kidneys perfused with HBOC-201 produced only very small amounts of urine. Possibly the hydrostatic pressure over the glomerular membrane is lower in the HBOC-201 group than in the RBC group or the tubular function is impaired. In contrast to the RBC group, fractional excretion of sodium was high and there was almost no sodium reabsorption in the HBOC-201 and the No Oxygen Carrier groups, indicating severely impaired tubular function. Creatinine clearance values were low in the HBOC-201 and No Oxygen Carrier groups indicating that glomerular function was also impaired. Interestingly, urinary NGAL, an early marker of renal tubular injury[26], was lower in the HBOC-201 group than in the RBC group,

however it showed a decrease over time in the RBC group. In contrast to renal function being superior in the RBC group, HBOC-201 kidneys did not seem to sustain more injury than the RBC group during NMP as regards to histological analysis, perfusate lactate and AST levels. The absence of a difference in histological findings between the groups may be due to the short duration of six hours NMP, which may not be long enough to detect histological changes between kidneys.

In liver transplantation, several studies have shown that the use of HBOC-201- and RBC-based perfusates yielded similar metabolic, functional and histological characteristics. Two studies reported significantly higher oxygen consumption in human livers perfused for 6 hours with HBOC-201 compared to RBCs.[20, 21] However, this appears not to be the case in kidney perfusion, as there were no significant differences in oxygen consumption between the kidneys perfused with RBCs or HBOC-201 in our study, nor in the study recently performed by Aburawi *et al*.[30] A potential explanation is the prolonged storage of RBCs. RBCs storage at 2-6°C for a prolonged period of time leads to reduced intracellular ATP and 2,3-diphosphoglycerate, leading to a left shift in the Hb-oxygen dissociation curve resulting in a higher oxygen affinity and when these cells are used for transfusion or during NMP are less capable of releasing oxygen. However this was not the case in the aforementioned human liver NMP studies.[31] Also in our study, there were no differences in oxygen consumption between kidneys perfused with No Oxygen Carrier and kidneys perfused with RBCs. Despite having similar oxygen consumption, perfusate AST and lactate were significantly increased in the No Oxygen Carrier group compared to the RBC group, indicating injury and anaerobic metabolism, respectively.[32] These results are in contrast with a study performed by Minor *et al.* In their experiments, lactate and AST levels did not differ and remained similar in porcine kidneys perfused by controlled rewarming and normothermic perfusion for two hours using an acellular perfusate without an oxygen carrier.[33] However, in this study NMP was only performed for 30 minutes which may have been too short to see differences between the groups.

In the study by Aburawi *et al.* a potential drawback of the use of HBOC-201, the accumulation of metHb, was briefly discussed but no data on concentrations were reported. Also, no control group without an oxygen carrier was reported. Although the perfusion solution containing the metHb could be flushed out prior to transplantation, the question remains if HBOC-201 would be suitable for prolonged periods of normothermic kidney perfusion, since there is a possibility that the percentage of metHb would increase even further with NMP durations more than six hours. HBOC-201, in contrast to erythrocytes, does not contain nicotinamide adenine dinucleotide hydrogen (NADH)-dependent enzyme methaemoglobin reductase, which converts metHb back to haemoglobin. Methaemoglobinemia impairs oxygen delivery to tissues by decreasing the oxygen-carrying capacity of blood, as the oxidised iron can no longer reversibly bind oxygen. In addition, the oxidation of iron atoms causes the conformation of haemoglobin to change, leading to an increase in oxygen affinity of the remaining ferrous haem groups.[34] Therefore, in perfusion solutions containing HBOC-201, but devoid of RBCs, metHb concentrations may increase, leading to a left shift of the oxygen dissociation curve. An effective treatment for the reduction of metHb is the intravenous administration of methylene blue. Methylene blue accelerates the enzymatic reduction of methaemoglobin by NADPH-methaemoglobin reductase and reduces to leucomethylene blue that, in turn, reduces metHb to haemoglobin.[35, 36] Vitamin C (ascorbic acid) or glutathione can be added to the perfusate to convert metHb back to haemoglobin but it requires high doses as it is less effective and the rate at which it reduces metHb is slower than methylene blue, making it less suitable when metHb levels increase above 30%.[37,38]

De Vries *et al*. reported a steady increase in metHb levels of approximately 10% during two hours of NMP with HBOC-201 without a liver in the perfusion circuit, despite the addition of vitamin C. In perfusions with a liver connected to the perfusion circuit the rise in metHb was much slower and became most prominent when the perfusion solution was oxygenated with 21% oxygen.[29] In our study metHb levels increased with a similar

rate, approximately 10% every two hours, as described by de Vries *et al*. in the perfusion without a liver in the circuit, leading to metHb concentrations of 45% in our experiments after six hours of NMP. We did not use vitamin C as this does not add to the standardisation of the perfusion solution across the three experimental groups and it affects the pH and osmolality of the perfusate. Although there is evidence that low doses of vitamin C could have nephroprotective effects, there are also longstanding concerns regarding possible renal complications after high dose vitamin C treatments, in the case of pre-existent impaired renal function.[39-42]

Newer generations of HBOCs, such as the HBOC-201 compound used in the present study, contain large molecules of polymerised or cross-linked haemoglobin in an attempt to avoid known complications of free haemoglobin tetramers, such as nephrotoxicity and extravasation. To date, however, HBOC-201 has only been licensed for commercial use in a limited number of countries (South Africa and Russia). Infusions of HBOCs in humans have been associated with several adverse events and safety concerns remain.[43] The mechanism of HBOC and free haemoglobin toxicity is still not fully understood and it has been suggested that haem-mediated mechanisms play a role. However, the evidence supporting a role for haem-mediated toxicity has been limited to biochemical studies conducted by a few investigators[44] and little evidence exists that such mechanisms are operative in cells under physiologic conditions or *in vivo*.[20, 45] With regards to nephrotoxicity, this would be the result of redox reactions related to iron availability and the generation of reactive oxygen species (ROS).[46, 47] In our study MDA, a product of lipid oxidative damage and measured for determination of oxidative stress by ROS[25], increased over time in the HBOC-201 group, but a steeper increase was seen in the RBC group. This may be explained by the significant amount of haemolysis detected in several kidneys in this group. Most likely the haemolysis seen in these experiments was related to the storage time of each individual RBC concentrate, as more haemolysis can be expected from RBCs that were older and had prolonged storage. This

finding is relevant for clinical perfusions, since RBC unit storage times used for NMP vary considerably in the clinical setting. MDA in the RBC group was higher than the No Oxygen Carrier group, which remained the same during perfusion. During an ischaemic period, only small amounts of ROS are produced compared to the entire ischaemia and reperfusion phase.[48, 49] The low levels of MDA in the No Oxygen Carrier group can indicate that there was not enough oxygen delivered in the tissue and going to the mitochondria leading to lower free radical production.[50, 51] Future studies should investigate the membrane potential to assess mitochondrial function, as well as assess mitochondrial DNA to determine oxidative damage and whether oxygen has been taken up by the tissue.[52]

Even though HBOC-201 presents the advantage of extended shelf life, ambient storage and similar outcomes in terms of oxygenation capacity, several studies have reported that it can cause significant interference in modern laboratory analysers that rely on colorimetric assays, due to the fact that the compound remains fully dissolved in the "plasma" after centrifugation.[53, 54] Korte *et al.* have reported that different routine analysers had a different sensitivity to the presence of HBOC-201.[54] In our study, a tenfold dilution was required to analyse samples that contained HBOC-201 with routine clinical biochemistry methods and to minimise assay inaccuracy. Unfortunately, it proved impossible to measure several relevant injury markers such as LDH in the HBOC-201 samples. Furthermore, for injury marker MDA a colorimetric assay was performed where MDA levels were already high prior to start of perfusion indicating that HBOC-201 can cause interference. For this reason the overall change (delta) in MDA was calculated and compared. To date, assessment of kidneys is based on the macroscopic appearance, blood flow and urine output during NMP.[55] A more in depth assessment, involving the detection of injury markers, appears to be more difficult and time consuming in samples containing HBOC-201, thereby limiting real time pre-transplant assessment of kidney viability during NMP.

A potential limitation of this study is the relatively small sample size, although our group sizes are comparable to other studies investigating NMP.[30, 56, 57] Secondly, we did not transplant kidneys after NMP. For a fully reliable comparison between the groups, which will also allow for follow-up and functional assessment *in vivo*, future studies should include auto-transplantation of these kidneys.

In conclusion, this study has demonstrated that healthy porcine donor kidneys perfused without an oxygen carrier or HBOC-201 as an oxygen carrier have significantly impaired renal function during NMP in comparison to kidneys perfused with allogenic concentrated RBCs. Although injury marker levels are relatively low in the HBOC-201 group, high metHb levels indicate that HBOC-201 might be less suitable as an oxygen carrier for prolonged periods of perfusion.

REFERENCES

1. Kaths JM, Cen JY, Chun YM, et al. Continuous Normothermic Ex Vivo Kidney Perfusion Is Superior to Brief Normothermic Perfusion Following Static Cold Storage in Donation After Circulatory Death Pig Kidney Transplantation. *Am J Transplant*. 2017;17:957-969.

2. Kaths JM, Paul A, Robinson LA, Selzner M. Ex vivo machine perfusion for renal graft preservation. *Transplant Rev (Orlando)*. 2018;32:1-9.

3. Hamar M, Urbanellis P, Kaths MJ, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. *Transplantation*. 2018;102:1262-1270.

4. Hosgood SA, Barlow AD, Dormer J, Nicholson ML. The use of ex-vivo normothermic perfusion for the resuscitation and assessment of human kidneys discarded because of inadequate in situ perfusion. *J Transl Med*. 2015;13:329-x.

5. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable but Resuscitated by Ex Vivo Normothermic Machine Perfusion. *Am J Transplant*. 2016;16:3282-3285.

6. Weissenbacher A, Lo Faro L, Boubriak O, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. *Am J Transplant*. 2019;19:178-192.

7. Kaths JM, Hamar M, Echeverri J, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. *Am J Transplant*. 2018;18:580-589.

8. Yong C, Hosgood SA, Nicholson ML. Ex-vivo normothermic perfusion in renal transplantation: past, present and future. *Curr Opin Organ Transplant*. 2016;21:301-307.

9. Harper S, Hosgood S, Kay M, Nicholson M. Leucocyte depletion improves renal function during reperfusion using an experimental isolated haemoperfused organ preservation system. *Br J Surg*. 2006;93:623-629.

10. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? *Transpl Int*. 2015;28:657-664.

11. Moritz ED, Winton CS, Tonnetti L, et al. Screening for Babesia microti in the U.S. Blood Supply. *N Engl J Med*. 2016;375:2236-2245.

12. Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. *N Engl J Med*. 1996;334:1685-1690.

13. Senay S, Toraman F, Gunaydin S, Kilercik M, Karabulut H, Alhan C. The impact of allogenic red cell transfusion and coated bypass circuit on the inflammatory response during cardiopulmonary bypass: a randomized study. *Interact Cardiovasc Thorac Surg*. 2009;8:93-99.

14. Sakota D, Sakamoto R, Sobajima H, et al. Mechanical damage of red blood cells by rotary blood pumps: selective destruction of aged red blood cells and subhemolytic trauma. *Artif Organs*. 2008;32:785-791.

15. Benedetti M, De Caterina R, Bionda A, et al. Blood--artificial surface interactions during cardiopulmonary bypass. A comparative study of four oxygenators. *Int J Artif Organs*. 1990;13:488-497.

16. Fontes PA. The Evolution of Oxygen Carrier Solutions for Machine Perfusion. *Transplantation*. 2017;101:2657-2658.

17. Levy JH, Goodnough LT, Greilich PE, et al. Polymerized bovine hemoglobin solution as a replacement for allogeneic red blood cell transfusion after cardiac surgery: results of a randomized, double-blind trial. *J Thorac Cardiovasc Surg*. 2002;124:35-42.

18. Mozzarelli A, Ronda L, Faggiano S, Bettati S, Bruno S. Haemoglobin-based oxygen carriers: research and reality towards an alternative to blood transfusions. *Blood Transfus*. 2010;8 Suppl 3:59.

19. Hemoglobin Oxygen Therapeutics L. Hemopure. . Accessed December, 2019.

20. Laing RW, Bhogal RH, Wallace L, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. *Transplantation*. 2017;101:2746-2756.

21. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl*. 2018;24:528-538.

22. de Vries Y, van Leeuwen OB, Matton APM, Fujiyoshi M, de Meijer VE, Porte RJ. Ex situ normothermic machine perfusion of donor livers using a haemoglobin-based oxygen carrier: a viable alternative to red blood cells. *Transpl Int*. 2018;31:1281-1282.

23. Fontes P, Lopez R, van der Plaats A, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. *Am J Transplant*. 2015;15:381-394.

24. Kerry Brandis. 2.4 Colloid Osmotic Pressure. 2019.

25. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev*. 2014;2014:360438.

26. Singer E, Marko L, Paragas N, et al. Neutrophil gelatinase-associated lipocalin: pathophysiology and clinical applications. *Acta Physiol (Oxf)*. 2013;207:663-672.

27. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation*. 2019;103:2057-2064.

28. Ronco C, Bellomo R, Kellum JA. Chapter 116. arterial and venous blood gases - oxygen content. In: *Critical Care in Nephrology.* 2nd ed. Saunders; 2008:608.

29. de Vries Y, Matton APM, Nijsten MWN, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. *Am J Transplant*. 2019;19:1202-1211.

30. Aburawi MM, Fontan FM, Karimian N, et al. Synthetic hemoglobin-based oxygen carriers are an acceptable alternative for packed red blood cells in normothermic kidney perfusion. *Am J Transplant*. 2019;19:2814-2824.

31. Yoshida T, Prudent M, D'alessandro A. Red blood cell storage lesion: causes and potential clinical consequences. *Blood Transfus*. 2019;17:27-52.

32. McNamara PJ, Khuffash A. 71 - oxygen transport and delivery. In: *Fetal and Neonatal Physiology (Fifth Edition).* ; 2017:724-737.

33. Minor T, von Horn C, Paul A. Role of erythrocytes in short-term rewarming kidney perfusion after cold storage. *Artif Organs*. 2019;43:584-592.

34. Agarwal N, Prcgal J. Chapter 49. methemoglobenia and ther dyshemoglobinemias. In: Lichtman MA, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT, eds. *Williams Hematology.* 8th edition ed. New York: McGraw-Hill; 2010.

35. McDonagh EM, Bautista JM, Youngster I, Altman RB, Klein TE. PharmGKB summary: methylene blue pathway. *Pharmacogenet Genomics*. 2013;23:498-508.

36. Umbreit J. Methemoglobin--it's not just blue: a concise review. *Am J Hematol*. 2007;82:134-144.

37. Rino PB, Scolnik D, Fustinana A, Mitelpunkt A, Glatstein M. Ascorbic acid for the treatment of methemoglobinemia: the experience of a large tertiary care pediatric hospital. *Am J Ther*. 2014;21:240-243.

38. Waller HD, Benohr HC, Tigges FJ. On the mechanism of ascorbic acid induced methemoglobin reduction of human erythrocytes (author's transl). *Klin Wochenschr*. 1977;55:955-964.

39. McHugh GJ, Graber ML, Freebairn RC. Fatal vitamin C-associated acute renal failure. *Anaesth Intensive Care*. 2008;36:585-588.

40. Lee KW, Park SY. High-dose vitamin C as treatment of methemoglobinemia. *Am J Emerg Med*. 2014;32:936.

41. McAllister CJ, Scowden EB, Dewberry FL, Richman A. Renal failure secondary to massive infusion of vitamin C. *JAMA*. 1984;252:1684.

42. Nankivell BJ, Murali KM. Renal Failure from Vitamin C after Transplantation. *N Engl J Med*. 2008;358:e4.

43. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics (Sao Paulo)*. 2009;64:803-813.

44. Alayash AI. Blood substitutes: why haven't we been more successful? *Trends Biotechnol*. 2014;32:177-185.

45. Taverne YJ, de Wijs-Meijler D, Te Lintel Hekkert M, et al. Normalization of hemoglobin-based oxygen carrier-201 induced vasoconstriction: targeting nitric oxide and endothelin. *J Appl Physiol (1985)*. 2017;122:1227-1237.

46. Alayash AI. Mechanisms of Toxicity and Modulation of Hemoglobin-based Oxygen Carriers. *Shock*. 2019;52:41-49.

47. Van Avondt K, Nur E, Zeerleder S. Mechanisms of haemolysis-induced kidney injury. *Nat Rev Nephrol*. 2019;15:671-692.

48. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol*. 2012;298:229-317.

49. Nieuwenhuijs-Moeke GJ, Pischke SE, Berger SP, et al. Ischemia and Reperfusion Injury in Kidney Transplantation: Relevant Mechanisms in Injury and Repair. *J Clin Med*. 2020;9:10.3390/jcm9010253.

50. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417:1-13.

51. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol*. 2015;6:524-551.

52. Kezic A, Spasojevic I, Lezaic V, Bajcetic M. Mitochondria-Targeted Antioxidants: Future Perspectives in Kidney Ischemia Reperfusion Injury. *Oxid Med Cell Longev*. 2016;2016:2950503.

53. Boteon YL, Laing RW, Schlegel A, et al. The impact on the bioenergetic status and oxidative-mediated tissue injury of a combined protocol of hypothermic and normothermic machine perfusion using an acellular haemoglobin-based oxygen carrier: The cold-to-warm machine perfusion of the liver. *PLoS One*. 2019;14:e0224066.

54. Korte EA, Pozzi N, Wardrip N, Ayyoubi MT, Jortani SA. Analytical interference of HBOC-201 (Hemopure, a synthetic hemoglobin-based oxygen carrier) on four common clinical chemistry platforms. *Clin Chim Acta*. 2018;482:33-39.

55. Hosgood SA, Barlow AD, Hunter JP, Nicholson ML. Ex vivo normothermic perfusion for quality assessment of marginal donor kidney transplants. *Br J Surg*. 2015;102:1433-1440.

56. Adams TD, Patel M, Hosgood SA, Nicholson ML. Lowering Perfusate Temperature From 37 degrees C to 32 degrees C Diminishes Function in a Porcine Model of Ex Vivo Kidney Perfusion. *Transplant Direct*. 2017;3:e140.

57. Hosgood SA, Shah K, Patel M, Nicholson ML. The effect of prolonged of warm ischaemic injury on renal function in an experimental ex vivo normothermic perfusion system. *J Transl Med*. 2015;13:207-4

.

Chapter 5

Dose and tracking of mesenchymal stromal cells during normothermic machine perfusion of porcine kidneys

ABBREVIATIONS

AST	aspartate aminotransferase
AUC	area under the curve
DAPI	4,6-diamidino-2-phenylindole dihydrochloride
HMP	hypothermic machine perfusion
HRP	horseradish peroxidase
IL	interleukin
IRI	ischaemia reperfusion injury
IV	intravenous
LDH	lactate dehydrogenase
MAP	mean arterial pressure
MAPC	multipotent adult progenitor cells
MSC	mesenchymal stromal cell
MSR	male specific repeat
NMP	normothermic machine perfusion
PGE2	prostaglandin E2
PBS	phosphate-buffered saline
QAS	quality assessment score
qPCR	quantitative real-time polymerase chain reaction
RBC	red blood cell
RT	room temperature
TMB	tetramethylbenzidine
UW-MPS	Belzer MPS® UW Machine Perfusion Solution
VEGF	vascular endothelial growth factor

5.1 INTRODUCTION

The development and availability of normothermic machine perfusion (NMP) technology has led to an increase in studies applying therapeutic agents to an isolated organ *ex vivo*, thus avoiding systemic therapies administered directly to the patient.[1-6] This new option offers the possibility of optimising the quality of marginal or higher risk donor kidneys. One therapeutic intervention during NMP of primary interest is the administration of mesenchymal stromal cells (MSCs). MSCs are multipotent cells that have the ability to modulate innate as well as adaptive immune responses at the site of tissue or cellular injury.[4, 5] In addition, MSCs have been shown to possess regenerative properties with different small and large animal studies reporting a beneficial effect on ischaemia reperfusion injury (IRI) with the possibility of acute repair in the kidney.[6, 7] MSCs most likely achieve this via interaction with host immune and progenitor cells, driven by the soluble factors they produce (secretome), leading to anti-inflammatory and anti-fibrotic, anti-apoptotic and pro-angiogenic influences.[8-11] In addition, MSCs have the potential to suppress oxidative stress and trigger tubular cell proliferation, which may minimise tissue injury and stimulate repair.[11] The exact mechanism of how MSCs migrate to the tissue, a process termed 'homing', is not clear yet. Chamberlain *et al.* proposed two different mechanisms. The first one involves active homing, in which MSCs actively tether to and roll on the activated vasculature leading to arrest and firm adhesion. The second mechanism is a passive mechanism where MSCs are passively retained in the narrow capillaries due to their large size.[12] The latter being confirmed by Sierra-Parraga *et al*., who showed similar living and dead MSC retention in the kidney tissue after intra-arterial administration.[13]

To date, research has mainly focused on MSC therapy after but not prior to kidney transplantation.[5, 14, 15] The efficacy of MSCs after delivery depends not only on the source of MSCs, but also the dose, timing, frequency and route of administration.[16] Intravenous

(IV) infusion of MSCs to transplant recipients has been the route of administration for most clinical studies and appears to be safe.[4, 17-19] However, this mode of systemic administration is associated with off-target distribution where IV infused MSCs are largely trapped in the lungs.[20, 21] For the MSCs to be physically present in the kidney with a greater number and in direct contact with the organ vasculature, infusion into the renal graft artery prior to transplantation might be the solution. A few preclinical studies have successfully tested the delivery of drugs or cells during cold machine perfusion.[22-24] Hypothermic conditions may impede the uptake of therapeutic agents, cellular signalling cascades may be altered, and the effector mechanisms blocked until the organ is re-warmed.[25] Therefore, NMP is an ideal platform for such an intervention. The use of MSCs during machine perfusion is promising; however, many factors are still unknown. Only a few studies are reported with MSC treatment during *sub*-NMP and NMP focussing on dose-response and traceability.[26-28] The dose of MSCs the authors investigated varied between 100 thousand and 200 million cells. Furthermore, little is known about the viability of the MSCs once they are delivered in the kidney.

This chapter aimed to determine the optimum MSC dose following administration to the kidney during NMP and to track the location of MSCs throughout organ perfusion. In addition, this chapter had the goal to study the 'secretome' released by MSCs during kidney NMP.

The objectives of this chapter are:

- To determine if administration of MSCs during NMP is feasible and safe;
- To determine the optimal dose of MSCs to be administered to assess the location, distribution and number of MSCs present in the kidney;
- To determine the optimal dose of MSCs to be administered to the kidney without adversely affecting renal perfusion dynamics;

- To determine the location of MSCs within the perfusion circuit and the kidney and whether they remain viable;
- To characterise cytokines and growth factors released by MSCs in perfusion fluid.

5.2 MATERIAL AND METHODS

5.2.1 Experimental design

A dose-response study was performed to determine the optimum dose of MSC administration during NMP. Pig kidneys were retrieved from a local abattoir with minimal warm ischaemia and were cold flushed as detailed in paragraph 5.2.2. Subsequently, kidneys were preserved using hypothermic machine perfusion (HMP) for approximately four hours and perfused on NMP for seven hours. After one hour of NMP, 2 million, 10 million or 50 million fluorescently labelled pig MSCs were administered via the renal artery.

This study was performed in two phases, where the first phase was a dose-response design to determine which MSC dose could be administered without detriment to perfusion and to identify the location and distribution of MSCs in the kidney during NMP. Furthermore, this was designed to establish the optimum dose of MSCs for the auto-transplantation study described in Chapter 7. Four experimental groups were defined (n=3, per group):

Group 1: NMP with an intra-arterial infusion of perfusate only (control);
Group 2: NMP with an intra-arterial infusion of 2 million MSCs (2 million MSC);
Group 3: NMP with an intra-arterial infusion of 10 million MSCs (10 million MSC);
Group 4: NMP with an intra-arterial infusion of 50 million MSCs (50 million MSC).

The second phase was to select the optimal group from phase one and increased the group size to n=6. The optimal dose was defined as the lowest MSCs dose with histological evidence of widespread of MSCs distribution throughout the renal cortex without adversely affecting renal perfusion parameters. The experimental groups were:

Group 1: NMP with an intra-arterial infusion of perfusate only (control);

Group 2: NMP with an intra-arterial infusion of 10 million MSCs (10 million MSC).

5.2.2 Organ and blood retrieval

Female porcine kidneys and autologous blood were obtained from a local abattoir. Pigs were terminated following standard Home Office procedures, which, in brief, involved stunning with a bi-temporal electric shock, followed by rapid exsanguination and then de-hairing in a hot wash at 60°C for five minutes. Autologous blood was collected in a heparinised container (20,000 IU, Wockhardt, Wrexham, United Kingdom) and both kidneys were retrieved, including vessels and ureter. Kidneys were immediately flushed with Marshall's hypertonic citrate solution (Soltran, Baxter Healthcare, Thetford, United Kingdom) supplemented with 5,000 IU of heparin. Warm ischaemia time was calculated from the time of exsanguination to the time of cold flush. Slaughterhouse waste material was used as an organ and blood source for these experiments, and therefore no animal ethics committee approval was needed.

5.2.3 Hypothermic machine perfusion

Kidneys were preserved on HMP with the portable Kidney Assist device (Kidney Assist Transport, XVIVO, Groningen, the Netherlands). The HMP circuit was primed with 500 mL of cold Belzer MPS® UW Machine Perfusion Solution (Bridge to Life Ltd., London, United Kingdom). The kidney was placed on the machine and was set up at a mean arterial pressure (MAP) of 25 mmHg. Hypothermia was maintained at 4°C by crushed ice surrounding the HMP circuit.

5.2.4 Normothermic machine perfusion

5.2.4.1 The normothermic machine perfusion setup

After HMP, the kidneys were cannulated and perfused for seven hours at a MAP of 70 mmHg on a pressure-controlled pulsatile pump (Kidney Assist, XVIVO, the Netherlands). The customised perfusion circuit consisted of an oxygenator (Hilte Lt 2500, Medos Medizintechnik AG, Stolberg, Germany), a centrifugal pump (DP3, Medos Medizintechnik AG) and a Life Port® organ chamber (Organ Recovery Systems, Illinois, USA). Flow was monitored using an ultrasonic clamp-on flow probe (Em-tec-Gmbh, Finning, Germany). Pressure was measured at the same height as the renal artery using a pressure transducer (TruWave disposable pressure transducer, Edwards Lifesciences, Irvine, California, USA). Perfusate temperature was kept at 37°C and oxygenated with 0.5 L/min carbogen (95% O_2 / 5% CO_2, BOC, Didcot, United Kingdom). Perfusate and urine samples were taken hourly, centrifuged at 1,800 x g for 12 minutes at room temperature (RT), and the supernatant stored at -80°C for further analysis. After the urine sample was collected, the remaining volume was re-circulated back into the NMP circuit. Perfusate without red blood cells (RBCs) was used to replace sample volume loss from perfusate and blood gas analysis during perfusion.

5.2.4.2 The perfusion solution

The perfusion circuit was primed with MePEP 3.0 perfusion solution that, in brief, consisted of 250 mL 5% human serum albumin (Alburex 50 g/L, CSL Behring, Lyngby, Denmark) and washed autologous pig RBCs to acquire a haematocrit of 25-30%. RBCs were isolated as described in paragraph 5.2.4.3. In addition, 5 mL 8.4% (w/v) sodium bicarbonate (B. Braun, Melsungen, Germany), 6 mL 5% (w/v) glucose (B. Braun), 5 IU insulin (Novo Nordisk A/S, Crawley, United Kingdom), 3 mL 10% (w/v) calcium gluconate (B. Braun), 10 mg mannitol (Sigma Aldrich) and 1000 µmol/L creatinine (Sigma Aldrich) were added to the perfusion solution. The perfusate was supplemented with a bolus of

300 mg amoxicillin-clavulanate (Bowmed, Wrexham, United Kingdom) at baseline with additional doses of 60 mg amoxicillin-clavulanate every hour during NMP. Furthermore, 0.25 mg/h verapamil (Mylan, Hatfield, United Kingdom) was continuously infused during NMP.

The following adjustments were made during perfusion: (i) 5% glucose was administered at a rate of 4 mL/h when glucose levels dropped < 4.0 mmol/L and the rate was increased to 5 mL/h if glucose levels were still below range at the next measurement. (ii) 1 mL 10% (w/v) calcium gluconate were added if ionized calcium levels were between 0.9 and 1.1 mmol/L and 2 mL 10% (w/v) calcium gluconate were added if ionized calcium were < 0.9 mmol/L.

5.2.4.3 Red blood cell isolation

Autologous blood was collected in a heparinised container at a local abattoir. The blood was poured into a catheter bag using a funnel and was filtered for leucocytes through a leucocyte filter (NPT/LCR2 filters, Macopharma, France). The filtered blood was centrifuged at 3,000 x g for 20 minutes at RT and the plasma supernatant was removed and discarded. The remaining RBCs were washed with 1x phosphate-buffered saline (PBS) at 3,000 x g for 20 minutes at RT. The PBS was removed with a syringe and remaining RBCs were used for the perfusion solution.

5.2.5 Perfusate and tissue collection

Perfusate samples for flow cytometry analysis were taken from five different locations in the NMP circuit to investigate whether MSCs could be identified within the circuit and these locations included: renal vein; the bottom of the reservoir; venous line before the pump; between the pump and oxygenator and the arterial line (Figure 5.1). Samples were treated for 10 minutes with 1x RBC lysis buffer (eBioscience™ 1X RBC Lysis

Buffer, Invitrogen by Thermofisher Scientific, Warrington, United Kingdom) in a 1:9 ratio protected from light according to the manufacturer's protocol. Afterwards, cells were washed twice with 1x PBS and analysed with flow cytometry as described in paragraph 5.2.11 for concentration and viability of the MSCs.

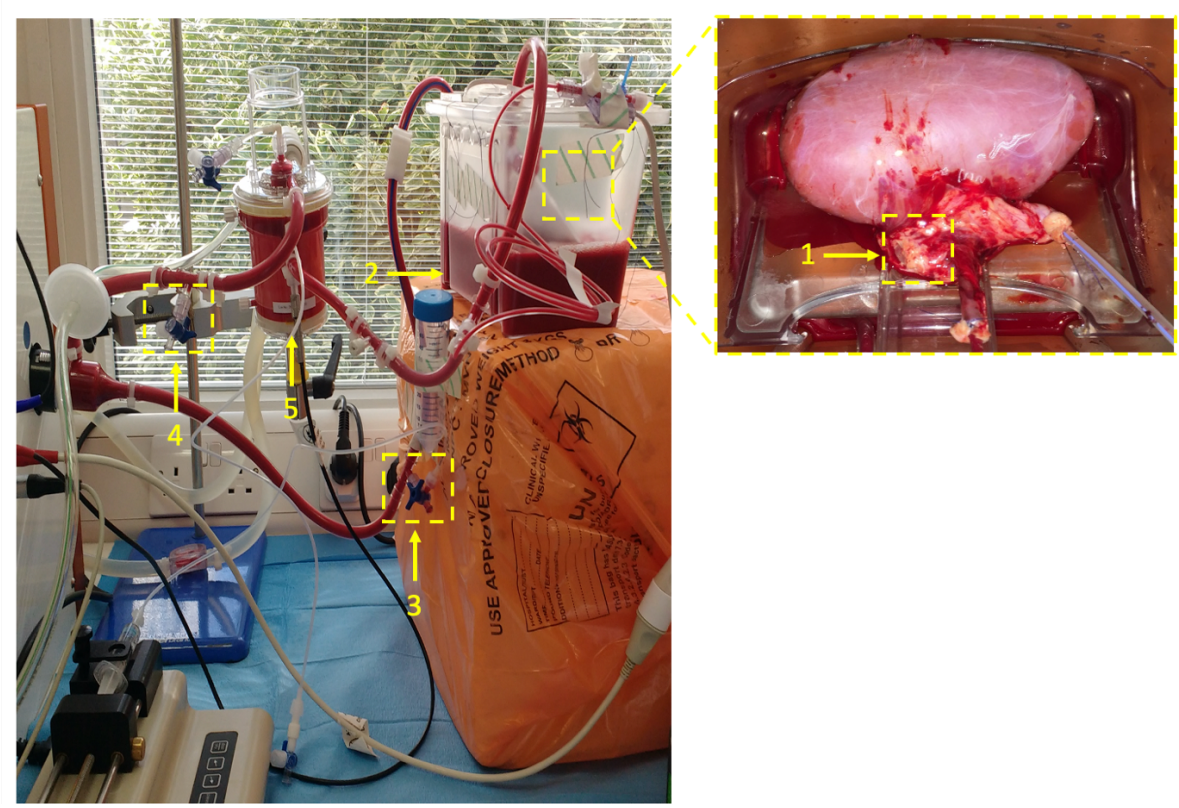


Figure 5.1. Sample locations of perfusate collection during NMP for flow cytometry analysis. Photographic images of the NMP circuit primed with perfusate and a kidney during NMP. After the injection of MSCs during NMP, perfusate was collected at the following locations: 1. renal vein; 2. bottom of the reservoir; 3. venous line; 4. between the pump and oxygenator and 5. arterial line.

Cortical biopsies were taken before and during NMP (t = 0, 1, 2 and 4 hours), while cortical and medulla biopsies were taken at the end of NMP (t = 7 hours) at various locations for flow cytometry and DNA analysis. Tissue samples were collected at various locations at the end of NMP (t = 7 hours), including upper cortex (UppC), upper medulla (UppM), lateral cortex (LatC), lateral medulla (LatM), lower cortex (LowC) and lower medulla (LowM). For histology and protein analysis, punch biopsies (5 mm, Stiefel, North Carolina, USA) were cut in half, with one half stored in 4% formalin while the other half was stored in RNA later (Thermofisher) and snap-frozen in liquid nitrogen, followed by

storage at -80°C. A 5 mm punch biopsy was also collected for flow cytometry analysis and stored in MACS Tissue Storage Solution overnight at 4°C (Miltenyi Biotec Ltd, Woking, United Kingdom) until dissociation of tissue described in paragraph 5.2.10. For DNA analysis, 3 mm x 23 mm cortical needle biopsies (Argon Medical Devices, Frisco, Texas, USA) were collected and stored at -20°C for further analysis.

5.2.6 Kidney assessment during NMP

Perfusion characteristics including renal blood flow, renal resistance and urine production were monitored during perfusion. Arterial and venous blood gas analysis was carried out hourly using the ABL90 FLEX blood gas analyser (Radiometer, Brønshøj, Denmark) to evaluate acid-base balance, pO_2, pCO_2, saturation, sodium, potassium, ionized calcium, glucose and lactate levels. Creatinine, sodium and albumin were measured in perfusate and urine samples obtained during perfusion using routinely automated test methodology carried out by the clinical biochemistry laboratory in the John Radcliffe Hospital, Oxford, United Kingdom. In addition to the tests mentioned above, cellular injury markers lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were measured in perfusate samples.

The kidneys were assessed using a published quality assessment score (QAS) at the end of NMP.[29] The score ranges from 1, indicating the least injury, to 5, indicating the most severe injury and is calculated using macroscopic appearance, renal blood flow and urine output. For macroscopic assessment, each kidney was categorised into one of three groups by two independent surgeons based on colour images taken at the end of NMP. Grade I, excellent perfusion (global pink appearance); grade II, moderate perfusion (patchy pink/purple appearance); or grade III, poor perfusion (global mottling and purple/black appearance). Macroscopic grades I, II and III were assigned scores of 1, 2 and 3, respectively. When the macroscopic appearance score was different, the highest score was selected to calculate the final QAS score. Kidneys with a mean renal

blood flow below 50 mL per min per 100g were given an additional score of 1. Kidneys producing less than 43 mL volume of urine were also given an additional score of 1. Kidneys with a QAS score of 3 or less, if taken in a clinical context, means they could be considered for transplantation.

Renal metabolic activity was approximated by calculation of renal oxygen consumption using arterial and venous blood gasses. The following formula was used to calculate oxygen consumption (mL O_2/min per 100 g)[30]:

$$Oxygen\ consumption: VO_2 = \frac{(CaO_2 - CvO_2)\ \text{x Q}}{g}\ \text{x } 100$$

$$Arterial\ oxygen\ content: CaO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ arterial) + (pO_2\ arterial\ x\ K))$$

$$Venous\ oxygen\ content: CvO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ venous) + (pO_2\ venous\ x\ K))$$

The number 0.024794 is the result of the equation of the oxygen binding capacity of RBCs, 1.54 mL O_2 at 37°C, 1.54 mL O_2 at 37°C[31], times the conversion factor of haemoglobin in g/L to mmol/L. Hb is the haemoglobin concentration in mmol/L, SO_2 is oxygen saturation in %, pO_2 is the partial oxygen pressure in kPa, K is the solubility constant of oxygen in water at 37ºC equals to 0.0225 (ml O_2 per kPa), Q is the renal blood flow in dL/min and *g* is the weight of the kidney in grams at the beginning of NMP.

5.2.7 Pig MSC expansion and characterisation

Since this project is part of the MePEP consortium, passage three porcine adipose-derived MSCs were provided from Aarhus University, Denmark. Male porcine adipose-derived MSCs from three donors (80 kg and approximately six months old) were isolated from subcutaneous adipose tissue and treated with collagenase type IV and the adherent cell fraction was expanded in culture medium. The culture medium consisted of minimum essential medium (Sigma Aldrich, Gillingham, United Kingdom) supplemented with 15% foetal bovine serum (Sigma Aldrich), 50 U/mL penicillin + 50 µg/mL streptomycin (Gibco

by Life Technologies, Warrington, United Kingdom), and 2 mM L-glutamine (Gibco by Life Technologies). Cells were expanded until passage three followed by cryopreservation. MSCs batches were > 95% positive for CD29, CD44, CD90 and negative for CD31 and CD45. Once the MSCs were received in Oxford, United Kingdom, they were thawed, cultured tri-weekly in culture medium and kept in a humidified 5% CO_2 atmosphere at 37°C. MSCs were cultured to passage four until confluence reached between 80-99%.

5.2.8 MSC labelling with Qtracker 655

In order to track the MSCs in the kidney and NMP circuit, MSCs were fluorescently labelled with Qtracker® 655 Cell Labeling Kit (ThermoFisher) according to the manufacturer's instructions. Briefly, cells were trypsinized and cell concentration and viability were counted using a haemocytometer. Twice the amount of MSCs were labelled due to the toxicity of the Qtracker. Component A and B were mixed 1:1 to obtain a 10nM solution, using 1.5 µL of each solution per million cells. This mixture was then incubated for five minutes at RT. Next, 200 µL culture medium per one million cells was added to the mixture and the cells were incubated for one hour with the Qtracker mixture at 37°C. After incubation, the cells were washed twice with culture medium and tested for viability using a haemocytometer. Fluorescence was measured immediately on a small fraction of cells after labelling with flow cytometry to establish the gating strategy to identify the MSCs. The rest of the cells were cryopreserved in Cryostore (Sigma Aldrich), first overnight in Mr Frosty Freezing container (ThermoFisher) containing isopropyl alcohol, followed by storage in the gas phase above liquid nitrogen until needed.

5.2.9 Preparation and infusion of MSC

Prior to each experiment, cryopreserved pre-labelled MSCs were thawed and re-suspended in 20 mL cultured medium followed by centrifugation for five minutes at 440 x g at RT. The cell pellet was re-suspended in 10 mL perfusate, filtered through a 70 μm cell strainer (Sigma Aldrich) and transferred to a 10 mL syringe. A small aliquot of cells was used for a control counting and inspection under the microscope to confirm a single cell solution. MSCs were stored on ice until infusion. After one hour of NMP, MSCs were infused at the arterial port at a rate of 60 mL/h. The infusion line was rinsed with 2 mL of Ringers Solution (Fresenius Kabi, Cheshire, United Kingdom) to ensure all the cells reached the kidney.

5.2.10 Tissue dissociation using the gentleMACS

In order to analyse the kidney biopsies, tissue was dissociated mechanically and enzymatically using the gentleMACS Dissociator (Miltenyi Biotec Ltd, Woking, United Kingdom). Renal tissue (5 mm biopsy) was cut up into small pieces and dissociated in 2.4 mL RMPI-1640 (ThermoFisher) supplemented with 100 μL enzyme D, 50 μL enzyme R, and 12.5 μL enzyme A of a Multi Tissue dissociation kit 1 (Miltenyi Biotec) and run with protocol 37C_Multi_B for one hour. After dissociation, RPMI-1640 was added to stop the reaction and samples were filtered using a 70 μm filter (Sigma). Cells were then centrifuged at 500 x g for seven minutes and the pellets were resuspended in PBS for flow cytometry analysis.

5.2.11 Flow cytometry detection of labelled MSCs in perfusate and kidney

5.2.11.1 MSCs and flow cytometry sensitivity

To test the sensitivity and feasibility of the flow cytometer to detect the Qtracker655 labelled MSCs in perfusate and tissue, dissociated tissue from the renal cortex and medulla from NMP perfused kidneys were mixed with 1 million, 500,000, 250,000 or

125,000 MSCs. The amount of MSCs were counted with counting beads (CountBright Absolute Counting Beads, ThermoFisher) on the flow cytometer as described in paragraph 5.2.11.2. Overall, approximately 25% MSCs (Qtracker655 positive cells) were detected in the dissociated tissue with flow cytometry (Appendix, Table S5.1).

5.2.11.2 Flow cytometry analysis

Flow cytometry analysis was used to detect and measure the viability of Qtracker labelled MSCs in the perfusate and the kidney. From the total dissociated kidney tissue and perfusate, 400 µL was filtered using the FACS tube filter (Falcon, Corning, New York, USA) and 300 µL was analysed. In order to determine MSCs concentration, 50 µL of counting beads (ThermoFisher) were added to each sample. Samples were stained 1:20 with 10 µg/mL 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, BD Biosciences, San Jose, California, USA) to test viability. DAPI is a fluorescent nucleic acid stain that binds to minor grove A-T rich regions of double-stranded DNA. It is essentially excluded from viable cells but can penetrate cell membranes of dead or dying cells. DAPI is used for evaluation of cell death and apoptosis of unfixed cells in flow cytometry.[32] Cells were measured on the BD LSR II (BD Biosciences). Unlabelled and unstained cells were used for gate setting. Qtracker was measured by excitation with a 405nm laser (violet laser) and detection with the 660/20 filter. The total number of MSCs in kidney tissue or blood was estimated from the number of events and the volume measured from each sample. Data were analysed using FlowJo software (version 10.5.3, FlowJo, Ashland, Oregon, USA). Non-detected cells are defined and calculated as cero (0) cells.

5.2.12 DNA isolation and detection of male pMSCs in the kidney tissue

5.2.12.1 DNA isolation

DNA was isolated from approximately 10 mg cortical tissue using a NucleoSpin Tissue DNA isolation kit (2017, revision 1 MACHEREY-NAGEL, Düren, Germany) according to

the manufacturer's protocol with a pre-lysis step. The pre-lysis consists of the incubation of tissue in T1 buffer supplemented with Proteinase K at 56°C. Pre-lysis was continued for a total of four hours for each sample while occasionally vortexing. DNA was eluted in 100 µl elution buffer and DNA was stored at -20°C until quantitative real-time polymerase chain reaction (qPCR) analysis.

5.2.12.2 Y-chromosome detection with quantitative real-time PCR

Y-chromosome was detected by qPCR using primers directed to the male specific repeat (MSR) located on the porcine Y-chromosome as done previously by Gruessner *et al.*[33] Primers used were: MSR upper 5'-CCA TCG GCC ATT GTT TTC CTG TTC A-3', MSR lower 5'-CCT CTG TGC CCA CCT GCT CTC TAC A-3', S100C upper 5'-ATG CTG GAA GGG ACG GTA ACA ACA-3', and S100C lower 5'-GCT CAG CTG CTG TCT TTC ACT CGT-3'. As a control for pig DNA, primers were used directed to the porcine S100C gene. qPCR mix consisted of 1 µL DNA, 10 pmol of each primer and 1x KiCStart SybrGreen qPCR ReadyMix (Sigma Aldrich) in an end volume of 25 µL. Samples were run in triplicates on Applied Biosystems ViiA 7 Real-Time PCR System (ThermoFisher). The thermocycling program included an initial step of 2 minutes at 50°C and 10 minutes at 95 °C, followed by a 40-time repeat two-step cycle at 95°C for 15 seconds and 60°C for 1 minute.

5.2.13 Tracking of MSCs in tissue with confocal microscopy

5.2.13.1 Immunofluorescent staining

Formalin-fixed and paraffin-embedded kidney biopsies were cut into 4µm thick slices. To observe whether MSCs were inside or outside of the renal capillaries, CD31 was stained on the renal tissue containing labelled infused MSCs. Immunofluorescent staining was performed by automated multiplex IF using the Ventana Benchmark Discovery (Ventana Medical Systems Inc., Roche, Almere, the Netherlands). In brief, following

deparaffinization and heat-induced antigen retrieval with CC1 (#950-500, Ventana Medical Systems Inc., Roche) for 64 minutes at 95°C, the tissue slides were incubated with CD31 (ab28364, 1:50, Abcam, Cambridge, United Kingdom) for 32 minutes at 37˚C followed by detection with Ultramap anti-rabbit HRP (#760-4315, Ventana Medical Systems Inc., Roche) for 12 minutes. Next, tissue slides were visualized with FAM for 24 minutes (#760-243, Ventana Medical Systems Inc., Roche), followed by incubation in PBS with DAPI for 15 minutes and covered with anti-fading medium (S3023, DAKO, Amstelveen, the Netherlands). Slides were scanned using the Zeiss LSM700 microscope and confocal images were created at 20x magnification.

5.2.13.2 MSCs identification

From each slide, six random fields were analysed for MSCs identification using a Leica TCS SP5 confocal microscope (Leica Microsystems B.V., Eindhoven, the Netherlands) equipped with Leica Application Suite – Advanced Fluorescence (LAS AF) software, 405nm laser using a 20x objective. In order to assess the presence of MSCs in renal tissue, the number of glomeruli containing MSCs from each microscopic field and the number of fluorescent MSCs outside glomeruli were counted in random order. The percentage of MSC-occupied glomeruli was calculated for each field and the number of fluorescent MSC outside glomeruli per field was averaged as MSC per microscopic field.

5.2.14 Prostaglandin E2 analysis

Prostaglandin E2 (PGE2) levels were measured in the perfusate using a competitive enzyme immunoassay (Prostaglandin E2 Parameter Assay Kit, KGE004B, R&D systems, Abingdon, United Kingdom) according to the manufacturer's protocol. In this assay, PGE2 competes with horseradish peroxidase (HRP)-labelled PGE2 for a limited number of binding sites on a mouse monoclonal antibody. Briefly, samples and a series of standard was added to a 96-well microplate pre-coated with goat anti-mouse

polyclonal antibody. Next, 50µL mouse monoclonal antibody to PGE2 was added to the wells and incubated for one hour at room temperature (RT) on a microplate shaker at 250 rpm. After incubation, 50µL of PGE2 conjugate HRP was added to the wells and the plate was incubated for two hours at RT on a microplate shaker at 250 rpm. Wells were washed and substrate solution containing stabilised hydrogen peroxide and tetramethylbenzidine (TMB) was added to the wells and the plate was incubated for 30 minutes at RT in the dark. The reaction was stopped with a stop solution was and the optical density was measured at 450 nm and 540 nm using a spectrophotometer. Readings were corrected by subtracting 450 nm readings from 540 nm readings. PGE2 concentration was determined by interpolation from the standard curve.

5.2.15 Interleukin 10 analysis

Levels of interleukin (IL)-10 were determined in the perfusate using a sandwich enzyme-linked immunosorbent assay (Porcine IL-10 Quantikine ELISA Kit, P1000, R&D system, Abingdon, United Kingdom) according to the manufacturer's protocol. In brief, samples and a series of standard were added to a pre-coated 96-well plate with a monoclonal antibody specific for pig IL-10 and incubated for two hours at RT on a microplate shaker. Wells were washed and then incubated with pig IL-10 conjugated HRP polyclonal antibody for two hours at RT on a microplate shaker. After washing, hydrogen peroxide and TMB substrate were added to the wells and the plate was incubated for 30 minutes at RT in the dark. The reaction was stopped with a stop solution and the optical density was measured at 450 nm and 540 nm using a spectrophotometer. Readings were corrected by subtracting 450 nm readings from 540 nm readings. Levels of IL-10 were determined by interpolation from the standard curve.

5.2.16 Angiopoietin-1 and vascular endothelial growth factor analysis

Levels of angiopoietin-1 and vascular endothelial growth factor-a (VEGF-A) were measured using porcine angiopoietin-1 (ab273219, Abcam, Cambridge, UK) and VEGF-A (ab218298, Abcam) ELISA kits according to the manufacture's protocol. In brief, samples and a series of standard were added to a pre-coated 96-well plate and incubated for 2.5 hours at RT on a microplate shaker. Wells were washed and then incubated with biotinylated pig antibody, specific for the markers, for one hour at RT on a microplate shaker. After washing, streptavidin was added to the well and incubated for 45 minutes at RT on a microplate shaker. Wells were washed again, TMB substrate was added to the wells after washing and the plate was incubated for 30 minutes at RT protected from light. The reaction was stopped with a stop solution was and the optical density was measured at 450 nm. Levels of angiopoietin 1 and VEGF-A was determined by interpolation from the standard curves.

5.2.17 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 8 (GraphPad Software Inc., USA). For all continuous longitudinally measured variables, the area under the curve (AUC) was calculated. The Shapiro–Wilk test was used to test variables for normal distribution. Normally distributed data were analysed using a student's *t*-test and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to compare more than two groups. Two-way ANOVA with Tukey's multiple comparison test was used to assess the interaction between two variables (MSCs quantity and time) of more than two groups. Non-parametric data were analysed using Mann Whitney U test and Kruskal-Wallis test with Dunn's multiple comparisons test to compare more than two groups. Categorical data were compared using Chi-square test Data are presented as mean ± standard deviation. P-value of ≤ 0.05 was considered statistically significant. One asterisk (*) indicates a level of significance of $p \leq 0.05$. Two asterisks (**) indicate a p-value of ≤ 0.01 and three asterisks (***) a p-value of $p \leq 0.001$.

5.3 RESULTS

5.3.1 Warm and cold ischaemia time, HMP duration and kidney weight

In phase one, the duration of warm ischaemia, time on HMP and kidney weight were similar between groups. Cold ischaemia time was significantly higher in the 2 million MSC (p=0.006, ANOVA) and 50 million MSC groups (p=0.0002, ANOVA) compared to the control group. (Table 5.1). There was no difference in warm and cold ischaemia time, time on HMP and kidney weight in phase two.

Table 5.1. Warm and cold ischaemia time, HMP duration and kidney weight from kidneys perfused on NMP with 2, 10 and 50 million MSCs administration. Data are presented as mean ± standard deviation. CIT, cold ischaemia time; HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cells; NMP, normothermic machine perfusion; WIT, warm ischaemia time.

Phase 1	**Control (n=3)**	**2 million MSC (n=3)**	**10 million MSC (n=3)**	**50 million MSC (n=3)**	**p-value**
WIT (minutes)	14 ± 4	14 ± 3	16 ± 2	14 ± 2	0.608
CIT (hours)	3.98 ± 0.28	4.91 ± 0.19	4.12 ± 0.25	5.62 ± 0.34	**0.0003***
HMP duration (hours)	3.20 ± 0.28	3.69 ± 0.26	3.27 ± 0.27	3.94 ± 0.51	0.089
Kidney weight (g)	198 ± 148	233 ± 20	251 ± 70	225 ± 70	0.901
Phase 2	**Control (n=6)**		**10 million MSC (n=6)**		**p-value**
WIT (minutes)	15 ± 2		16 ± 2		0.476
CIT (hours)	4.20 ± 0.58		4.69 ± 0.94		0.307
HMP duration (hours)	3.41 ± 0.63		3.80 ± 0.93		0.422
Kidney weight (g)	210 ± 53		267 ± 57		0.100

* 2 million MSC and 50 million MSC groups significantly higher than the control group.

5.3.2 Phase one: Dose-response study

In the first phase, we performed a dose-response study to track the location and distribution of MSCs in the kidney and to monitor renal perfusion parameters during NMP for evidence of dysfunction. Kidneys were perfused for seven hours on NMP and after one hour, *either* vehicle (control, n=3) *or* treatment with 2 million MSC (n=3), 10 million MSC (n=3) or 50 million MSC (n=3) was administered intra-arterially and compared.

5.3.2.1 Detection of pre-labelled MSC with flow cytometry

5.3.2.1.1 The distribution of MSCs in the tissue is heterogeneous

To determine if and where MSCs home in the kidney, labelled MSCs were processed and tracked in biopsies with flow cytometry. After intra-arterial infusion, MSCs (Qtracker 655 positive cells) were present in the renal cortex and medulla after one hour (T2), three hours (T4) and six (T7) hours of administration and showed a heterogeneous distribution in all three groups (Figure 5.1). The number of MSCs present in the tissue increased with dose but the overall percentage was unchanged across the three doses. Measured MSCs during perfusion ranged from 0 to 6,260, 0 to 11,707 and 0 to 90,452 MSCs per gram of dissociated tissue in the 2 million MSC, 10 million MSC and 50 million MSC groups, respectively. Extrapolation of measured concentrations of MSCs using total kidney weight indicates that an average of 14, 9 and 8% of MSCs were found back in the kidney after administration of 2, 10 and 50 million MSCs, respectively.

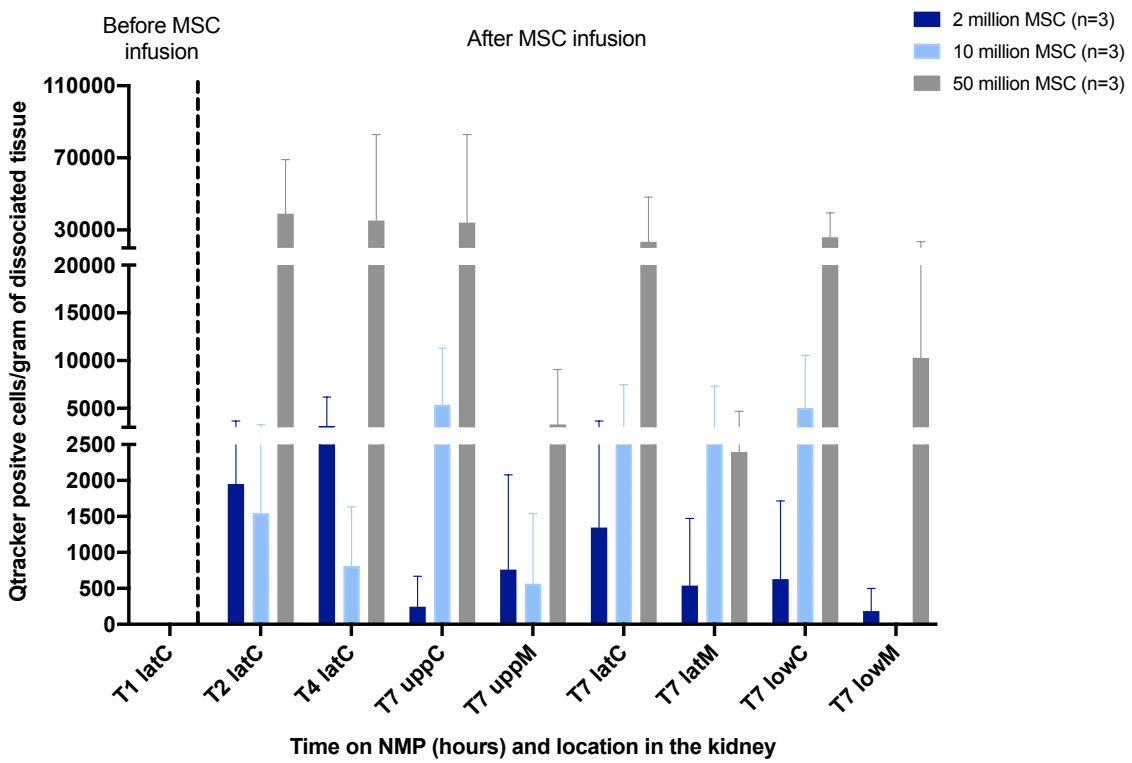


Figure 5.1. Qtracker positive cells measured in renal tissue by flow cytometry undergoing NMP with 2 million MSC, 10 million MSC and 50 million MSC infusion. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were collected during and after NMP and processed into single cell suspension with the GentleMACS. Samples were analysed with flow cytometry. MSCs were present in the 2 million MSC group, 10 million MSC group and 50 million MSC group and showed a heterogenous distribution. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; UppC, upper cortex; UppM, upper medulla, LatC: lateral cortex, LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

Viability, assessed by staining with DAPI, of the detected MSCs in the renal cortex measured by flow cytometry at the end of NMP had a combined average of 12 ± 11% live MSCs in the 2 million MSC group, 26 ± 12% live MSCs in the 10 million MSC group and 54 ± 13% live MSCs in the 50 million MSC group. The number of live cells in the renal medulla had a combined average of 4 ± 6 % in the 2 million MSC group, 13 ± 18% in the 10 million MSC group and 32 ± 19% in the 50 million MSC group. Viable MSCs were detected in all kidneys perfused in the 50 million MSC group and at every timepoint (Figure 5.2).

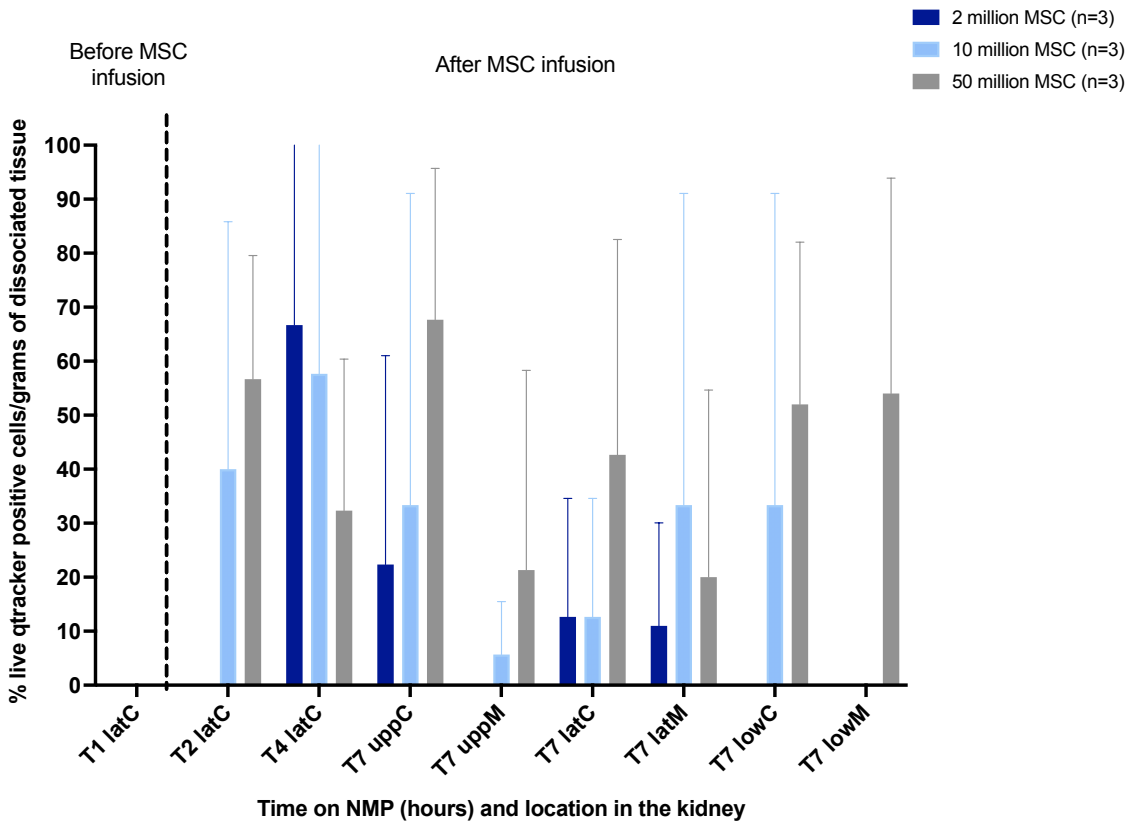


Figure 5.2. Viability of Qtracker positive cells measured in renal tissue by flow cytometry undergoing NMP with 2 million MSCs, 10 million MSCs and 50 million MSCs infusion. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were collected during and after NMP and processed into single cell suspension with the GentleMACS. Samples were analysed with flow cytometry. There was a relatively large difference in the percentage of viable cells detected between the samples at every timepoint in all groups. Kidneys perfused in the 50 million MSC group had viable cells at every timepoint. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; UppC, upper cortex; UppM, upper medulla, LatC: lateral cortex, LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

5.3.2.1.2. Low amounts of Qtracker positive cells are found in the perfusate

Perfusate samples from different locations in the circuit were collected to determine if MSCs were retained in the kidney or if MSCs pass through the kidney and circulate in the perfusate. After 10 minutes of MSC infusion, an average of 42 ± 79 MSC/mL, 21 ± 13 MSC/mL and 29 ± 45 MSC/mL were measured exiting the kidney through the renal vein in the 2 million MSC, 10 million MSC and 50 million MSC groups respectively (Figure 5.3A). After 20 minutes of MSC infusion, lower amounts of circulating MSCs were measured in the perfusate at every location in the circuit and time points in all groups (Figure 5.3A–5.3E).

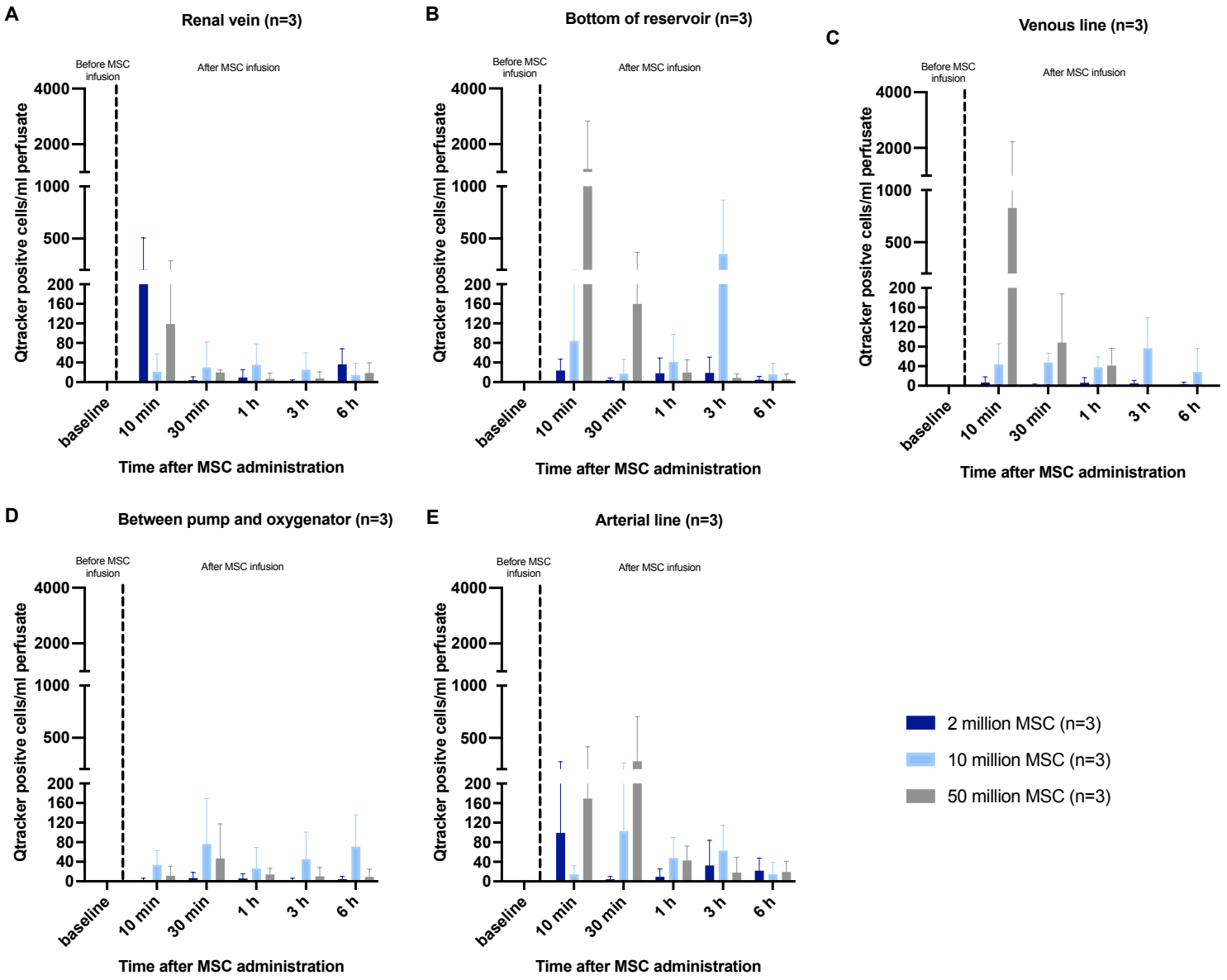


Figure 5.3 Qtracker positive cells measured by flow cytometry in the perfusate of kidneys undergoing NMP with 2 million MSCs, 10 million MSCs and 50 million MSCs infusion. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Perfusate was collected and treated with RBC-lysis buffer and subsequently analysed with flow cytometry. In all groups low amounts of Qtracker positive cells were found in the perfusate at different locations in the circuit during NMP (A = renal vein, B = bottom of reservoir, C = venous line, D = between pump and oxygenator, E = arterial line). Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; UppC, upper cortex; UppM, upper medulla, LatC: lateral cortex, LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

5.3.2.2 qPCR confirmed MSC retention in the tissue

Biopsies confirmed successful delivery of MSCs by Y-chromosome qPCR in the cortex and medulla during and after NMP in all groups (Figure 5.4). There was a significantly higher number of MSCs present in the upper cortex ($p=0.025$, 2-way ANOVA), lateral cortex ($p<0.0001$; 2-way ANOVA) and lower cortex ($p=0.0004$; 2-way ANOVA) after NMP (t=7 hours) compared to baseline in the 50 million MSC group.

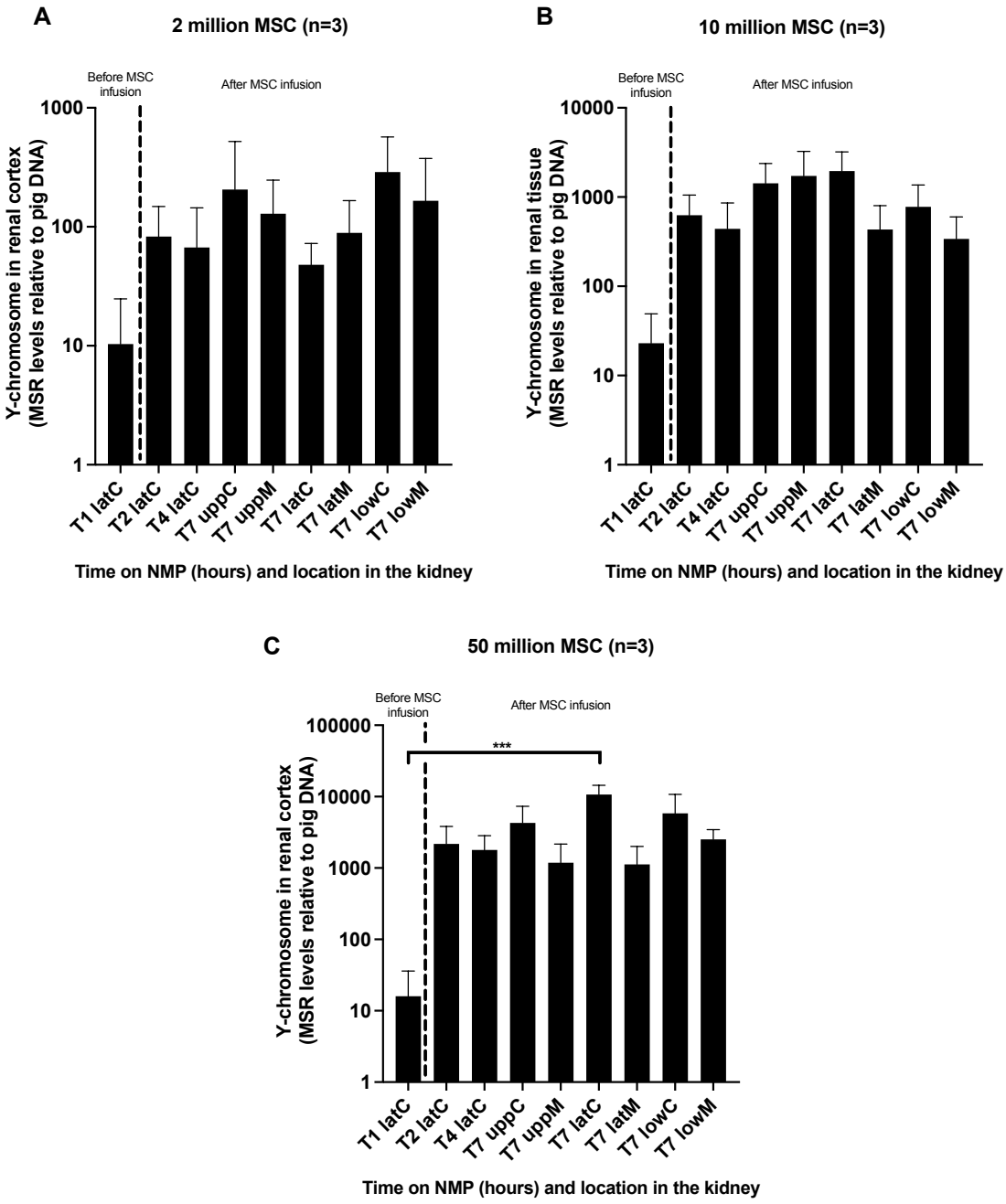


Figure 5.4. Relative Y-chromosome compared with total pig DNA in kidney tissue after NMP with 2 million MSCs, 10 million MSCs and 50 million MSCs infusion. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were collected during and after NMP. DNA was isolated and PCR was performed against the male specific repeat (MSR) on the Y-chromosome. Relative Y-chromosome compared with total pig DNA in kidney tissue after NMP with (A) 2 million MSCs, (B) 10 million MSCs and (C) 50 million MSCs infusion. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP: normothermic machine perfusion; qPCR, real-time quantitative polymerase chain reaction; UppC, upper cortex; UppM, upper medulla; LatC, lateral cortex; LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

When comparing the groups for detected Y-chromosome at different locations in the kidney, kidneys in the 50 million MSC group had significantly higher amounts of MSC present in the upper cortex ($p=0.004$. 2-way ANOVA), lateral cortex and lower cortex ($p<0.0001$, 2-way ANOVA) compared with the 2 million MSC group. Kidneys perfused in the 50 million MSC group had also significantly higher MSCs present in the lateral cortex ($p<0.0001$; 2-way ANOVA) and lower cortex ($p=0.0003$. 2-way ANOVA) compared to the 10 million MSC group (Figure 5.5).

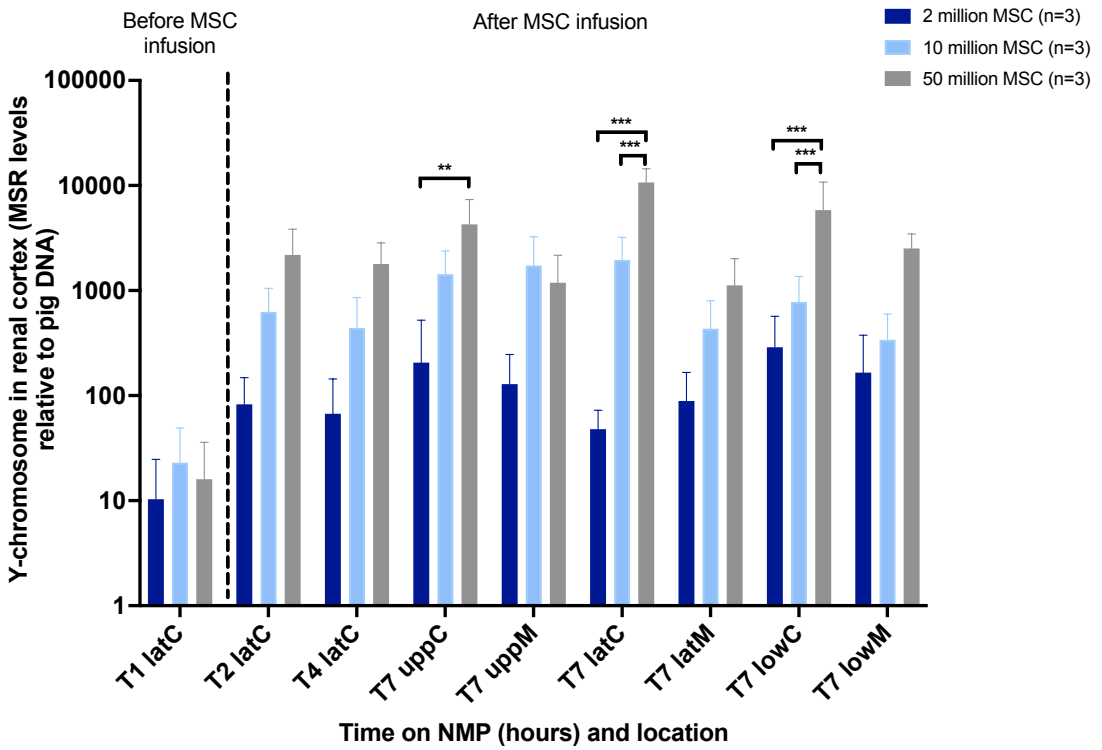


Figure 5.5. Comparison of relative Y-chromosome detected in kidney tissue undergoing NMP with 2, 10 and 50 million MSCs infusion. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were taken during and after NMP. DNA was isolated and qPCR was performed against the male specific repeat (MSR) on the Y-chromosome. Relative Y-chromosome compared with total pig DNA in kidney tissue during and after NMP. Kidneys perfused in the 50 million MSC group had significantly higher levels of Y-chromosome present at the end of NMP compared to kidneys in the 2 million MSC group in the upper, lateral and lower cortex. Kidneys in the 50 million MSC group had also significant higher Y-chromosome present than the kidneys in the 10 million MSC group in the lateral and upper cortex. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP: normothermic machine perfusion; qPCR, real-time quantitative polymerase chain reaction; UppC, upper cortex; UppM, upper medulla; LatC, lateral cortex; LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

5.3.2.3 MSCs are mainly localised in the glomeruli

Confocal microscopy demonstrated that MSCs (Qtracker 655 positive cells) were mainly localised in the luminal part of the glomerular structures in the renal cortex (Figure 5.6).

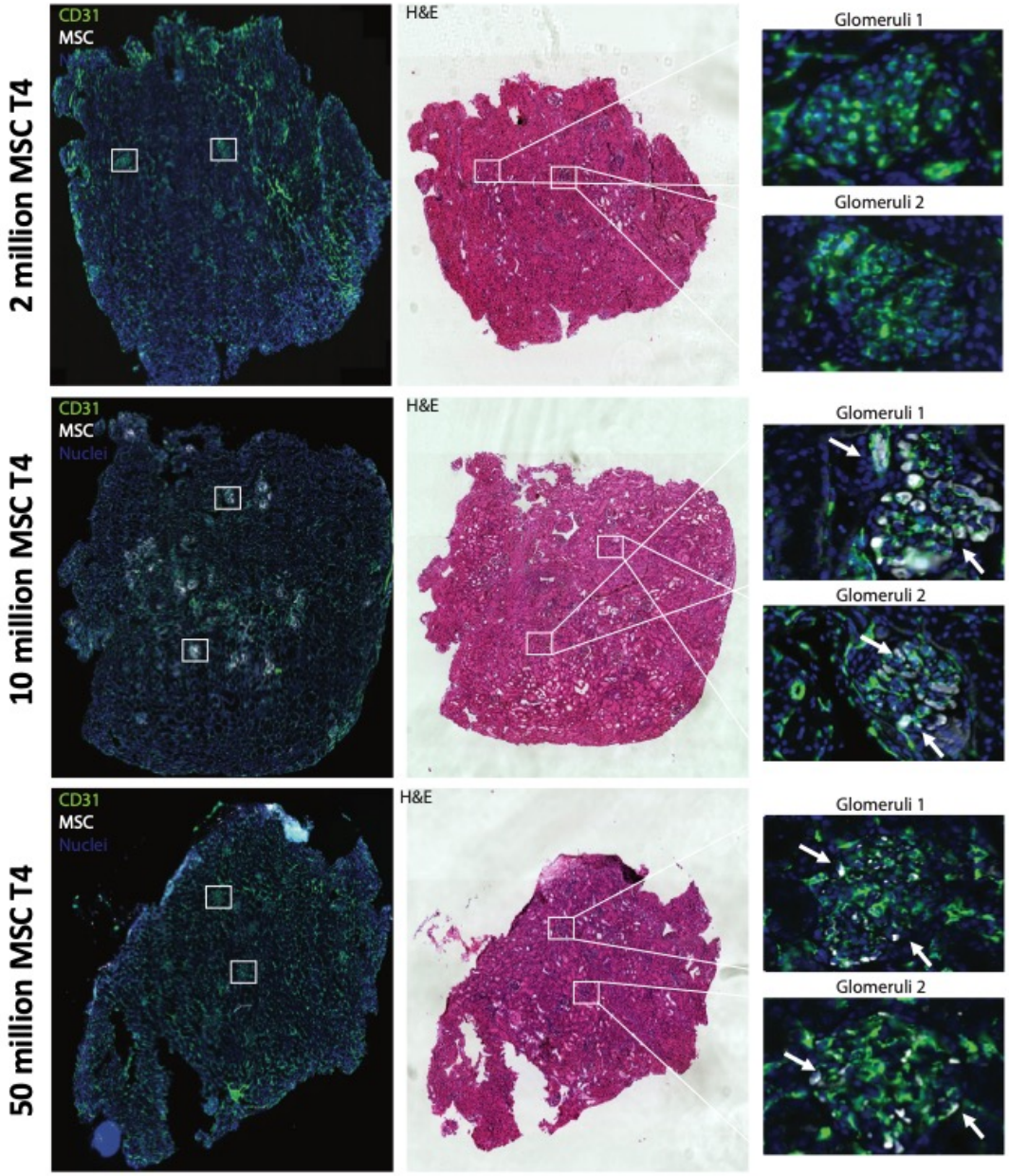


Figure 5.6. Detected MSCs with confocal microscopy from kidneys perfused with 2, 10 and 50 million MSCs. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were taken before, during and after NMP. Tissue samples were fixed in 4% formaldehyde, processed, and embedded in paraffin wax for sectioning. Sections were stained with CD31 (green) to detect endothelial cells and DAPI (blue) to indicate the nucleus. The picture shows detected MSCs (Qtracker 655 positive cells, white) in the glomeruli after 3 hours of 2,10 and 50 million MSCs administration. DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

Mean values of MSCs in the tissue were 27± 33% in the 2 million MSC group, 73 ± 26 % in the 10 million MSC group and 85 ± 28% in the 50 million MSC group (Figure 5.7A). There was a significant increase in the percentage of localised MSCs observed in the glomeruli per macroscopic field in the 10 million MSC group ($p=0.002$; Kruskal-Wallis test) and 50 million MSC group ($p<0.0001$; Kruskal-Wallis test) compared to the 2 million MSC group (Figure 5.7A). No significant differences were observed between the 10 and 50 million MSC groups ($p=0.768$; Kruskal-Wallis test). Low numbers of MSCs (Qtracker 655 positive cells) were observed outside of the glomeruli at the end of NMP, Figure 5.7B.

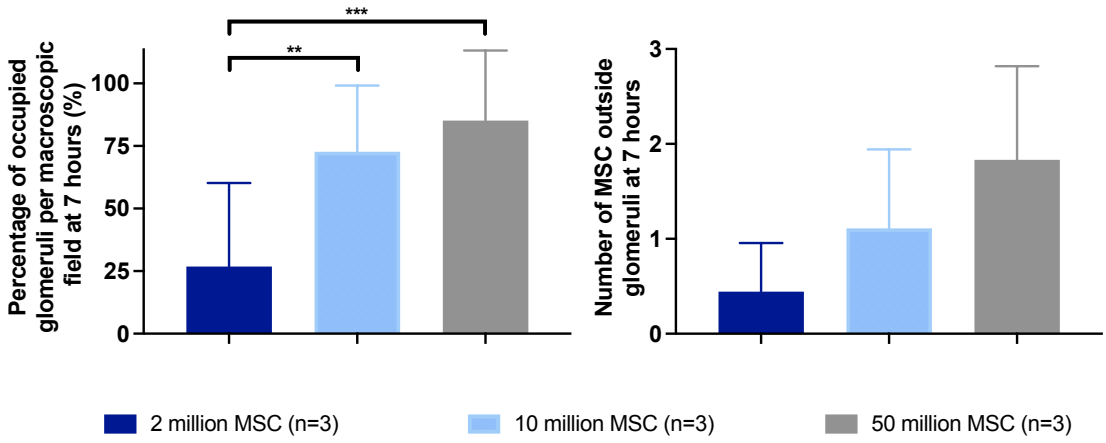


Figure 5.7. MSCs are mainly found in the glomeruli. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were taken from the lateral cortex before, during and after NMP. Tissue samples were fixed in 4% formaldehyde, processed, and embedded in paraffin wax for sectioning. (A) Significant percentage of MSCs (Qtracker 655 positive cells) were observed in the glomeruli per macroscopic field in the 10 and 50 million groups compared to the 2 million MSC group at the end of NMP. (B) There were low numbers of MSCs (Qtracker 655 positive cells) observed outside of the glomeruli. Data are presented as mean ± standard deviation. DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

5.3.2.4 NMP perfusion characteristics and injury markers during NMP

5.2.3.4.1 Perfusion characteristics and kidney quality

Renal blood flow increased during the first hour of NMP and slowly decreased afterwards. Mean renal blood flow values were 100 ± 40 mL/min/100g in the control group, 74 ± 10 mL/min/100g in the 2 million MSC group, 63 ± 17 mL/min/100g in the 10 million MSC group and 82 ± 53 mL/min/100g in the 50 million MSC group (Figure 5.8A). There were no differences between kidneys perfused with MSCs and the control group (p=0.840, Kruskal-Wallis test). There were also no differences in flow values at the end of NMP (control group: 71 ± 42 mL/min/100g, 2 million MSC group: 40 ± 13 mL/min/100g, 10 million MSC group: 48 ± 12 mL/min/100g and 50 million MSC group: 76 ± 60 mL/min/100g, p=0.613, ANOVA). Mean renal resistance was 0.44 ± 0.13 mmHg/mL/min in the control group, 0.46 ± 0.10 mmHg/mL/min in the 2 million MSC group, 0.52 ± 0.03 mmHg/mL/min in the 10 million MSC group and 0.51 ± 0.18 mmHg/mL/min in the 50 million MSC group and there were no significant differences between the groups (p=0.849, ANOVA; Figure 5.8B). At the end of perfusion there were also no differences between the groups (control group: 0.62 ± 0.30 mmHg/mL/min, 2 million MSC group: 0.79 ± 0.25 mmHg/mL/min, 10 million MSC group: 0.63 ± 0.11 mmHg/mL/min and 50 million MSC group: 0.55 ± 0.24 mmHg/mL/min, p=0.577, ANOVA). There was no difference in urine output between the groups during NMP; however, there was one of the five kidneys in the 50 million group that produced high amount of urine (control group: 10 ± 15 mL, 2 million MSC group: 21 ± 30 mL, 10 million MSC group: 26 ± 13 mL and 50 million MSC group 65 ± 56, p=0.282, ANOVA; Figure 5.8C). After administration of the MSCs, no effect on renal blood flow and renal resistance were observed.

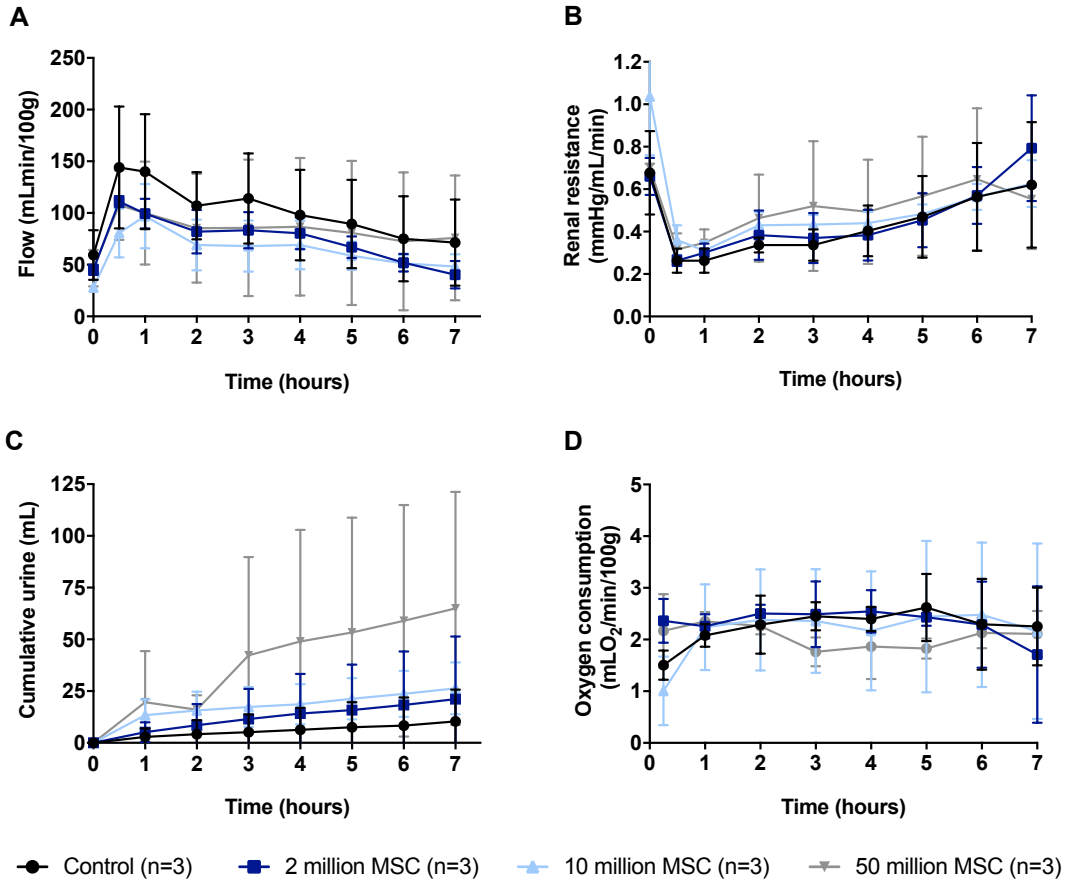


Figure 5.8. Perfusion characteristics, urine production and oxygen consumption during NMP with NMP with administration of no MSCs, 2 million, 10 million and 50 million MSCs. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. There were no differences in (A) mean renal blood flow per 100 grams and (B) mean renal resistance between the groups. (C) There was a relatively large difference in cumulative urine between the groups. (D) All kidneys consumed oxygen in a similar manner during NMP. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

Kidney quality and metabolic activity were assessed by oxygen consumption and the QAS score. All kidneys consumed oxygen similarly and there were no differences between the groups (p=0.905, ANOVA). At the end of perfusion (t = 7 hours), mean oxygen consumption levels were 2.25 ± 0.75 mLO_2/min/100g in the control group, 1.71 ± 1.32 mLO_2/min/100g in the 2 million MSC group, 2.16 ± 1.70 mLO_2/min/100g in the 10 million MSC group and 2.11 ± 0.45 mL O_2/min/100g in the 50 million MSC group and there were no differences between the groups (p=0.941, ANOVA; Figure 5.8D).

Two of the three kidneys in the 2 million MSC group had a QAS more than 3. One of the three kidneys perfused in the control and 10 million MSC groups had a QAS more than 3. All kidneys in the 50 million MSC group had a QAS equal or less than 3 (Table 5.2). There were no differences in QAS between the groups (p=0.392, Chi-square test).

Table 5.2. Quality assessment score (QAS) of kidneys undergoing NMP with either a vehicle or 2, 10 and 50 million MSC infusion. The QAS is based on urine production, renal blood flow and macroscopic appearance at the end of NMP.

Kidney	Urine	Flow	Macroscopic appearance	QAS score
Control 1	1	0	2	3
Control 2	1	0	1	2
Control 3	1	1	2	4
2 million MSC 1	0	0	2	2
2 million MSC 2	1	1	3	5
2 million MSC 3	1	1	3	5
10 million MSC 1	1	0	1	2
10 million MSC 2	1	1	3	5
10 million MSC 3	1	0	2	3
50 million MSC 1	1	0	2	3
50 million MSC 2	0	0	2	2
50 million MSC 3	0	1	2	3

5.2.3.4.2 Injury markers lactate, LDH and AST

After seven hours of NMP, mean lactate values were 6.9 ± 2.3 mmol/L in the control group, 7.4 ± 3.9 mmol/L in the 2 million MSC group, 11.2 ± 7.8 mmol/L in the 10 million MSC group and 10.0 ± 7.6 mmol/L in the 50 million MSC group. There were no differences in lactate between the groups (p=0.761, Kruskal-Wallis test; Figure 5.9A). Similarly, injury marker LDH increased in all groups throughout NMP and was similar at the end of NMP (p=0.418, ANOVA; Figure 5.9B). There were no differences in injury marker AST in the perfusate between kidneys perfused with MSCs compared to the control group. (p=0.370, Kruskal-Wallis test; Figure 5.9C).

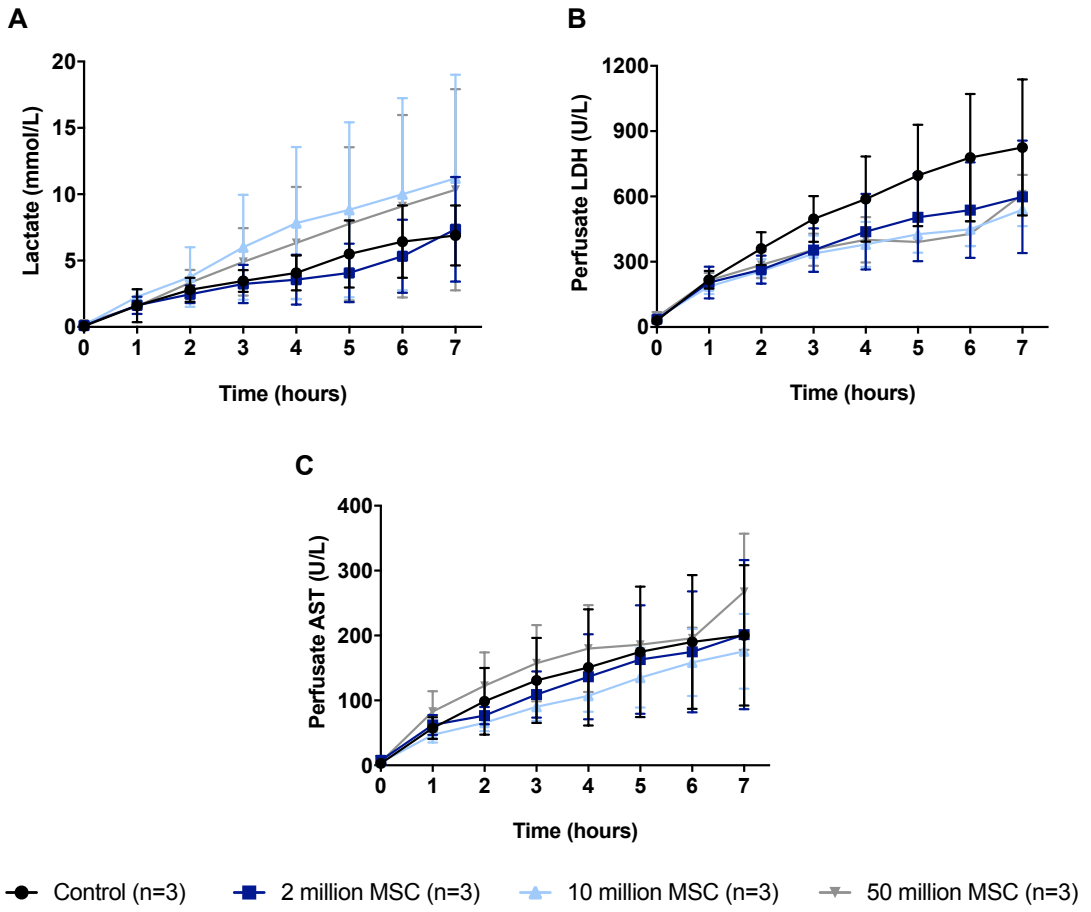


Figure 5.9. Kidney injury markers LDH and AST as well as lactate during NMP with administration of no MSCs, 2 million, 10 million and 50 million MSCs. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million (n=3), 10 million (n=3) or 50 million (n=3) of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Perfusate (A) lactate, (B) LDH and (C) AST increased in all groups during NMP and there are no differences between the groups. Data are presented as mean ± standard deviation. AST, aspartate aminotransferase; HMP, hypothermic machine perfusion; LDH, lactate dehydrogenase; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

5.3.3 Optimal MSC dose

The addition of 2, 10 and 50 million MSCs did not adversely affect perfusion parameters as renal blood flow, renal resistance, oxygen consumption and injury markers LDH and AST were similar between the groups. Furthermore, significantly more MSC was observed in the glomeruli per macroscopic field when administrating 10 and 50 million MSCs compared to 2 million MSCs. No difference was observed between the 10 million MSC and 50 million MSC groups. Given these results and the significant additional cost and time expanding 50 million MSCs compared to 10 million MSCs, 10 million MSC was defined as optimal and was selected for phase two.

5.3.4 Phase two: Completion of 10 million MSC dose and control groups

In the second phase, we increased the number of experiments in the optimal 10 million MSC group and the control group to n=6 per group. The optimal dose was defined as the lowest MSCs dose with histological evidence of widespread of MSCs distribution throughout the renal cortex without adversely affecting renal perfusion parameters. Kidneys were perfused on NMP with a vehicle (control, n=6) and with 10 million MSC (n=6) administration were compared.

5.3.4.1 Detection of pre-labelled MSCs with flow cytometry

5.3.4.1.1 Detection of MSCs in the tissue

MSCs were primarily present in the renal cortex however, lower amounts of MSCs were also detectable in the medulla and there was a heterogeneous distribution of the MSCs in the tissue. (Figure 5.10A). After NMP, a combined average of 4525 ± 1630 MSCs were detected in the cortex and 17 ± 8% were still viable (Figure 5.10B). There was a combined average of 1240 ± 818 MSC detected in the medulla, and 8 ± 11% were still viable (Figure 5.11B).

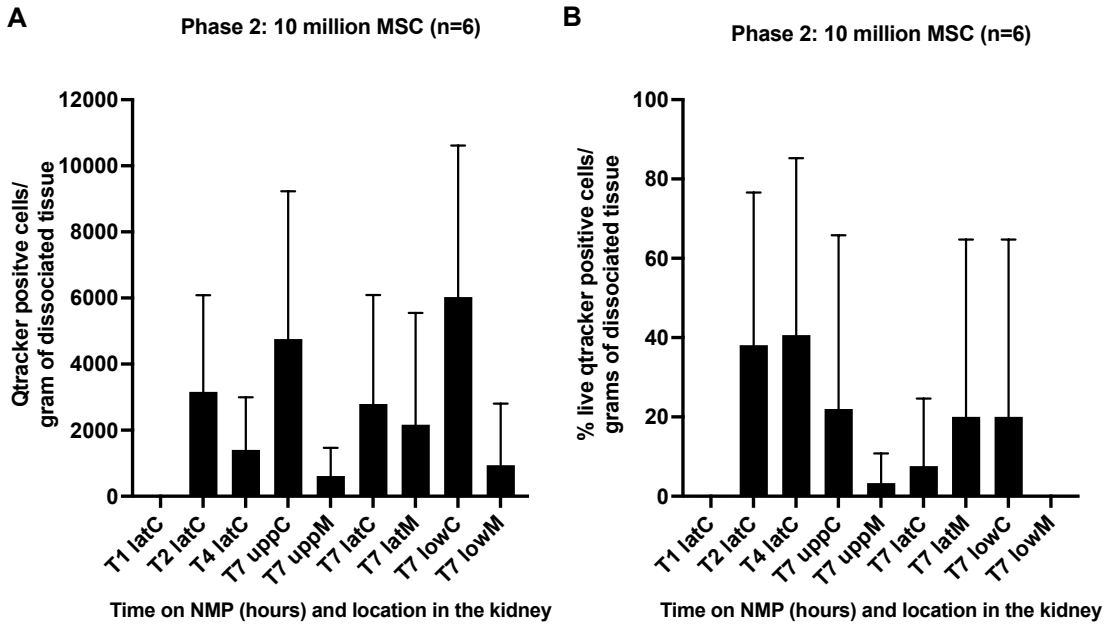


Figure 5.10. Detected Qtracker positive cells and percentage of viable Qtracker positive cells measured by flow cytometry in renal tissue during NMP with 10 million MSCs administration. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 10 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were collected during and after NMP and processed into single cell suspension with the GentleMACS. Samples were analysed with flow cytometry. (A) MSC were present in the renal cortex and medulla after NMP and showed a heterogeneous distribution. (B) The combined average of detected live MSC at seven hours were below 17% in the cortex and 8% in the medulla. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; UppC, upper cortex; UppM, upper medulla, LatC: lateral cortex, LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

5.3.4.1.2 Detection of MSCs in the perfusate

For the second phase, we decided to take an additional perfusate sample after five minutes to determine if high amounts of MSCs exit the kidney during the administration. Low amounts of MSCs were detected in renal vein samples in the kidney effluent during the administration of MSCs (after five minutes), and this amount slowly decreased during NMP (Figure 5.11A). Minimal amounts of MSC, less than 1000, were in the perfusate at the different locations in the circuit (Figure 11A-11E). Similarly, as in phase one, overall, only low amounts of MSCs were circulating in the perfusate at the different locations at the specific time points (Figure 5.11A-11E).

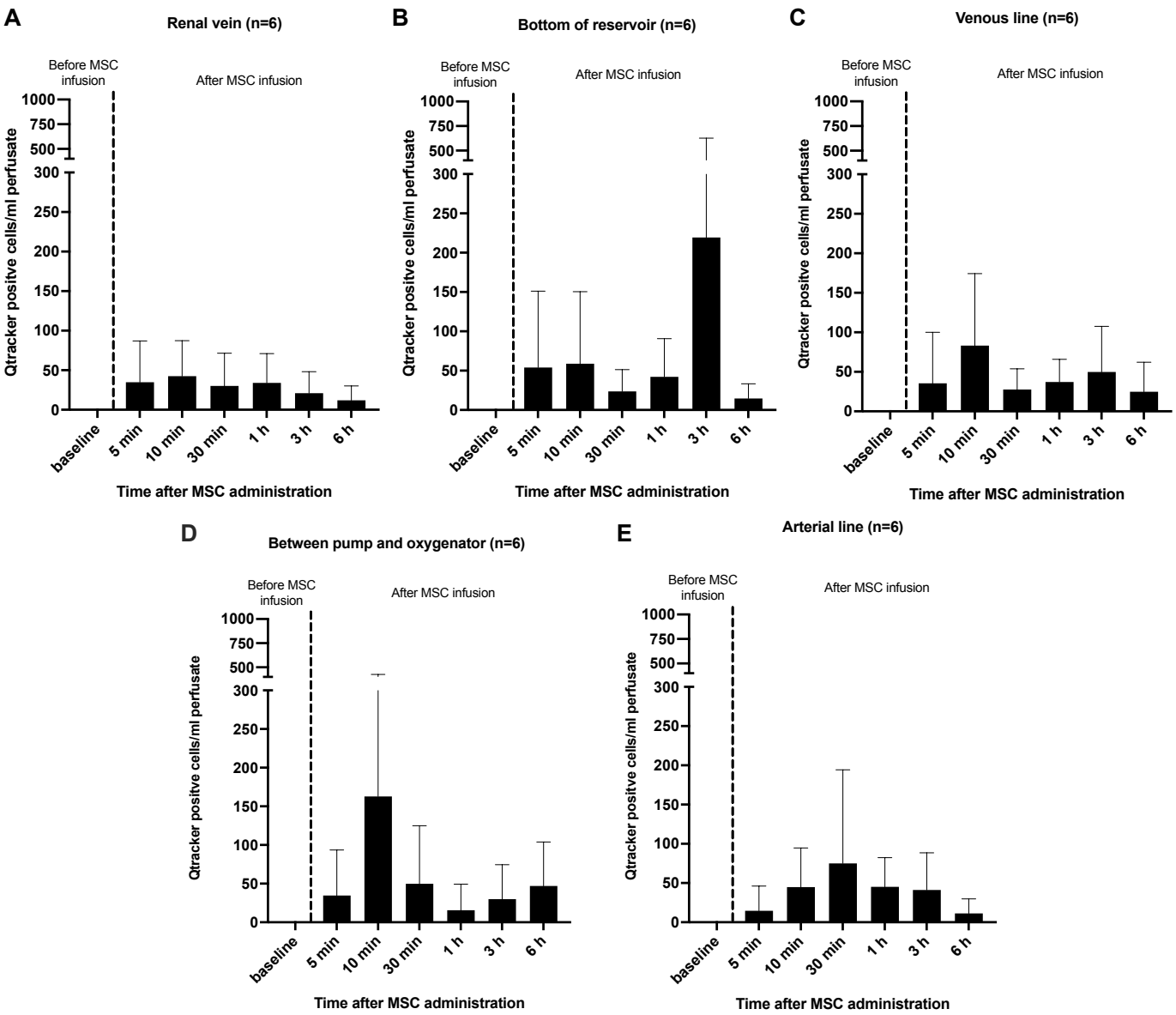


Figure 5.11 Qtracker positive cells measured by flow cytometry in the perfusate of kidneys undergoing NMP with 10 million MSCs administration. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 10 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were collected during and after NMP and processed into single cell suspension with the GentleMACS. Samples were analysed with flow cytometry. Perfusate samples were collected and treated with RBC-lysis buffer and subsequently analysed with flow cytometry. (A-E) Low amounts of Qtracker positive cells were found in the perfusate at different locations in the circuit during NMP. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; UppC, upper cortex; UppM, upper medulla, LatC: lateral cortex, LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

5.3.4.2 Detection of male pMSCs in tissue with PCR

Y-chromosome levels measured in kidney biopsies taken after one, three and six hours of NMP were consistent with the findings from phase one and confirmed successful delivery of MSCs to the renal cortex and medulla (Figure 5.12).

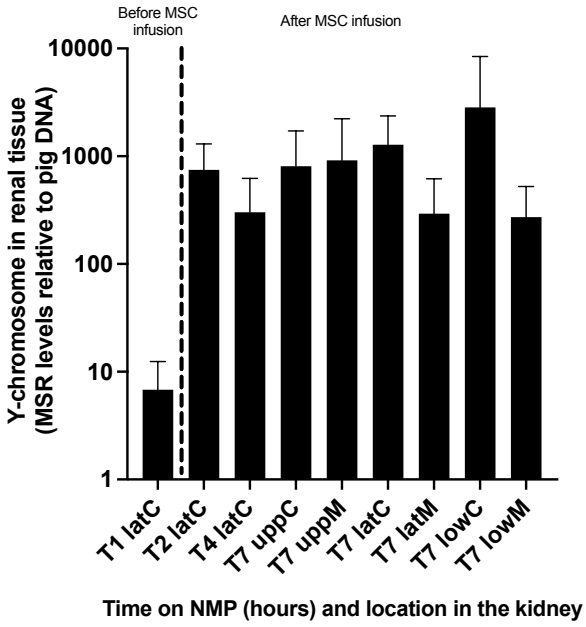


Figure 5.12. Relative Y-chromosome compared with total pig DNA in renal tissue during NMP with 10 million MSCs administration. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 10 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Kidney biopsies were collected during and after NMP and processed into single cell suspension with the GentleMACS. Samples were analysed with flow cytometry. qPCR confirmed successful delivery of MSCs in the kidney tissue after NMP with 10 million MSCs and infusion. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP: normothermic machine perfusion; qPCR, real-time quantitative polymerase chain reaction; UppC, upper cortex; UppM, upper medulla; LatC, lateral cortex; LatM, lateral medulla; LowC, lower cortex; LowM, lower medulla.

5.3.4.3 No adverse effects were observed after MSCs administration during NMP

5.3.4.3.1 Perfusion characteristics and kidney quality

Overall mean blood flow was 106 ± 49 mL/min/100g in the control group and 71 ± 16 mL/min/100g in the 10 million MSC group and there were no differences in mean blood flow per 100 grams between the groups (p=0.153, student's t-test; Figure 5.13A). There were no differences in mean renal resistance (0.41 ± 0.12 vs 0.43 ± 0.11 mmHg/mL/min, p=0.889, student's t-test) between the control and the 10 million MSC groups (Figure 5.13B). Total urine production after seven hours of NMP was 45 ± 83 mL in the control group and 189 ± 337 mL in the 10 million MSC group and there were no differences between the groups (p=0.087, Mann-Whitney test; Figure 5.13C). In both groups, kidneys consumed oxygen similarly and there were no differences between the groups (p=0.262, student's t-test; Figure 5.12D). At seven hours, mean oxygen consumption was 2.53 ± 0.54 mLO_2/min/100g in the control group and 2.09 ± 0.70 mLO_2/min/100g in the 10 million MSC groups, p=0.245 (student's t-test). QAS was similar for kidneys perfused in the control and 10 million MSC groups (p<0.999, Chi-square test). In both groups, one of the six kidneys had a QAS more than 3 (Table 5.3).

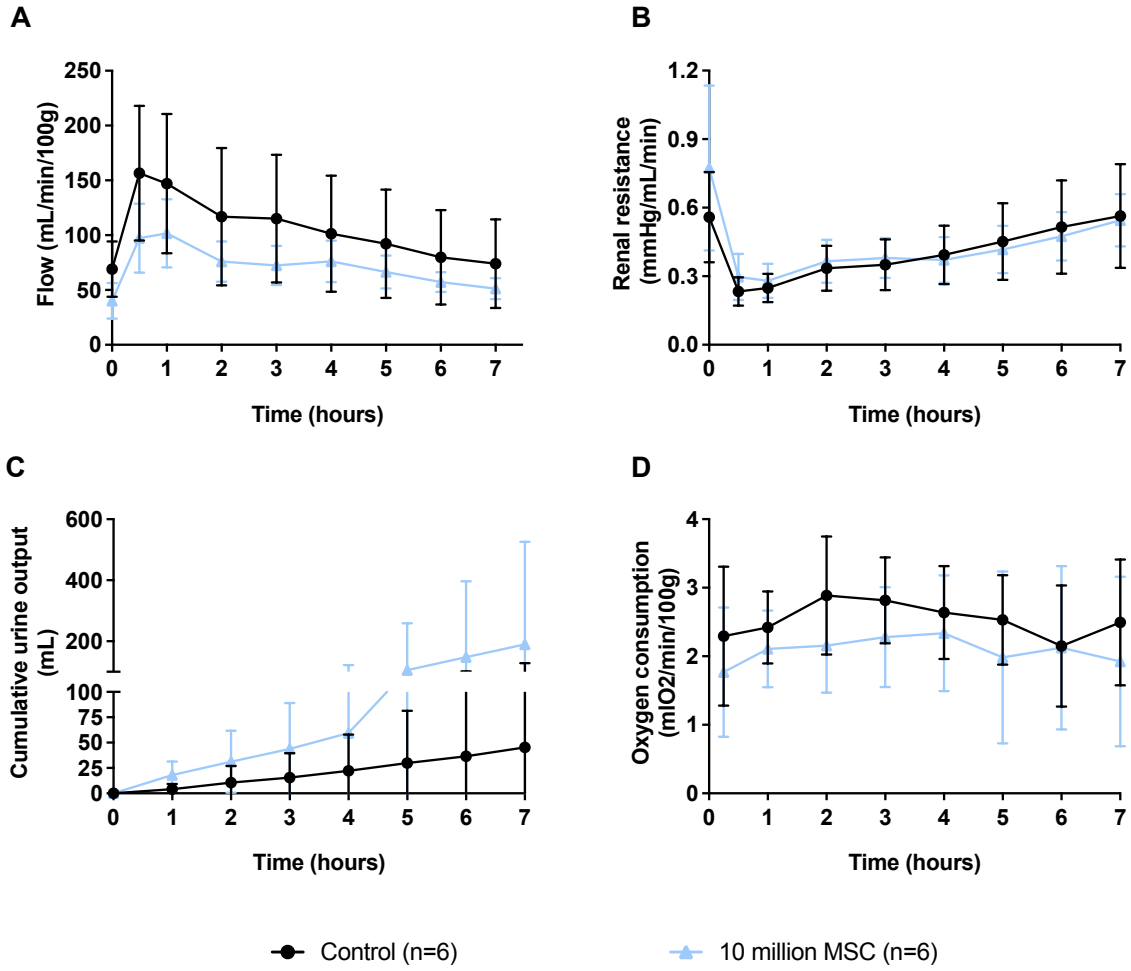


Figure 5.13. Perfusion characteristics, urine production and oxygen consumption during NMP with 10 million MSCs administration. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 10 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. There were no differences in (A) mean renal blood flow per 100 grams and (B) mean renal resistance between the groups. (C) There was a relatively large difference in cumulative urine between the groups. (D) All kidneys consumed oxygen in a similar manner during NMP. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

Table 5.3. Quality assessment score (QAS) of kidneys undergoing NMP with either a vehicle or 2, 10 and 50 million MSC infusion. The QAS is based on urine production, renal blood flow and macroscopic appearance at the end of NMP.

Kidney	Urine	Flow	Macroscopic appearance	QAS score
Control 1	1	0	2	3
Control 2	1	0	1	2
Control 3	1	1	2	4
Control 4	1	0	2	3
Control 5	0	1	2	3
Control 6	1	0	1	2
10 million MSC 1	1	1	3	5
10 million MSC 2	1	0	2	3
10 million MSC 3	1	0	2	3
10 million MSC 4	0	0	2	2
10 million MSC 5	0	0	2	2
10 million MSC 6	1	0	2	2

5.3.4.3.2 Injury markers lactate, LDH and AST

Perfusate lactate increased from baseline until the end of perfusion in all groups. At the end of NMP, mean values were 9.9 ± 9.1 mmol/L in the control group and 12.8 ± 7.9 mmol/L in the 10 million MSC group and there were no differences between the groups (p=0.610, student's t-test; Figure 5.14A). The cellular injury marker, LDH, increased throughout perfusion with end values of 826 ± 416 U/L in the control group and 577 ± 75 in the 10 million MSC group and there were no differences between the groups (p=0.157, student's t-test; Figure 5.14B). Similarly, there were no differences in perfusate AST levels between the control and the 10 million MSC groups (p=0.331, student's t-test; Figure 5.14C).

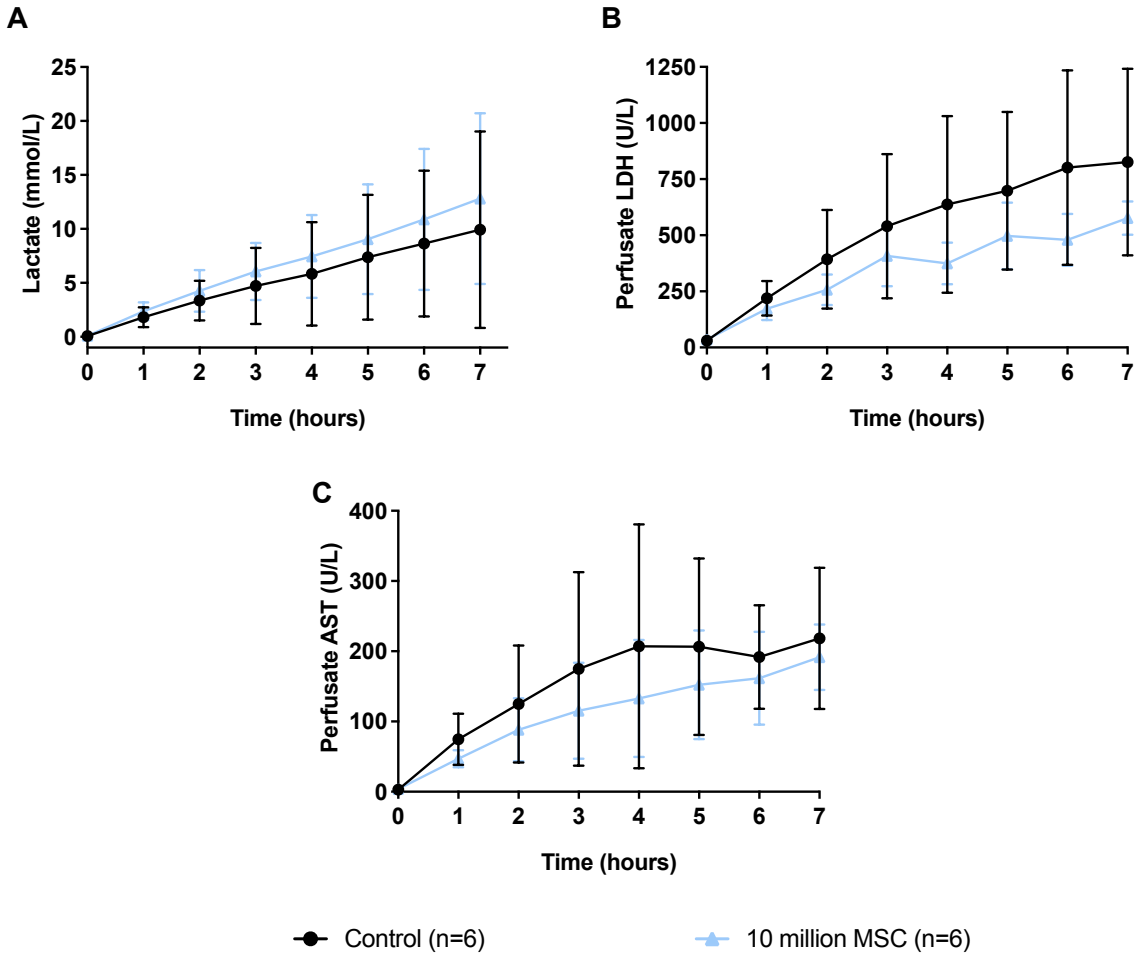


Figure 5.14. Kidney injury markers LDH and AST as well as lactate during NMP with 10 million MSCs administration. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 10 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. Perfusate (A) lactate, (B) LDH and (C) AST increased in all groups during NMP and there are no differences between the groups. Data are presented as mean ± standard deviation. AST, aspartate aminotransferase; HMP, hypothermic machine perfusion; LDH, lactate dehydrogenase; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

5.3.5 Perfusate IL-10 levels increase over time with the addition of MSCs

In phase one, IL-10 levels increased over time in the perfusate during NMP; however, there were no significant differences between the groups (p=0.068, 2-way ANOVA; Figure 5.15A). In phase two, the secretion of IL-10 in the perfusate increased over time in the 10 million MSC group (p=0.010 (T1 vs T7), 2-way ANOVA) and there was a significantly higher production of IL-10 in the perfusate at the end of NMP in the 10 million MSC group compared to the control group (p=0.029, 2-way ANOVA; Figure 5.15B).

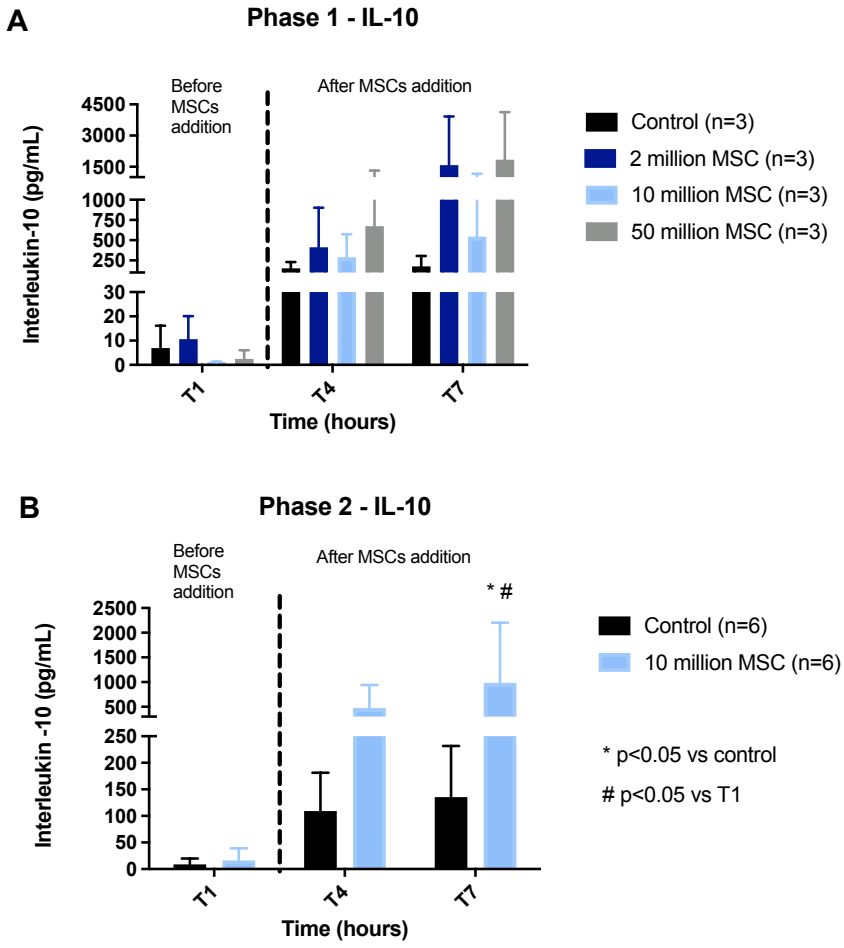


Figure 5.15. Perfusate interleukin-10 during NMP with administration of no MSCs and different doses of MSCs in phase one and phase two. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million, 10 million or 50 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. (A) IL-10 increased over time with the addition of MSCs and there were no significant differences between the groups. (B) IL-10 increased over time in the 10 million MSC group in comparison with the control group. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; IL, interleukin; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

5.3.6 Perfusate angiopoietin-1, VEGF-A and PGE2 increases over time in the presence or absence of MSCs

In phase one, perfusate angiopoietin-1 significantly increased in the control and 50 million MSC groups (p=0.016 (T1 vs T7) and p=0.017 (T1 vs T7), respectively, 2-way ANOVA) and no differences were found between the groups (p=0.274, 2-way ANOVA; Figure 5.16A). In phase two, an increase in angiopoietin-1 levels in the perfusate was observed in both groups, however, this was only significant in the control group (p=0.037 (T1 vs T7), 2-way ANOVA; Figure 5.16B). Notably, in phase two, there was an increased secretion of perfusate angiopoietin-1 in the 10 million MSC group after 3 hours of MSCs administration (T4) compared to the control group; however, this did not reach statistical significant (p=0.057, 2-way ANOVA; Figure 5.16B).

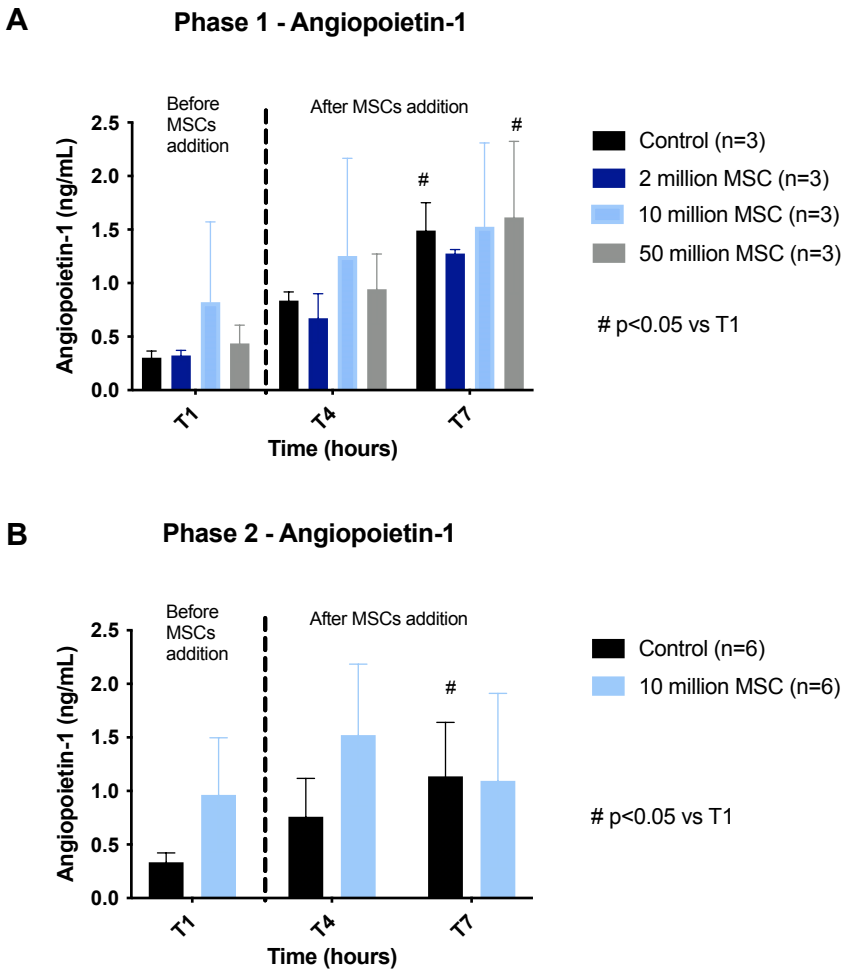


Figure 5.16. Perfusate angiopoietin-1 during NMP with administration of no MSCs and different doses of MSCs in phase one and phase two. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million, 10 million or 50 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. In phase one (A) and phase two (B), angiopoietin 1 increases overtime with and without the administration of MSCs and there are no differences between the groups. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion.

Similarly, perfusate VEGF-A levels increased over time in all groups in phase one, however, this was only significant in the 10 million MSC group (p=0.011 (T1 vs T7) and p=0.012 (T4 vs T7), 2-way ANOVA; Figure 5.17A). In phase two, VEGF-A levels significantly increased over time in the control group (p=0.0098 (T1 vs T7 and T4 vs T7), 2-way ANOVA) and the 10 million MSC group (p<0.0001 (T1 vs T7 and T4 vs T7), 2-way ANOVA; Figure 5.17B). In both phase one and phase two, secretion of VEGF-A was similar between the groups (p=0.620 and p=0.226, respectively).

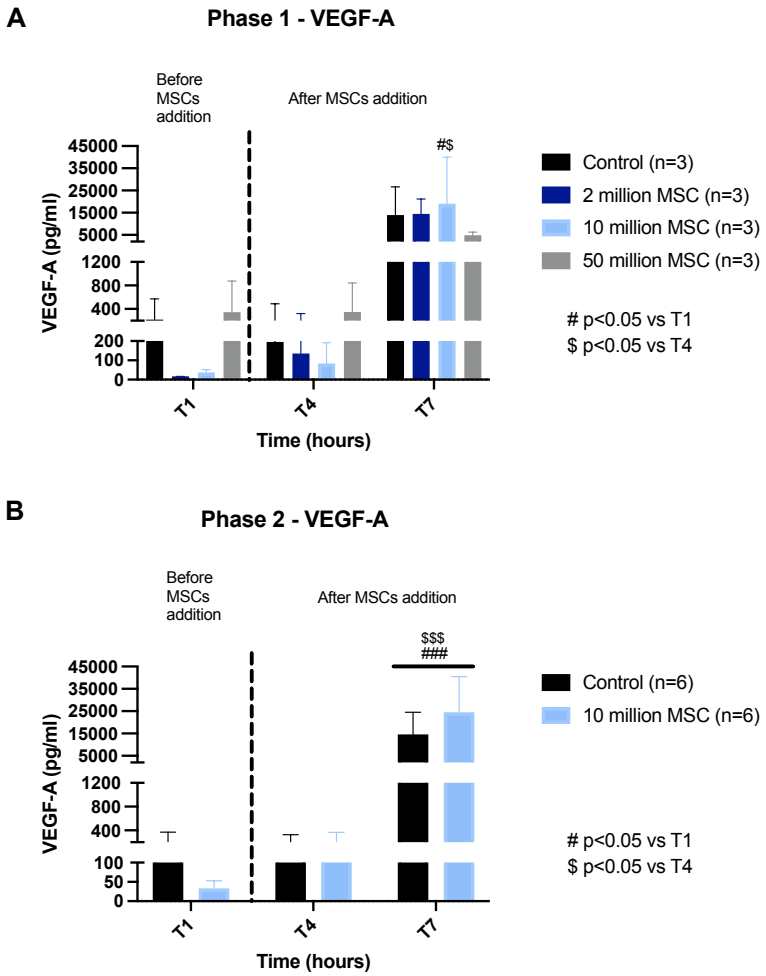


Figure 5.17. Perfusate VEGF-A during NMP with administration of no MSCs and different doses of MSCs in phase one and phase two. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million, 10 million or 50 million fluorescently labelled adipose derived pig MSCs were intra-arterially infused. In phase one (A) and phase two (B) perfusate VEGF-A levels increased over time with and without the addition of MSCs and there were no significant differences between the groups. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; VEGF, vascular endothelial growth factor.

In phase one and phase two, perfusate PGE2 significantly increased over time in the perfusate, throughout NMP in all groups ($p<0.0001$, (T1 vs T7 and T4 vs T7), 2-way ANOVA; Figure 5.18). In phase one, there was significantly more PGE2 secretion after 4 hours of NMP in the 10 million MSC group compared to the control group ($p= 0.045$, 2-way ANOVA).

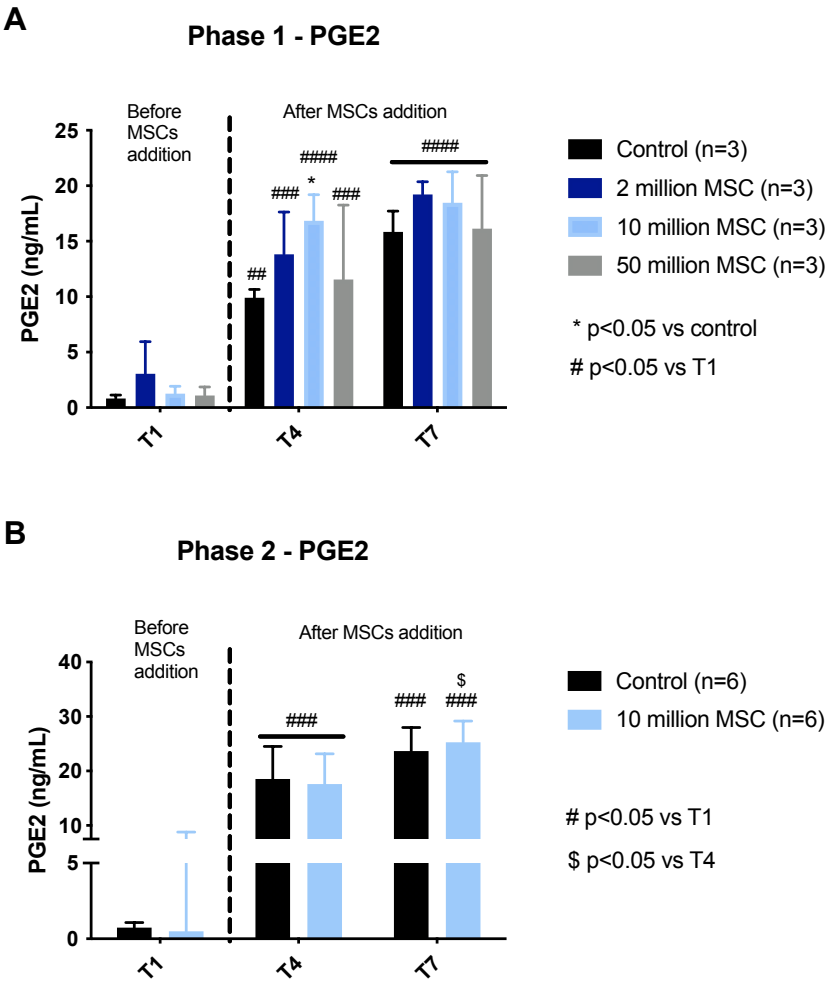


Figure 5.18. Perfusate PGE2 during NMP with administration of no MSCs and different doses of MSCs in phase one and phase two. Female pig kidneys retrieved from an abattoir underwent four hours of HMP and were perfused on NMP for seven hours. After one hour of NMP 2 million, 10 million or 50 million of fluorescently labelled adipose derived pig MSCs were intra-arterially infused. (A) PGE2 significantly increased over time in all groups and there were a significant difference between the 10 million MSC group and control group after 4 hours of NMP. (B) PGE2 increase over time with and without the addition of MSCs and there were no differences between the group. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; PGE2, prostaglandin E2

5.4 DISCUSSION

In the past decade, the transplant community has attempted to develop different strategies to reduce IRI and thus optimise utilisation of marginal donor organs for transplantation. One therapeutic intervention of primary interest is the administration of MSCs during NMP; however, many factors such as optimal dose and viability of the MSCs after they reach the kidney are unknown. In this study, we investigated the optimum MSC dose following administration to the injured donor pig kidney during NMP and determined the location of MSCs throughout perfusion.

We have shown that administration of 2, 10 and 50 million MSCs during NMP was safe and feasible as that there was no detrimental effect on perfusion characteristics or injury markers such as LDH and AST. These findings are consistent with other studies that administered similar doses of MSCs during pig and human kidney *sub*-NMP and NMP.[26, 28, 34] Thompson *et al.* reported improved microvascular perfusion, assessed with *ex vivo* ultrasound, to damaged cells and increased urine production during seven hours of human discarded kidney NMP with the administration of multipotent adult progenitor cells (MAPCs).[28] MAPCs belong to the family of MSCs but have different culture and expansion characteristics resulting in higher proliferative capacity and a broader differentiation potential. However, data suggest that they are the same in terms of immunomodulatory and cytoprotective capacity[35], although MAPCs may have superior angiogenic properties.[36, 37] A high amount of urine production has previously been regarded, together with renal blood flow and macroscopic appearance, as parameters of a more viable graft.[29] However, as discussed in Chapter 3, urine production should not be considered synonymous with kidney function in an NMP setting as it will be influenced by the perfusate oncotic pressure and can also function as a marker of tubular injury i.e. polyuria in acute tubular necrosis. Furthermore, urine production does not mean there is evidence of creatinine clearance or tubular reabsorption of sodium. Kaths *et al.* showed

no correlation between the amount of urine produced during NMP and graft function after auto-transplantation of pig kidneys.[38] Also, Ferdinand *et al*. assessed transcriptomic data from 33 donation after circulatory death kidneys, undergoing one-hour of NMP as part of a randomised clinical trial (ISRCTN15821205), and showed that a high amount of urine production is associated with higher expression of immune pathway genes and that delayed graft function is more prevalent in kidneys that produce higher amounts of urine during NMP.[39]

MSCs were easily identifiable using confocal microscopy and were mainly found in the luminal part of the glomeruli after seven hours of NMP. No dose-response was observed, but there were notably higher and similar percentages of identified MSCs in the 10 and 50 million MSC group. This was further confirmed with qPCR analysis of the MSR in the tissue, which showed presence of MSCs in the cortical and medullary tissue. In phase two, MSCs were also present in the medullary tissue but in much lower numbers. During renal transplant-related IRI, renal proximal tubular cells, located in the outer medulla of the kidney, and endothelial cells are the primary target of injury. Proximal tubular cells are packed with mitochondria and are highly dependent on mitochondrial oxidative phosphorylation and lack antioxidant and anti-apoptotic proteins.[40, 41] However, MSCs reparative and regenerative effects do not rely only on cell-to-cell interaction but also on the paracrine interaction of MSCs with the injured tissue.[5, 21, 42, 43]

In this study, we used a semi-quantitative flow cytometry method to quantify the number of MSCs in the tissue and perfusate. Flow cytometry analysis showed that the distribution of the MSCs in the tissue was heterogeneous and that a range of 0 to 6,260, 0 to 11,707 and 0 to 90,452 MSCs per gram of dissociated tissue in the 2 million MSC, 10 million MSC and 50 million MSC groups, respectively. Extrapolation of measured concentrations of MSCs using total kidney weight indicates that only an average of 14, 9 and 8% of MSCs were found back in the kidney after administration of 2, 10 and 50

million MSCs, respectively. The distribution of the MSCs was similar when MSCs were administrated during *ex vivo* kidney and liver NMP[44, 45] and might be due to the small capillary networks in the organs. In our study, 12%, 26% and 54% of the identified MSCs were viable and found in the kidney cortex after administration with 2, 10 and 50 million MSCs, respectively. MSCs are generally short-lived after IV administration (2-24 hours)[20, 21, 46, 47] and studies showed that intra-arterial administration of MSCs could prolong the survival of MSCs (3 days–5 weeks).[48-52] However, there is evidence that the potential beneficial effect of MSCs does not necessarily depend on their viability and that inactivated MSCs might initiate immunoregulatory and regenerative processes after disappearance of MSC.[21, 53]

Also, in our study, only low amounts of MSCs were circulating in the perfusate circuit, which raises the question where the remaining MSCs are located. We first hypothesised that the MSCs might have adhered to the polyethene tubing of the NMP circuit and plastic reservoir, as this is an inherent property of MSCs.[54] To study whether this thought was correct, in phase two, we rinsed the NMP circuit including the reservoir for 30 minutes with trypsin at 37°C after removal of the kidneys and were not able to detect any cells using flow cytometry. It is also a possibility that the MSCs do not adhere to the injured tissue and die whilst circulating in the NMP circuit. Sierra Parraga *et al.* showed that the perfusion solution affects the adhesive properties of MSCs and reduced survival rates in thawed MSCs.[55] Another possible explanation is that the MSCs might be lost during the tissue dissociation and analysis process. We performed a sensitivity and feasibility test prior to the main experiment to determine if we could detect MSCs in dissociated tissue using flow cytometry. After processing the tissue, we obtained a higher yield of tissue than the actual experiments (Appendix, Figure S5.1). Obtaining flow cytometry data proved to be time-consuming and challenging; despite setting the total recording events at 3 million events, only an average of 204,226 events (range: 9,739 to 888,271) were recorded for the tissue and an average of 1,222,762 events (range: 10,483 to 3,000,000)

in the perfusate (*data not shown*). A fluorescence threshold can be applied to the flow cytometer settings in future experiments to avoid unwanted cells and debris and speed up the measuring process.

The MSCs used in this study were harvested from adipose tissue. MSCs can be harvested from multiple locations in the body and can secrete different factors having different therapeutic potential.[56-58] Kusama *et al*. and Tsuji *et al*. suggested that this is most likely caused by the different native microenvironments the cells reside in and the different signals they receive. During culture, the cells reside in additional '*in vitro*' microenvironment and corresponding conditions (e.g. oxygen tension) and this can also influence the characteristics of the MSCs.[59, 60] Therefore, standardising multiple factors like cell source, culture conditions, and passage number is crucial to minimise the differences caused by microenvironments in upcoming preclinical and clinical research. In this study we chose adipose tissue-derived MSCs as they are easily accessible, the harvesting approach is safe and a significantly high number of cells can be obtained from the same amount of tissue compared to MSCs harvested from other sources.[61, 62]

One potential limitation of our study is the small sample size group in phase 1. However, this phase aimed to determine the optimum dose and location of MSCs throughout the kidney during NMP, which are variables that have not been unravelled yet. Also and important, this experiment was designed to identify the optimum dose of MSCs to be used later in the pig auto-transplantation study described in Chapter 6. In Phase two, a subsequent group of n=6 would be preferential to two groups of n=3. However, time and resources did not allow this and therefore we chose the latter option. In our study, MSCs were fluorescently labelled with Qtracker which introduces quantum dots into the cytoplasm which can be cytotoxic. MSCs stained with Qtracker might therefore be less capable of secreting cytokines[63], indicating that the dye can reduce MSC viability. Pool *et al*. described that detection of the release of cytokines of pre-labelled MSCs with

Qtracker and PKH during NMP was not possible when compared to administration of unlabelled MSCs during pig kidney NMP[64]. However, in our study we were able to detect secretome released from pre-labelled MSC with Qtracker.

Another limitation of this study might be the use of cryopreserved/thawed and allogenic MSCs, as opposed to usage of autologous MSCs and fresh MSCs directly from cell culture as other experimental studies have done.[13, 20, 45] We decided to opt for cryopreserved/thawed and allogeneic MSCs from a logistical and translational point of view. Although the autologous and fresh approach may have advantages in terms of elevated MSC activity[55, 65, 66], the method we chose appears to be more in line with eventual clinical practice.

Summarising, we showed that administration of 2, 10 and 50 million MSCs to the donor pig kidneys during NMP is safe and feasible. MSCs are found predominantly in the glomeruli after seven hours of NMP. The ability to deliver MSCs to the tissue and the detection of viable MSCs indicates that regenerative and reparative processes may be initiated during NMP.

REFERENCES

1. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable but Resuscitated by Ex Vivo Normothermic Machine Perfusion. *Am J Transplant*. 2016;16:3282-3285.

2. Hamar M, Urbanellis P, Kaths MJ, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. *Transplantation*. 2018;102:1262-1270.

3. Weissenbacher A, Lo Faro L, Boubriak O, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. *Am J Transplant*. 2019;19:178-192.

4. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation*. 2018;102:35-43.

5. Morigi M, Benigni A. Mesenchymal stem cells and kidney repair. *Nephrol Dial Transplant*. 2013;28:788-793.

6. Furuichi K, Shintani H, Sakai Y, et al. Effects of adipose-derived mesenchymal cells on ischemia-reperfusion injury in kidney. *Clin Exp Nephrol*. 2012;16:679-689.

7. Togel F, Zhang P, Hu Z, Westenfelder C. VEGF is a mediator of the renoprotective effects of multipotent marrow stromal cells in acute kidney injury. *J Cell Mol Med*. 2009;13:2109-2114.

8. Reinders ME, Leuning DG, de Fijter JW, Hoogduijn MJ, Rabelink TJ. Mesenchymal stromal cell therapy for cardio renal disorders. *Curr Pharm Des*. 2014;20:2412-2429.

9. Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int*. 2006;70:121-129.

10. Ueno T, Nakashima A, Doi S, et al. Mesenchymal stem cells ameliorate experimental peritoneal fibrosis by suppressing inflammation and inhibiting TGF-beta1 signaling. *Kidney Int*. 2013;84:297-307.

11. Chen YT, Sun CK, Lin YC, et al. Adipose-derived mesenchymal stem cell protects kidneys against ischemia-reperfusion injury through suppressing oxidative stress and inflammatory reaction. *J Transl Med*. 2011;9:51-51.

12. Chamberlain G, Smith H, Rainger GE, Middleton J. Mesenchymal stem cells exhibit firm adhesion, crawling, spreading and transmigration across aortic endothelial cells: effects of chemokines and shear. *PLoS One*. 2011;6:e25663.

13. Sierra-Parraga JM, Munk A, Andersen C, et al. Mesenchymal Stromal Cells Are Retained in the Porcine Renal Cortex Independently of Their Metabolic State After Renal Intra-Arterial Infusion. *Stem Cells Dev*. 2019;28:1224-1235.

14. Perico N, Casiraghi F, Remuzzi G. Clinical Translation of Mesenchymal Stromal Cell Therapies in Nephrology. *J Am Soc Nephrol*. 2018;29:362-375.

15. Peired AJ, Sisti A, Romagnani P. Mesenchymal Stem Cell-Based Therapy for Kidney Disease: A Review of Clinical Evidence. *Stem Cells Int*. 2016;2016:4798639.

16. Chen C, Hou J. Mesenchymal stem cell-based therapy in kidney transplantation. *Stem Cell Res Ther*. 2016;7:16-6.

17. Franquesa M, Hoogduijn MJ, Reinders ME, et al. Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: lessons learned from first clinical trials. *Transplantation*. 2013;96:234-238.

18. Lalu MM, McIntyre L, Pugliese C, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One*. 2012;7:e47559.

19. Thompson M, Mei SHJ, Wolfe D, et al. Cell therapy with intravascular administration of mesenchymal stromal cells continues to appear safe: An updated systematic review and meta-analysis. *EClinicalMedicine*. 2020;19:100249.

20. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol*. 2012;3:297.

21. de Witte, S. F. H., Luk F, Sierra Parraga JM, et al. Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. *Stem Cells*. 2018;36:602-615.

22. Snoeijs MG, Vaahtera L, de Vries EE, et al. Addition of a water-soluble propofol formulation to preservation solution in experimental kidney transplantation. *Transplantation*. 2011;92:296-302.

23. Sedigh A, Larsson R, Brannstrom J, et al. Modifying the vessel walls in porcine kidneys during machine perfusion. *J Surg Res*. 2014;191:455-462.

24. Gregorini M, Corradetti V, Pattonieri EF, et al. Perfusion of isolated rat kidney with Mesenchymal Stromal Cells/Extracellular Vesicles prevents ischaemic injury. *J Cell Mol Med*. 2017;21:3381-3393.

25. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? *Transpl Int*. 2015;28:657-664.

26. Brasile L, Henry N, Orlando G, Stubenitsky B. Potentiating Renal Regeneration Using Mesenchymal Stem Cells. *Transplantation*. 2019;103:307-313.

27. Pool M, Eertman T, Sierra Parraga J, et al. Infusing Mesenchymal Stromal Cells into Porcine Kidneys during Normothermic Machine Perfusion: Intact MSCs Can Be Traced and Localised to Glomeruli. *Int j mol sci*. 2019;20.

28. Thompson ER, Bates L, Ibrahim IK, et al. Novel delivery of cellular therapy to reduce ischemia reperfusion injury in kidney transplantation. *Am J Transplant*. 2020.

29. Hosgood SA, Barlow AD, Hunter JP, Nicholson ML. Ex vivo normothermic perfusion for quality assessment of marginal donor kidney transplants. *Br J Surg*. 2015;102:1433-1440.

30. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation*. 2019;103:2057-2064.

31. Ronco C, Bellomo R, Kellum JA. Chapter 116. arterial and venous blood gases - oxygen content. In: *Critical Care in Nephrology.* 2nd ed. Saunders; 2008:608.

32. BD PharmingenTM. Technical Data Sheet: DAPI Solution. 2020.

33. Gruessner RW, Levay-Young BK, Nakhleh RE, et al. Portal donor-specific blood transfusion and mycophenolate mofetil allow steroid avoidance and tacrolimus dose reduction with sustained levels of chimerism in a pig model of intestinal transplantation. *Transplantation*. 2004;77:1500-1506.

34. Pool MBF, Vos J, Eijken M, et al. Treating Ischemically Damaged Porcine Kidneys with Human Bone Marrow- and Adipose Tissue-Derived Mesenchymal Stromal Cells During Ex Vivo Normothermic Machine Perfusion. *Stem Cells Dev*. 2020;29:1320-1330.

35. Khan RS, Newsome PN. A Comparison of Phenotypic and Functional Properties of Mesenchymal Stromal Cells and Multipotent Adult Progenitor Cells. *Front Immunol*. 2019;10:1952.

36. Roobrouck VD, Clavel C, Jacobs SA, et al. Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts,

and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. *Stem Cells*. 2011;29:871-882.

37. LoGuidice A, Houlihan A, Deans R. Multipotent adult progenitor cells on an allograft scaffold facilitate the bone repair process. *J Tissue Eng*. 2016;7:2041731416656148.

38. Kaths JM, Hamar M, Echeverri J, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. *Am J Transplant*. 2018;18:580-589.

39. Ferdinand JR, Hosgood SA, Moore T, et al. Cytokine absorption during human kidney perfusion reduces delayed graft function-associated inflammatory gene signature. *Am J Transplant*. 2020.

40. Smith SF, Hosgood SA, Nicholson ML. Ischemia-reperfusion injury in renal transplantation: 3 key signaling pathways in tubular epithelial cells. *Kidney Int*. 2019;95:50-56.

41. Chevalier RL. The proximal tubule is the primary target of injury and progression of kidney disease: role of the glomerulotubular junction. *Am J Physiol Renal Physiol*. 2016;311:145.

42. Sierra-Parraga JM, Merino A, Eijken M, et al. Reparative effect of mesenchymal stromal cells on endothelial cells after hypoxic and inflammatory injury. *Stem Cell Res Ther*. 2020;11:352-3.

43. de Vries DK, Schaapherder AF, Reinders ME. Mesenchymal stromal cells in renal ischemia/reperfusion injury. *Front Immunol*. 2012;3:162.

44. Verstegen MMA, Mezzanotte L, Ridwan RY, et al. First Report on Ex Vivo Delivery of Paracrine Active Human Mesenchymal Stromal Cells to Liver Grafts During Machine Perfusion. *Transplantation*. 2020;104:e5-e7.

45. Pool M, Eertman T, Sierra Parraga J, et al. Infusing Mesenchymal Stromal Cells into Porcine Kidneys during Normothermic Machine Perfusion: Intact MSCs Can Be Traced and Localised to Glomeruli. *Int J Mol Sci*. 2019;20:10.3390/ijms20143607.

46. Saat TC, van den Engel S, Bijman-Lachger W, et al. Fate and Effect of Intravenously Infused Mesenchymal Stem Cells in a Mouse Model of Hepatic Ischemia Reperfusion Injury and Resection. *Stem Cells Int*. 2016;2016:5761487.

47. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. *Stem Cells Dev*. 2013;22:2825-2835.

48. Moghadasali R, Azarnia M, Hajinasrollah M, et al. Intra-renal arterial injection of autologous bone marrow mesenchymal stromal cells ameliorates cisplatin-induced acute kidney injury in a rhesus Macaque mulatta monkey model. *Cytotherapy*. 2014;16:734-749.

49. Zonta S, De Martino M, Bedino G, et al. Which is the most suitable and effective route of administration for mesenchymal stem cell-based immunomodulation therapy in experimental kidney transplantation: endovenous or arterial? *Transplant Proc*. 2010;42:1336-1340.

50. Eirin A, Zhu XY, Krier JD, et al. Adipose tissue-derived mesenchymal stem cells improve revascularization outcomes to restore renal function in swine atherosclerotic renal artery stenosis. *Stem Cells*. 2012;30:1030-1041.

51. Eirin A, Zhu XY, Ferguson CM, et al. Intra-renal delivery of mesenchymal stem cells attenuates myocardial injury after reversal of hypertension in porcine renovascular disease. *Stem Cell Res Ther*. 2015;6:7.

52. Lee KW, Kim TM, Kim KS, et al. Renal Ischemia-Reperfusion Injury in a Diabetic Monkey Model and Therapeutic Testing of Human Bone Marrow-Derived Mesenchymal Stem Cells. *J Diabetes Res*. 2018;2018:5182606.

53. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol*. 2014;5:148.

54. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315-317.

55. Sierra Parraga JM, Rozenberg K, Eijken M, et al. Effects of Normothermic Machine Perfusion Conditions on Mesenchymal Stromal Cells. *Front Immunol*. 2019;10:765.

56. Wilson A, Hodgson-Garms M, Frith JE, Genever P. Multiplicity of Mesenchymal Stromal Cells: Finding the Right Route to Therapy. *Front Immunol*. 2019;10:1112.

57. Mattar P, Bieback K. Comparing the Immunomodulatory Properties of Bone Marrow, Adipose Tissue, and Birth-Associated Tissue Mesenchymal Stromal Cells. *Front Immunol*. 2015;6:560.

58. Najar M, Raicevic G, Fayyad-Kazan H, et al. Impact of different mesenchymal stromal cell types on T-cell activation, proliferation and migration. *Int Immunopharmacol*. 2013;15:693-702.

59. Kusuma GD, Carthew J, Lim R, Frith JE. Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine

Signaling: Opportunities to Engineer the Therapeutic Effect. *Stem Cells Dev*. 2017;26:617-631.

60. Tsuji K, Kitamura S, Wada J. Secretomes from Mesenchymal Stem Cells against Acute Kidney Injury: Possible Heterogeneity. *Stem Cells Int*. 2018;2018:8693137.

61. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*. 2002;13:4279-4295.

62. Czapla J, Matuszczak S, Kulik K, et al. The effect of culture media on large-scale expansion and characteristic of adipose tissue-derived mesenchymal stromal cells. *Stem Cell Res Ther*. 2019;10:235-9.

63. Soenen SJ, Demeester J, De Smedt SC, Braeckmans K. The cytotoxic effects of polymer-coated quantum dots and restrictions for live cell applications. *Biomaterials*. 2012;33:4882-4888.

64. Pool M. *Towards ex vivo repair of damaged donor kidneys.* University of Groningen; 2020.

65. Hoogduijn MJ, de Witte SF, Luk F, et al. Effects of Freeze-Thawing and Intravenous Infusion on Mesenchymal Stromal Cell Gene Expression. *Stem Cells Dev*. 2016;25:586-597.

66. Chinnadurai R, Garcia MA, Sakurai Y, et al. Actin cytoskeletal disruption following cryopreservation alters the biodistribution of human mesenchymal stromal cells in vivo. *Stem Cell Reports*. 2014;3:60-72.

APPENDIX

Table S5.1. Qtracker 655 positive cells detected with flow cytometry in dissociated cortex and medulla spiked with 1 million, 500 thousand, 250 thousand or 125 thousand MSCs.

Location + amount of MSC added	Total live MSC	Difference (%)
Cortex 1 million MSCs	1,249,864	25
Cortex 500 thousand MSCs	459,866	-8
Cortex 250 thousand MSCs	192,268	-23
Cortex 125 thousand MSCs	92,990	-26
Medulla 1 million MSCs	743,058	-26
Medulla 500 thousand MSCs	374,401	-25
Medulla 250 thousand MSCs	172,111	-31
Medulla 125 thousand MSCs	79,272	-37

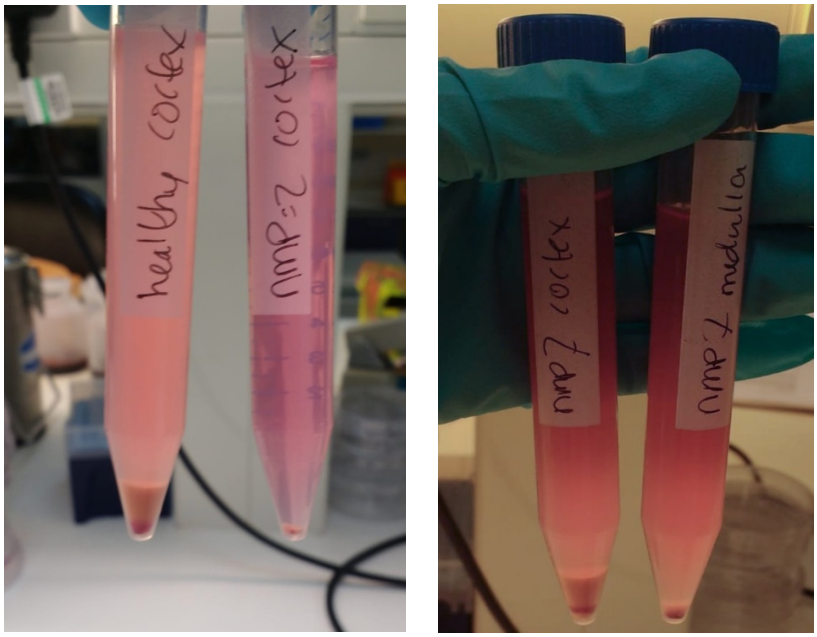


Figure S5.1. Photographic image of kidney tissue after mechanical and enzymatic digestion from the sensitivity and feasibility test. Prior to the experiments describe in this chapter, a sensitivity and feasibility test was performed to determine if we could detect MSCs in dissociated tissue using flow cytometry. After processing the 0.500 g of cortex and medulla tissue after 7 hours of NMP, we obtained a higher yield of tissue than the actual experiments included in this chapter.

Chapter 6

Mesenchymal stromal cell treatment during *ex vivo* normothermic kidney perfusion: A porcine renal auto-transplantation study

ABBREVIATIONS

AST	aspartate aminotransferase
A-MSCs	adipose tissue derived MSCs
BM-MSCs	bone marrow derived MSCs
DCD	donation after circulatory death
ECD	extended criteria donor
FBS	foetal bovine serum
FFPE	formalin fixed paraffin embedded
GFR	glomerular filtration rate
HMP	hypothermic machine perfusion
hMSC	human mesenchymal stromal cell
IV	intravenous
MAP	mean arterial pressure
MEM-α	minimal essential medium-α
MSC	mesenchymal stromal cell
NMP	normothermic machine perfusion
PBS	phosphate-buffered saline
p-creatinine	plasma creatinine
p-NGAL	plasma neutrophil gelatinase-associated lipocalin
pMSC	porcine mesenchymal stromal cell
qPCR	quantitative polymerase chain reaction
SCS	static cold storage
^{99}Tc-DTPA	^{99}Tc-diethylenetriamine pentaacetic acid
UW-CSS	Belzer UW® Cold Storage Solution
UW-MPS	Belzer MPS® UW Machine Perfusion Solution

6.1 INTRODUCTION

In the last decade higher risk donor kidneys such as those recovered from donation after circulatory death (DCD) have been increasingly accepted to decrease waiting time for a transplant.[1-3] DCD kidneys are more prone to ischaemia reperfusion injury, affecting early kidney graft function and survival. Organs from DCD donors inevitably sustain warm ischaemic damage between circulatory arrest and initiation of flush-out when cooling the organs [4, 5] To date, most European transplant centres still use conventional static cold storage for the preservation of donor organs, but with increased numbers of DCD kidneys a growing need for improved preservation techniques has emerged. Alternative approaches, such as hypothermic machine perfusion (HMP), are being introduced in clinical practice and large clinical trials have shown that HMP is superior to static cold storage, reducing delayed graft function and significantly improving long term graft survival[6, 7], which has prompted centres in some countries to adopt HMP as their new standard.[8, 9] However, while better able to maintain viability than simple cold storage there is only limited evidence that HMP can improve the quality of older and higher risk donor kidneys.[10, 11]

Normothermic machine perfusion (NMP) is a technique that allows delivery of therapies that may help condition or repair the organ prior to transplantation.[12-14] There is also an increasing interest in mesenchymal stromal cells (MSCs) as cellular therapy, due to their immunomodulatory and regenerative properties.[15, 16] Studies have shown that MSCs may be able to ameliorate ischaemia reperfusion injury. Clinical trials have used intravenous (IV) infusions of MSCs to transplant recipients and this appears to be safe.[15, 17-19] With this mode of systemic administration, however, the cells will most likely never reach the kidney as IV infused MSCs are largely trapped in the lungs and have a poor delivery to any other target organs.[20, 21] In contrast, the administration of MSCs to donor kidneys in an *ex vivo* isolated organ perfusion system will deliver the MSCs directly to

the injured organ of choice. Also, target delivery in the kidney enhances the possibility of achieving therapeutic effects with relatively low numbers of MSCs compared to systemic administration that has been used so far in clinical studies. Only a few attempts have tried MSC treatment in combination with *ex vivo* NMP, mainly focussing on traceability and dose-response. In the aforementioned studies and in Chapter 5 it was demonstrated that MSCs are mainly located in the glomeruli capillaries after NMP. To date, no other studies incorporated actual transplantation after cellular therapy, leaving questions on possible post-transplant effects unanswered.[22-24]

Therefore, the aim of this chapter was to determine feasibility and safety of MSCs therapy during NMP while evaluating any functional effect of MSCs after transplantation in a DCD pig auto-transplant model.

The objectives of this chapter are:

- To determine the safety of intra-arterial administration of 10 million MSCs to injured porcine kidneys during NMP, followed by auto-transplantation;
- To determine whether the addition of MSCs during NMP had a beneficial effect on kidney function after transplantation with up to 14 days follow-up.

6.2 MATERIALS AND METHODS

6.2.1 Study design

Kidneys were randomly assigned to four experimental groups (n=7 per group). After 75 minutes of warm ischaemia, kidneys were subjected to different preservation strategies. Control group kidneys underwent oxygenated HMP (oxHMP) for 14 hours followed by auto-transplantation. In the other three groups, oxHMP was followed by four hours of NMP with an intra-arterial infusion after one hour of either only vehicle (NMP+vehicle), 10 million porcine adipose tissue derived MSCs (NMP+pMSC) or 10 million human adipose tissue derived MSCs (NMP+hMSC) (Figure 6.1) followed by auto-transplantation. All personnel were blinded to the NMP regimen, except for two people handling the MSCs, who had no other direct involvement in the execution of the experiments or post-transplant animal care. Due to technical problems, one kidney suffered anoxia during NMP and had to be excluded from further analysis. This experiment has been repeated to complete the groups.

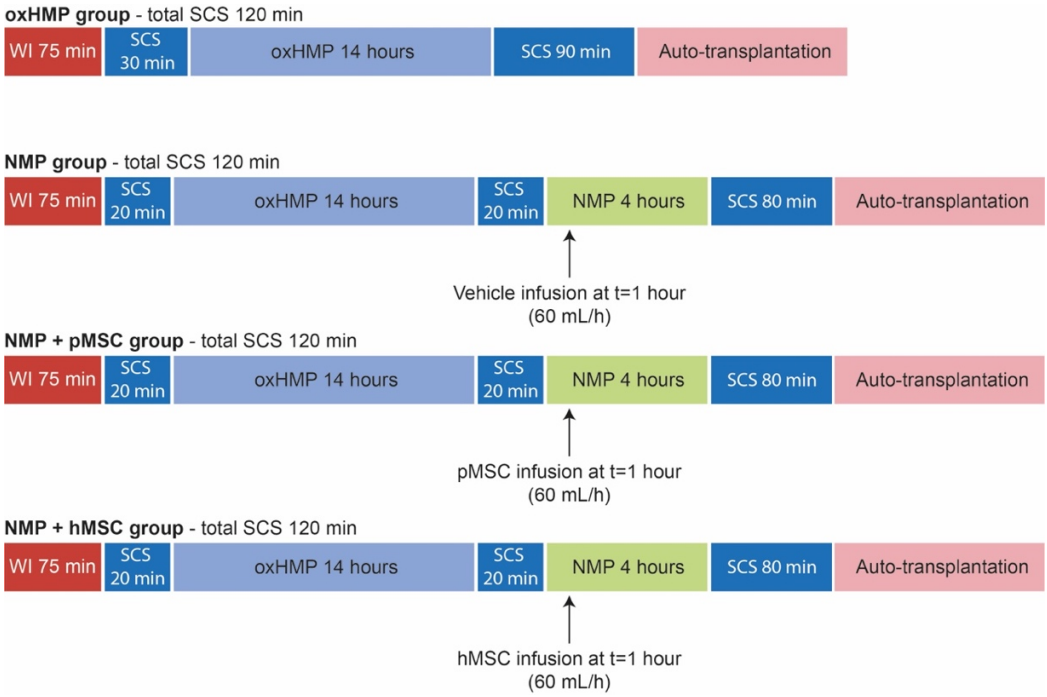


Figure 6.1. Preservation strategies of the four experimental groups. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

6.2.2 Ethics and animals

In this series of experiments, female laboratory pigs (Danish Landrace and Yorkshire crossbreed) weighing approximately 50 kg were used. All animal care and animal procedures followed guidelines by the European Union (directive 2010/63/EU) and local regulations. The study was approved by The Animal Experimentation Council of the University of Aarhus, Denmark (reference-number 2016-15-0201-01145). All personnel involved in the animal experiments had Federation of European Laboratory Animal Science Associations licenses. The operation rooms were at the same site as the husbandry and no transportation of the animals was needed; nor was restrain or sedation of the pigs used when blood and urine were collected post-operatively.

6.2.3 Surgical procedures

Operative procedures were performed as described in Chapter 4 section 4.2.3. Briefly, after induction, anaesthesia was maintained by sevoflurane and intravenous remifentanil (0.03 - 0.06 mg/kg/h for analgesics). On postoperative days zero to four, intramuscular buphrenorphine was administrated three times a day (0.02 mg/kg until day three and 0.01 mg/kg on day three and four). In a sterile setting, a permanent semi-central venous catheter (5 Fr, 20 cm, Careflow, BD, NJ, USA) was placed through an ear vein into the jugular vein. After midline incision, left retroperitoneal nephrectomy (day -1) was performed and the kidney was exposed to 75 minutes of warm ischaemia at 37°C. The midline incision was closed in two layers and local anaesthetic was administered (ropivacain 20 mL, 7.5 mg/mL). After warm ischaemia, the graft was flushed with 500 mL cold Belzer UW® Cold Storage Solution (UW-CSS, Bridge to Life, London, UK) and was subjected to either HMP for 14 hours (control) or 14 hours of HMP plus 4 hours of NMP (described in section 6.2.5). After *ex vivo* perfusion (day 0), the kidneys undergoing NMP were disconnected from the device, flushed and cooled with 500 mL cold UW-CSS. The kidneys undergoing HMP alone were disconnected, the cannulas were removed and the organs were placed on ice ready for transplant. Following right nephrectomy, the left

kidney graft, wrapped in a gauze containing ice, was autotransplanted end-to-end to the right renal artery and vein. Ureteric anastomosis was performed, also end-to-end, to recipient ureter with insertion of a JJ-stent (Urosoft 7/16, Angiomed GmbH, Karlsruhe, Germany) to overcome postoperative oedema. A cortical punch biopsy was taken one hour after reperfusion. The midline incision was closed and the pig was returned to the stables for 14 days of observation with recurrent blood and urine sampling.

On day 14, pigs were anaesthetised again following the aforementioned procedures. After removal of the JJ-stent, a feeding tube was placed in the ureter for continuous urine collection. For a total of three hours, glomerular filtration rate (GFR) was measured by the urinary clearance of ^{99}Tc-diethylenetriamine pentaacetic acid (^{99}Tc-DTPA).

$$GFR = \frac{U(cpm/ml) \text{ x Uvol } (ml/min)}{\frac{(Pl1 + Pl2)\,(cpm/\text{ml})}{2}}$$

Where U is urine, Pl is plasma, and cpm is counts per minute. A priming dose of 75 MBq was administered intravenously followed by a constant ^{99}Tc-DTPA infusion of 37 MBq/h. One hour of equilibration was completed. Hereafter, serial blood and urine samples were collected every 30 minutes for the first 2 hours and a last sample after 3 hours. The radioactivity measurements were, in 2 mL plasma samples and 1 mL urine samples, performed on the 2480 Wizard Gamma counter (PerkinElmer, Waltham, MA).

Lastly, the graft was removed and the pigs were euthanised under general anaesthesia.

6.2.4 Collection of allogeneic erythrocytes

All experimental animals and blood donor pigs were blood typed prior to the start of this series of experiments (Ortho BioVue™ Card, Ortho Clinical Diagnostics, Raritan, USA), and only blood type A pigs were used. Under sterile conditions, a 12 Fr central sheet (Radifocus® Introducer II, Terumo Europe, Leuven, Belgium) was inserted into the external jugular vein of anaesthetised porcine blood donors weighing between 60 to 80 kg. Blood was collected in quadruple-blood bags (Macopharma, Mouvaux, France), containing 63 mL of anticoagulant citrate-phosphate-dextrose solution. Subsequently, the blood was fractionated (Baxter Optipress II Blood Component Separator, Baxter, Chicago, USA). Next, the red blood cells (RBCs) were leucocyte filtered, transferred into bags containing saline-adenine-glucose-mannitol storage solution and stored for a maximum of 14 days at 4°C. RBCs were washed twice in phosphate-buffered Saline (PBS, Dulbecco's, Thermo Fisher) one hour prior to the start of NMP.

6.2.5 Machine perfusion

6.2.5.1 Machine perfusion setup

Kidneys preserved during both oxHMP and NMP were perfused with a pressure controlled pulsatile perfusion system with no venous cannulation. This setup was controlled by a custom-made electronic interface and software (LabVIEW Software, National Instruments Netherlands BV, Woerden, the Netherlands). The sterile perfusion circuit consisted of an oxygenator (CAPIOX® FX05, Terumo Cardiovascular, Herlev, Denmark), a centrifugal pump unit (Medos Deltastream Pumpdrive DP2, Medos Medizintechnik AG, Stolberg, Germany) and a LifePort® organ chamber (Organ Recovery Systems, Illinois, USA) with a 3mm LifePort® disposable straight cannula (Organ Recovery Systems). Flow was monitored using an ultrasonic clamp-on flow probe (Transonic Systems Europe BV, Elsloo, the Netherlands). Pressure was measured

directly at the straight cannula using a pressure transducer (TruWave disposable pressure transducer, Edwards Lifesciences, Irvine, California, USA).

6.2.5.2 Hypothermic machine perfusion

The HMP circuit was primed with 500 mL of cold Belzer MPS® UW Machine Perfusion Solution (UW-MPS, Bridge to Life Ltd., Colombia, South Carolina, USA) and temperature was kept between 4 and 7°C. The perfusate was oxygenated with 0.1 L/min 100% oxygen. The kidneys were perfused in a pressure controlled, sinusoid pulsatile fashion at a mean arterial pressure (MAP) of 25 mmHg.

6.2.5.3 Normothermic machine perfusion

The NMP circuit was primed with a perfusion solution consisting of 250 mL 5% human serum albumin (Alburex 50 g/L, CSL Behring, Lyngby, Denmark) and 170 mL allogeneic erythrocytes to acquire a haematocrit of 25-30%. Additionally, 5 mL 8.4% sodium bicarbonate (B. Braun, Melsungen, Germany), 6 mL 5% glucose (B. Braun), 5 IU insulin (Novo Nordisk A/S, Bagsværd, Denmark), 3 mL 10% calcium gluconate (B. Braun), 10 mg mannitol (Sigma-Aldrich, Soeborg, Denmark) and 1000 µmol/L creatinine (Sigma-Aldrich) were added to the perfusion solution. The perfusate was supplemented with a bolus of 300 mg amoxicillin-clavulanate (Bowmed, Wrexham, United Kingdom) at baseline with additional doses of 60 mg amoxicillin-clavulanate every hour during NMP. Furthermore, a bolus dose of 1.25 mg verapamil (Orion Pharma, Copenhagen, Denmark) was given at baseline and 0.25 mg/h verapamil was infused continuously during NMP. Perfusate temperature was kept at 37°C and oxygenated with 0.5 L/min carbogen (95% O_2 / 5% CO_2). After oxHMP, the kidney was flushed with 50 mL cold Ringer's acetate (Fresenius Kabi, Bad Homburg, Germany) to remove UW-MPS solution, connected to the circuit and perfused for four hours in a sinusoid pulsatile fashion at a MAP of 70 mmHg. Arterial and venous blood gas analysis was carried out every 30 minutes on the

ABL90 FLEX blood gas analyser (Radiometer, Bronshoej, Denmark). Perfusate and urine samples were taken every hour, centrifuged and stored at -80°C for further analysis. After 5 mL of urine collection, the remaining volume was re-circulated back into the NMP circuit. Ringer's acetate was used to replace sample volume.

6.2.6 Tissue collection

Nephrectomy was performed on day 14 and the kidney was sliced coronally. For histology, tissue samples were taken from the upper and lower poles and contained both medulla and cortex. The samples were placed in 4% formalin immediately and processed as mentioned below (section 6.2.10). For DNA analysis, a cortical biopsy (Miltex Biopsy Standard Punches 3mm, ThermoFischer, Hvidovre, Denmark) from the upper pole was collected directly after oxHMP, NMP, one hour after reperfusion and at day 14. A quarter of the biopsy was stored at -20°C in RNAlater (ThermoFisher) prior to DNA isolation.

6.2.7 Blood and urine collection

6.2.7.1 Pre- and post-operative

Blood and urine at baseline and day 14 were collected perioperatively. During observation, blood and urine were collected while the pig was awake and in the stable. Blood was aspirated from the CVC followed by a flush with 5 mL of saline containing 500 IU heparin to prevent clotting. An ostomy bag was attached around the pig's external genitals to collect urine non-invasively and the bag was removed after urination.

6.2.7.2 NMP

Perfusion characteristics including renal blood flow, renal resistance and urine production were monitored regularly. Perfusion characteristics were registered immediately before the infusion of MSCs. Creatinine, sodium, lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) levels were measured in the perfusion fluid

with routine automated test methodology carried out by the clinical biochemistry laboratories at Aarhus University Hospital in Denmark and John Radcliffe Hospital in Oxford, United Kingdom. Creatinine clearance and fractional excretion of sodium (FENa) were calculated to determine renal function. Levels of neutrophil gelatinase-associated lipocalin (NGAL), one of the earliest induced proteins that indicate acute kidney injury[25] were determined in perfusate and urine using an enzyme linked immunosorbent assay (pig NGAL ELISA kit 044, BioPorto, Hellerup, Denmark) according to the manufacturer's protocol. We calculated the absolute amount of NGAL in the urine every hour.

Oxygen consumption (mL O_2/min per 100 g) was calculated as follows[26]:

$$Oxygen\ consuption: VO_2 = \frac{(CaO_2\ -\ CvO_2)\ \text{x Q}}{g}\ \text{x } 100$$

$$Arterial\ oxygen\ content: CaO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ arterial) + (pO_2\ arterial\ x\ K))$$

$$Venous\ oxygen\ content: CvO_2 = ((0.024794\ x\ Hb\ x\ SO_2\ venous) + (pO_2\ venous\ x\ K))$$

The number 0.024794 is the result of the equation of the oxygen binding capacity of RBCs, 1.54 mL O_2 at 37°C[34], times the conversion factor of haemoglobin from g/L to mmol/L. Hb is the haemoglobin concentration in mmol/L, SO_2 is oxygen saturation in %, pO_2 is the partial oxygen pressure in kPa, K is the solubility constant of oxygen in water at 37ºC, equal to 0.0225 (ml O_2 per kPa), Q is the renal blood flow in dL/min and *g* is the weight of the kidney in grams at the beginning of NMP.

6.2.8 MSC expansion, characterisation and preparation

6.2.8.1 Porcine MSCs

Male porcine adipose tissue-derived MSCs (pMSCs) from three individual donors (approximately six months old) were isolated, expanded and characterised as described in Chapter 5 section 5.2.7. Briefly, subcutaneous adipose tissue was treated with collagenase type IV and the adherent cell fraction was expanded in minimum essential medium (MEM-α, Sigma-Aldrich) supplemented with 15% foetal bovine serum (FBS, Sigma-Aldrich), 50 U/mL penicillin + 50 µg/mL streptomycin (ThermoFisher), and 2 mM L-glutamine (ThermoFisher). Cells were expanded until passage three, followed by cryopreservation. MSC batches were >95% positive for CD29, CD44, CD90 and negative for CD31 and CD45. Prior to MSC infusion 10 million cryopreserved MSCs were thawed and resuspended in 20 mL culture medium followed by centrifugation (440 g, five minutes, RT). Cell pellet was resuspended in 10 mL perfusate, filtered through a 70 µm cell strainer and transferred to a 10 mL syringe. A small aliquot of cells was used for a control counting and inspection under the microscope to confirm a single cell solution.

6.2.8.2 Human MSCs

Human MSCs (hMSCs) were isolated from male and female perirenal fat, a clinical waste product of living donor nephrectomies in the University Medical Center Groningen, the Netherlands. Adipose tissue was processed only when donors had not objected to anonymous usage of medical waste products. The resulting cell products were stored in such a fashion that these could not be traced back to their individual donors. Isolation and culture expansion were performed as described previously.[22] In brief, the adipose tissue was mechanically disrupted and enzymatically digested using collagenase type IV (Life Technologies Limited, Bleiswijk, Netherlands). The cell fraction was expanded in MEM-α supplemented with 20% FBS, 1% p/s (100 U/mL penicillin and 100 mg/mL

streptomycin) and 2 mM L-glutamine (Life Technologies Limited). Cryopreservation and characterisation were identical to the porcine MSCs, as described above.

6.2.9 Detection of male pMSCs in kidney tissue

DNA was isolated from cortical tissue using a NucleoSpin Tissue DNA isolation kit (Macherey-nagel) according to the manufacturer's protocol. In total, 10 mg of tissue was used for day 14 samples and approximately 1-2 mg were used for day 0 samples. DNA was eluted in 100 µL elution buffer and Y-chromosome was detected by qPCR using primers directed to the male specific repeat (MSR) located on the porcine Y-chromosome as described previously by Gruessner *et al*.[27] As a control for pig DNA, primers were used directed to the porcine S100C gene. Primers used were: MSR upper 5'-CCA TCG GCC ATT GTT TTC CTG TTC A-3', MSR lower 5'-CCT CTG TGC CCA CCT GCT CTC TAC A-3', S100C upper 5'-ATG CTG GAA GGG ACG GTA ACA ACA-3', and S100C lower 5'-GCT CAG CTG CTG TCT TTC ACT CGT-3'.. Quantitative polymerase chain reaction (qPCR) mix consisted of 0.5 µL DNA, 10 pmol of each primer and 1x KiCStart SybrGreen qPCR ReadyMix (Sigma-Aldrich) in a final volume of 25 µL. Samples were run in duplicate on Applied Biosystems 7300 Real-Time PCR machine (ThermoFisher). The thermocycling program included an initial step of 2 minutes at 50°C, subsequently 10 minutes at 95 °C, followed by a 40-time repeat two-step cycle at 95°C for 15 seconds and 60°C for 1 minute.

6.2.10 Immunohistochemical staining and histomorphological assessment

Tissue samples from the renal upper and lower poles were fixed in 4% formalin immediately after collection. Next, tissue was stored in formalin for 24 hours followed by storage in PBS until dehydrated and embedded in paraffin (FFPE). Two-µm sections of FFPE harvested tissue were stained with haematoxylin and eosin, masson-trichrome as well as periodic acid–Schiff and slides were analysed by an experienced renal

pathologist, blinded to the intervention and cases. Several parameters were assessed: inflammation, tubular atrophy, fibrosis, tubular and glomerular damage. For each section, each parameter was scored semi-quantitatively on a scale of zero to four based on the degree of injury: zero if < 2% was affected, one (2% - 5%), two (6% - 25%), three (26% - 50%) and four (51% - 100%).

6.2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 8 (GraphPad software Inc., La Jolla, CA, USA). For all continuous longitudinally measured variables, the area under the curve (AUC) was calculated and a one-way ANOVA with multiple comparisons was used to compare AUC values between groups if the data were normally distributed (Shapiro Wilk test). If data failed these assumptions the Kruskal-Wallis test with Dunn's multiple comparisons test was used. Categorical data were compared using Chi-square test. One pig in the NMP+vehicle group was terminated on posttransplant day six, and its data is included in all analyses until its death. Data are presented as mean ± standard deviation. Two-sided p-values of ≤ 0.05 were considered to indicate statistical significance. To indicate the level of significance in the graphs, asterisks were plotted in the graphs. One asterisk (*) indicates a level of significance of $p \leq 0.05$. Two asterisks (**) indicate a p value of ≤ 0.01 and three asterisks (***) a p value of $p \leq 0.001$.

6.3 RESULTS

6.3.1 Animal characteristics, basic information and perioperative data

The duration of oxygenated HMP, NMP and total cold ischaemic time were similar between groups. Kidney weight at baseline was lower in the oxHMP group compared to NMP+pMSC group (p=0.026, ANOVA) but not in comparison with the other two groups. Perioperatively, all groups had similar duration of warm and cold ischaemia, surgical duration as well as anastomosis time (Table 6.1). Postoperative graft survival across groups was similar, only one pig was terminated (NMP+vehicle group) on day six due to acute renal failure and uraemia.

Table 6.1. Animal characteristics and selected perioperative data. *Table adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

	oxHMP (n=7)	NMP+vehicle (n=7)	NMP+pMSC (n=7)	NMP+hMSC (n=7)	p-value
Body weight, baseline (kg)	51.6 ± 3.4	51.7 ± 2.9	52.4 ± 2.0	54.4 ± 1.7	0.167
Body weight gain, peak-baseline (kg)	3.9 ± 2.2	6.0 ± 2.1	7.0 ± 3.1	6.3 ± 3.9	0.256
Kidney weight (g)	155 ± 20	174 ± 20	188 ± 21	175 ± 19	**0.035***
WIT (min)	75.0 ± 0.0	75.1 ± 0.4	75.0 ± 0.0	75 ± 0.0	0.926
Total CIT (hh:mm)	16:00 ± 00:11	16:02 ± 0:08	16:06 ± 0:11	16:00 ± 0:02	0.812
Time on HMP (hh:mm)	14:04 ± 0:12	13:59 ± 0:01	14:00 ± 0:01	13:59 ± 0:01	0.126
Time on NMP (hh:mm)	N/A	04:00 ± 00:00	04:00 ± 00:00	04:01 ± 00:01	0.838
Total storage time (hh:mm)	16:00 ± 00:11	20:02 ± 0:08	20:06 ± 0:11	20:00 ± 0:02	**0.001****
Anastomosis duration, day 0 (min)	53 ± 9	61 ± 18	59 ± 10	53 ± 10	0.484
Peroperative fluid (iv), total (L)	5.4 ± 0.4	5.3 ± 0.7	5.7 ± 0.8	5.8 ± 1.2	0.745

* oxHMP significantly lower than NMP+pMSC, ** oxHMP significantly lower than NMP+vehicle, NMP+pMSC and NMP+hMSC.

6.3.2 HMP and NMP perfusion characteristics

During oxHMP all kidneys showed similar flow patterns with starting values ranging between 11 and 27 mL/min/100g and an increase over time to a maximum of 39 mL/min/100g at the end of oxHMP. Temperatures stabilised quickly after the start of oxHMP and ranged from 4 to 7°C. During the first hour of NMP, renal blood flow increased in all groups and remained stable until the end of perfusion. There were no significant differences in flow progression over time between groups (p=0.104, ANOVA; Figure 6.2A). However, post-hoc comparison revealed that kidneys perfused in the NMP+pMSC group had already significantly lower renal blood flow values and higher renal resistance at the start of NMP before MSCs were administrated (Table 6.2) compared to NMP+vehicle group.

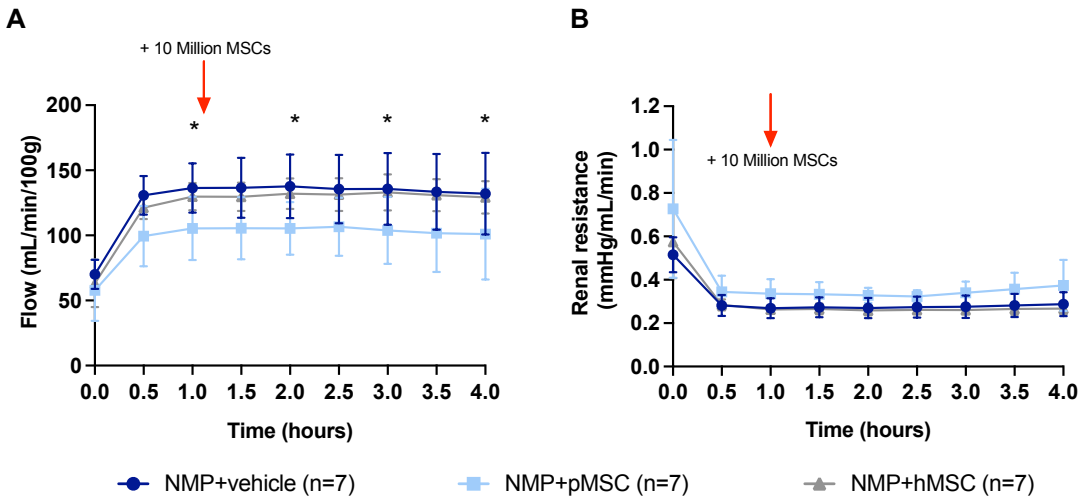


Figure 6.2. Flow and renal resistance during NMP. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. (A) Mean renal blood flow per 100 grams and (B) Mean renal resistance were stable during perfusion and there were no overall differences over time between the groups. Data are presented as mean ± standard deviation. * $p \leq 0.05$ NMP+pMSC vs NMP+vehicle. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Table 6.2. Hourly renal blood flow during NMP with administration of no MSCs or 10 million pig MSC. *Table adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Group comparison	Flow (mL/min/100g)	Time (hours)	p-value
NMP+pMSC vs NMP	105 ± 24 vs 136 ± 19	1	0.012
NMP+pMSC vs NMP	105 ± 20 vs 138 ± 24	2	0.018
NMP+pMSC vs NMP	104 ± 26 vs 136 ± 27	3	0.010
NMP+pMSC vs NMP	101 ± 35 vs 132 ± 31	4	0.012

During the first hour of NMP, renal vascular resistance decreased and remained stable until the end of perfusion and there were no significant differences over time between the groups (p=0.329, ANOVA; Figure 6.2B). MSC administration did not have a significant effect on renal blood flow immediately around the time of infusion (Table 6.3). During NMP, concentrations of electrolytes and acid-base balance remained stable and within physiological range (data not shown).

Table 6.3. Renal blood flow from one to two hours during NMP with administration of no MSCs, 10 million pig MSC and 10 million human MSC. *Table adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Time (hours)	Flow (mL/min/100g)		
	NMP+vehicle (n=7)	**NMP+pMSC (n=7)**	**NMP+hMSC (n=7)**
1:00	136 ± 19	105 ± 24	130 ± 10
1:05	132 ± 14	104 ± 25	130 ± 16
1:10	136 ± 19	102 ± 25	125 ± 11
1:15	137 ± 20	102 ± 26	126 ± 10
1:20	138 ± 21	104 ± 25	126 ± 11
1:25	136 ± 23	104 ± 25	127 ± 11
1:30	136 ± 23	105 ± 24	130 ± 11
1:35	135 ± 25	105 ± 22	128 ± 12
1:40	135 ± 25	104 ± 22	129 ± 12
1:45	135 ± 25	105 ± 21	130 ± 11
1:50	135 ± 25	105 ± 21	130 ± 12
1:55	136 ± 25	105 ± 21	130 ± 12
2:00	138 ± 24	105 ± 20	132 ± 12

6.3.3 Metabolic activity, viability and injury markers during NMP

Metabolic activity and function of the kidney during NMP were assessed by calculating oxygen consumption, urine production, creatinine clearance and fractional excretion of sodium. Kidneys in all groups produced urine during NMP and there were no significant differences in cumulative urine output between the groups (p=0.552, ANOVA; Figure 6.3A). With regards to creatinine clearance during NMP, kidneys in all groups showed very low rates, with end values of 0.97 ± 0.83 mL/min for NMP+vehicle, 0.65 ± 0.60 mL/min for NMP+pMSC and 0.95 ± 0.96 mL/min for NMP+hMSC. No significant differences were found between groups over time (p=0.698, ANOVA; Figure 6.3B). Fractional excretion of sodium improved in all groups during NMP, with end values of 36 ± 21% for NMP+vehicle, 28 ± 13% for NMP+pMSC and 24 ± 12% for NMP+hMSC, but no significant differences were found between the groups over time (p=0.168, ANOVA; Figure 6.3C). Kidneys in all groups consumed oxygen in a similar manner with values ranging from 0.26 to 2.33 mL O_2/min per 100g and no significant differences were found between the groups (p=0.165, ANOVA; Figure 6.3D).

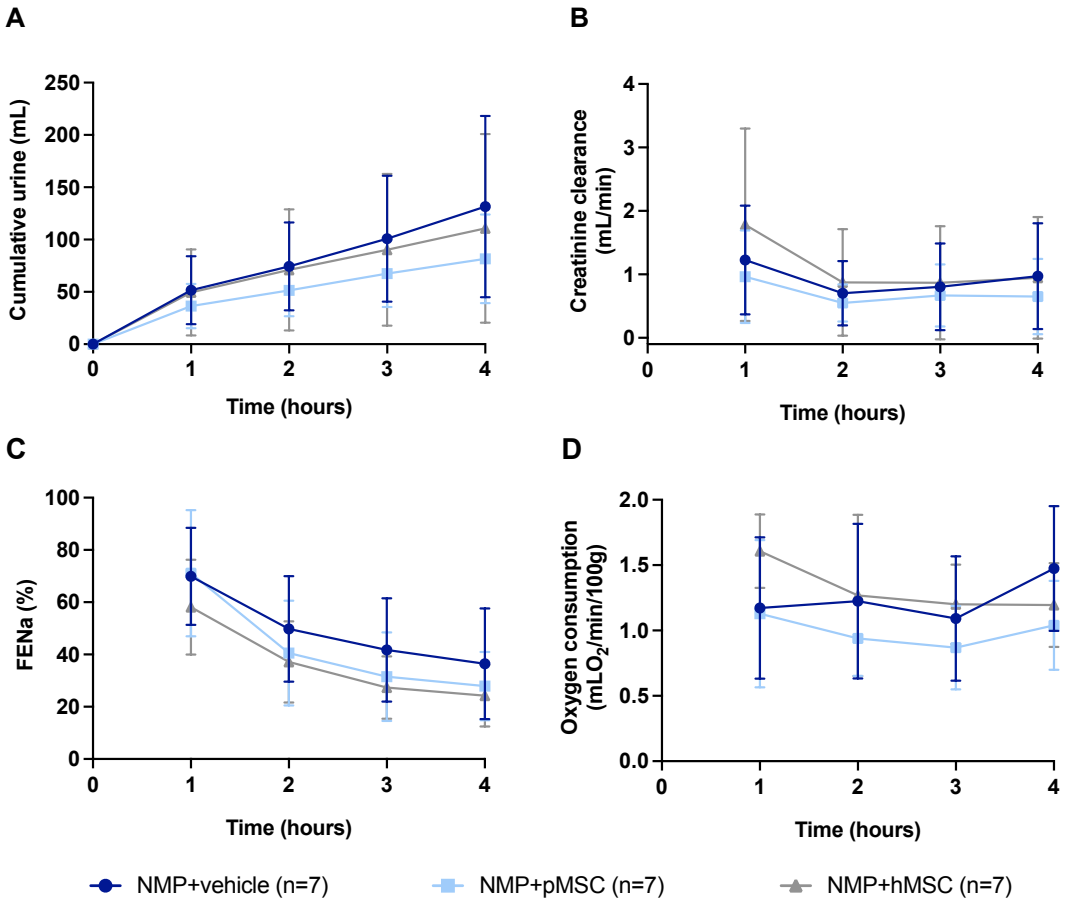


Figure 6.3. Renal function during NMP. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. (A) Cumulative urine output, (B) Creatinine clearance, (C) Fractional excretion of sodium (FENa) and (D) Oxygen consumption were similar between the groups during NMP. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Perfusate lactate increased from baseline during the first two hours of NMP in all groups and stabilised in the remaining hours of NMP. After four hours of NMP, mean values were 2.46 ± 0.63 mmol/L for the NMP+vehicle group, 2.57 ± 0.60 mmol/L for the NMP+pMSC group and 2.21 ± 0.49 mmol/L for the NMP+hMSC group. There were no significant differences over time between groups (p=0.147, ANOVA; Figure 6.4A). Perfusate LDH increased during the first hour of NMP in all groups with end values of 378 ± 86 U/L in the NMP+vehicle group, 365 87 U/L in the NMP+pMSC group and 390 ± 151 U/L in the NMP+hMSC group and there were no differences between the groups (p=0.595, Kruskal-Wallis test; Figure 6.4B). Similarly, perfusate AST increased during the first hour of perfusion and remained stable until the end of NMP (Figure 6.4C). There were no significant differences between the groups (p=0.818, ANOVA).

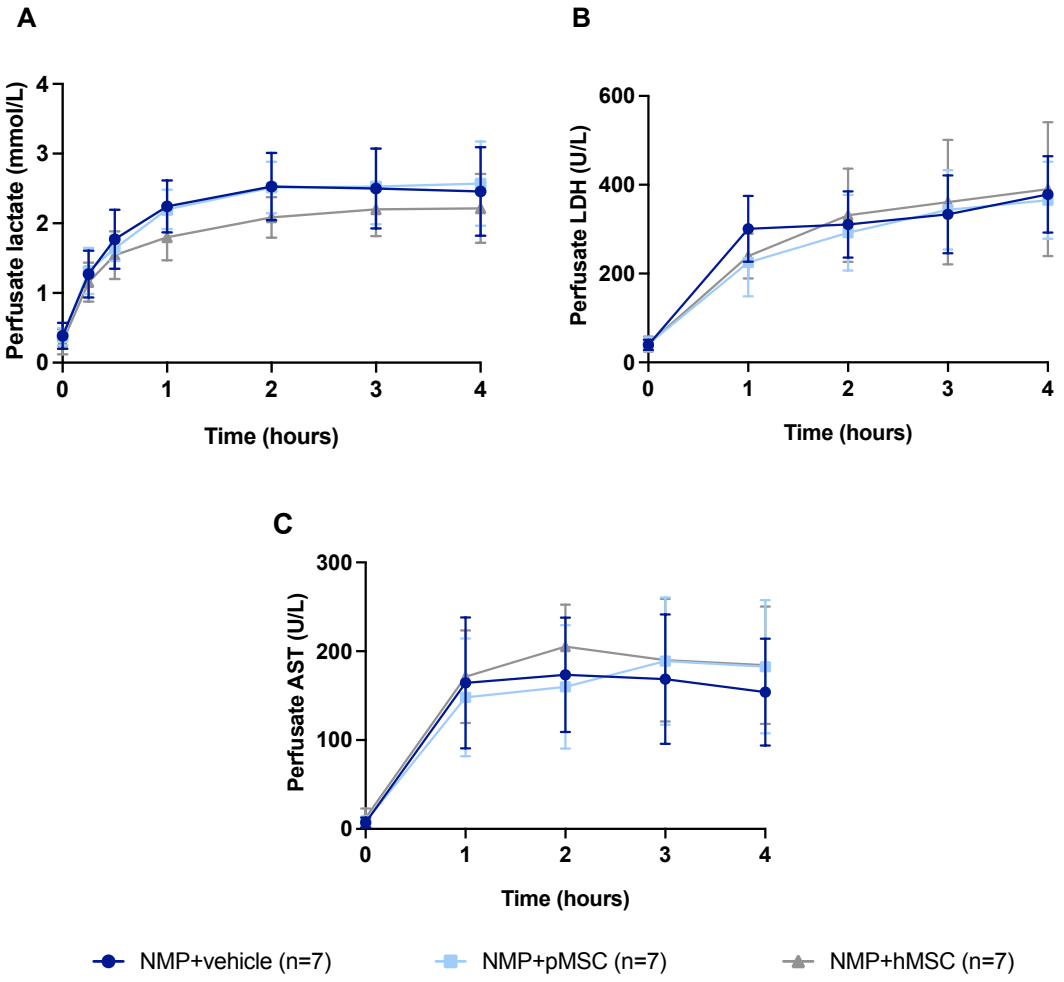


Figure 6.4. Kidney injury markers lactate, LDH and AST during NMP. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. There were no differences in (A) Perfusate lactate, (B) Perfusate LDH and (C) Perfusate AST between the groups during NMP. Data are presented as mean ± standard deviation. AST, aspartate aminotransferase; HMP, hypothermic machine perfusion; LDH, lactate dehydrogenase; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Kidney injury marker urinary NGAL was relatively low throughout NMP (0–5.58 µg in all groups) and levels were similar between the groups over time (p=0.986, ANOVA; Figure 6.5A). Perfusate NGAL increased significantly in all groups during the first hour of NMP and was similar between the groups until the end of NMP (p=0.069, ANOVA; Figure 6.5B).

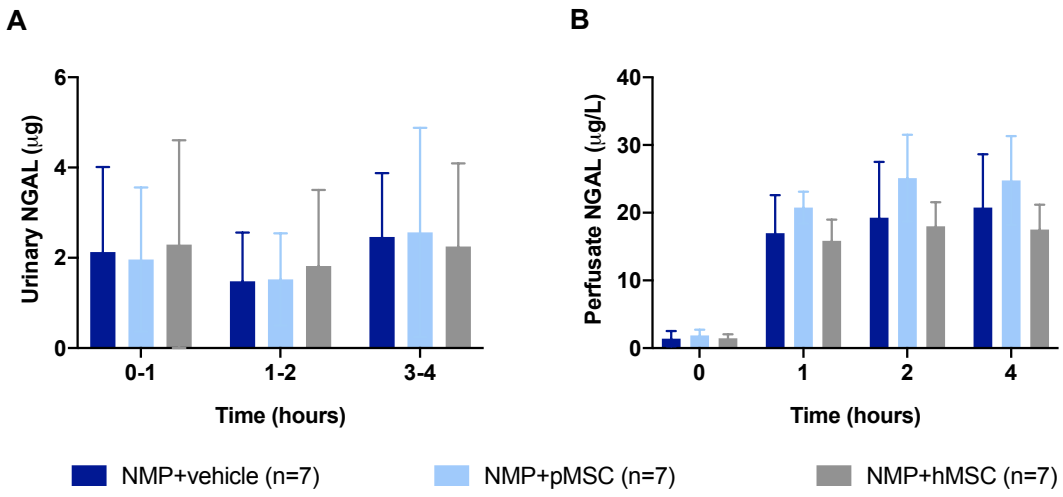


Figure 6.5. Perfusate and urinary NGAL during NMP. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. There were no differences in (A) Absolute urinary NGAL (NGAL concentration x volume of urine produced) and (B) Perfusate NGAL between the groups during NMP. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; NGAL, neutrophil gelatinase associated lipocalin; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

6.3.4 Renal MSC retention after NMP and post-transplant

Biopsies confirmed successful delivery of MSCs during NMP to the seven porcine MSC-treated grafts and Y chromosomes were detected at unaltered levels by qPCR until one hour after reperfusion. On day 14, Y-chromosome DNA was still present in the renal cortex, the amount was reduced about twenty fold compared to the amount found directly after NMP ($p<0.0001$, ANOVA) and transplantation ($p=0.003$, ANOVA; Figure 6.6).

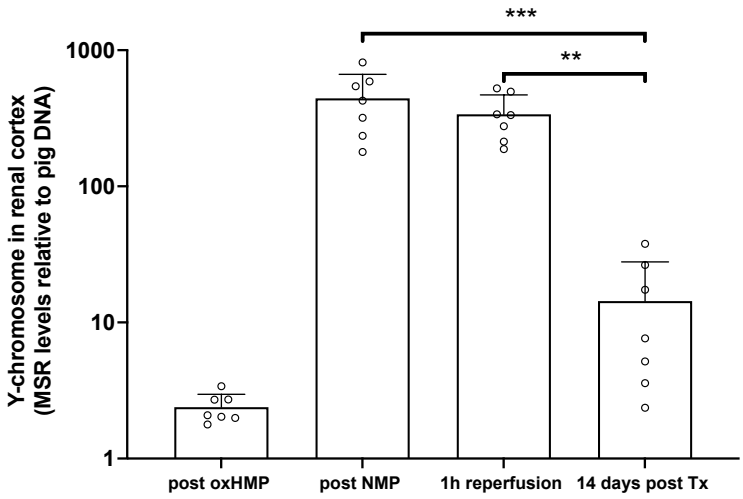


Figure 6.6. Relative Y-chromosome levels compared to total pig DNA in kidney tissue after oxHMP, after NMP, after one hour reperfusion and fourteen days after transplantation. Female porcine kidneys subjected to 75 minutes of warm ischaemia were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP) followed by4 hours NMP with an intra-arterial infusion of ten million male pig MSCs (NMP+pMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. On day 14, Y-chromosome was still present in the renal cortex, but in lower amount in comparison with directly after NMP and transplantation. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; MSR, male specific repeat; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

6.3.5 Renal function and injury post-transplant

As expected in this porcine DCD auto-transplantation model, pigs developed acute kidney injury post-transplant and injury markers increased during the first 72 hours. Pigs did not produce urine until 24 to 48 hours after transplantation. Except for the pig that was terminated, the animals' welfare scores were acceptable, however, they retained considerable amounts of fluid two to three days post-transplant, as displayed by the weight gain in Table 6.1. Their body weight started to decrease when urine production was restored. Glomerular filtration rate (GFR) was measured as urinary clearance of ^{99}Tc-DTPA on day 14 and was significantly higher in the NMP+vehicle group compared to oxHMP (p=0.048, ANOVA; Figure 6.7). Mean values were 42 ± 12 mL/min for the oxHMP group, 55 ± 3 mL/min for the NMP+vehicle group, 52 ± 8 mL/min for the NMP+pMSC group and 48 ± 7 mL/min for the NMP+hMSC group.

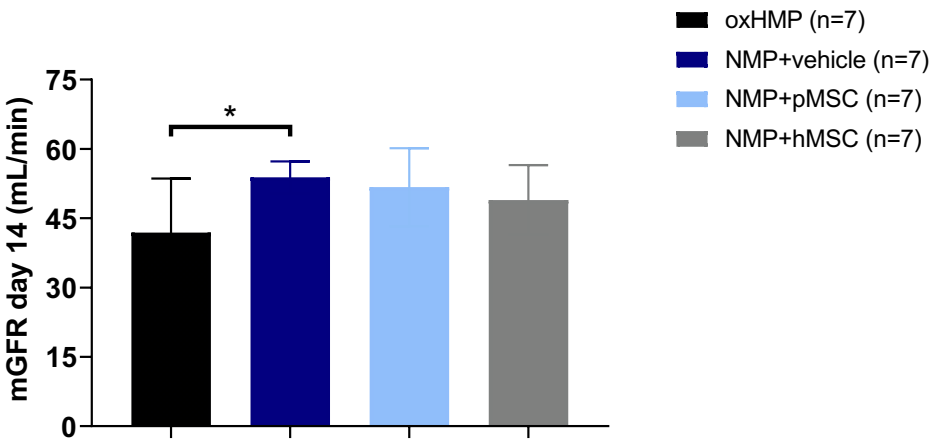


Figure 6.7. Measured GFR (mGFR) 14 days after transplantation. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. Kidneys in the NMP+vehicle group had higher mGFR than kidneys in the oxHMP group. There were no differences between the MSCs treated kidneys with kidneys in the NMP and oxHMP groups. Data are presented as mean ± standard deviation. * $p \leq 0.05$ oxHMP vs NMP+vehicle. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Postoperative plasma creatinine (p-creatinine) increased in all animals during the first days and peaked on either day three or day four with mean peak levels of 1041 ± 358 µmol/L (oxHMP), 1261 ± 334 µmol/L (NMP+vehicle), 1234 ± 246 µmol/L (NMP+pMSC) and 1076 ± 195 µmol/L (NMP+hMSC) (Figure 6.8). There were no significant differences between groups over time (p=0.371, ANOVA) nor in peak plasma creatinine levels (p=0.408, ANOVA).

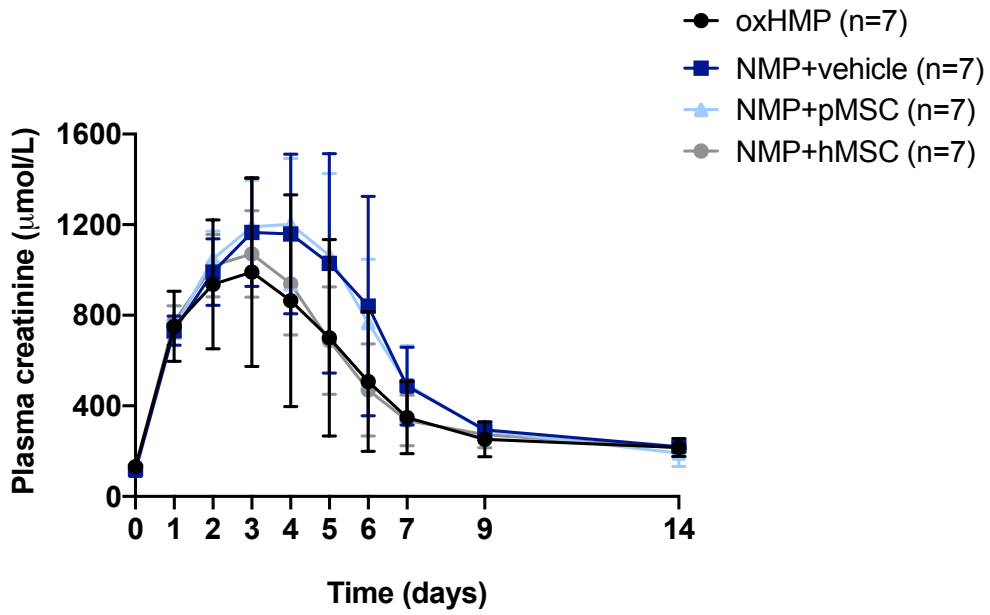


Figure 6.8. Postoperative plasma creatinine. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. There were no differences in plasma creatinine between the groups during the 14 days of follow-up. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

Plasma NGAL (p-NGAL) increased in all animals in the 2 days post-transplant, and during the 14 days of follow up the levels were similar between groups (p=0.581, ANOVA; Figure 6.9). p-NGAL peaked on day two in all groups with mean peak values of 1858 ± 1259 µg/L for the oxHMP group, 1939 ± 522 µg/L for the NMP+vehicle group, 2033 ± 370 µg/L for the NMP+pMSC group and 2264 ± 509 µg/L for the NMP+hMSC group.

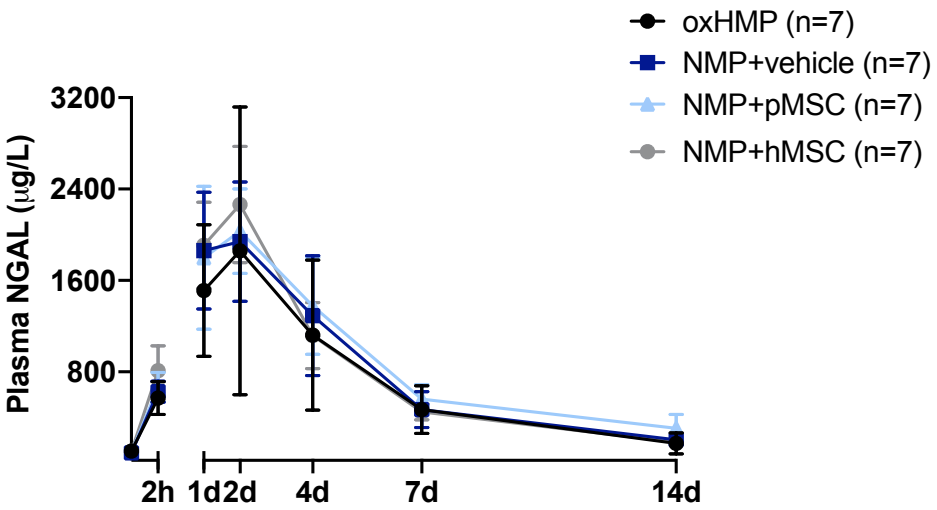


Figure 6.9. Postoperative plasma neutrophil gelatinase associated lipocalin (NGAL). Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. There were no differences in plasma NGAL between the groups during the 14 days of follow-up. Data are presented as mean ± standard deviation. HMP, hypothermic machine perfusion; NGAL, neutrophil gelatinase associated lipocalin; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

6.3.6 Histology

Sections of biopsies taken from the kidney's upper pole were scored on a scale of zero to four for the presence of inflammation, tubular atrophy and fibrosis (Figure 6.10). The histological assessment revealed presence of inflammation, tubular atrophy and fibrosis with a tendency of higher scores in the MSC treated kidneys without reaching statistical significance (p=0.648, p=0.304, p=403, respectively; Chi-square test).

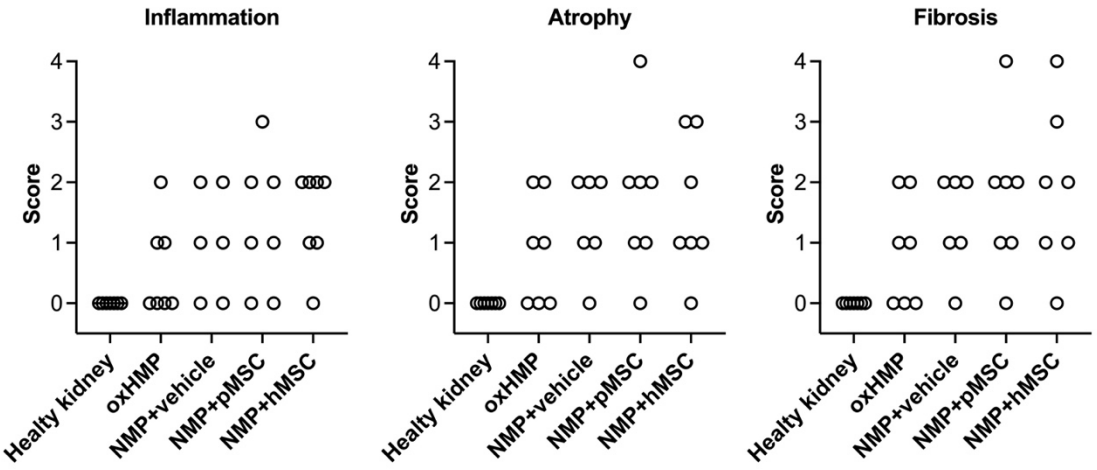


Figure 6.10. Histological scoring of biopsies collected after 14 days of transplantation. Female porcine kidneys subjected to 75 minutes of warm ischaemia (four experimental groups, n=7 per group) were retrieved and preserved for 14 hours by oxygenated HMP (oxHMP). In three groups this was followed by four hours of NMP with an intra-arterial infusion of vehicle (NMP+vehicle), ten million pig MSCs (NMP+pMSC) or ten million human MSCs (NMP+hMSC). All kidneys were autotransplanted with immediate contralateral nephrectomy and pigs were observed for 14 days. There were no differences in histological scoring of inflammation, atrophy and fibrosis after 14 days of transplantation between the groups. HMP, hypothermic machine perfusion; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion. *Figure adapted from Lohmann S et al. Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study. Am J Transplant. 2021;21(7):2348-2359.*

6.4 DISCUSSION

In this study we subjected ischaemically injured kidneys to MSC therapy during continuous *ex vivo* normothermic machine perfusion followed by transplantation, using a porcine kidney auto-transplantation model. The study aimed to demonstrate feasibility and safety of cell therapy during NMP while evaluating potential beneficial effects of *ex vivo* MSC treatment up to two weeks after transplantation. To our knowledge, this is the first study to undertake a systematic approach assessing a number of aspects of *ex vivo* MSC administration in the context of kidney transplantation. We found that renal *ex vivo* MSC therapy is indeed feasible and likely to be safe, but we did not observe beneficial effects during the two-week post-transplant observation period.

The first study to report renal infusion of MSCs during *sub*-NMP at 32°C with an acellular perfusate, but without transplantation, was performed by Brasile *et al.* In their experiments, MSC therapy was administered to human discarded kidneys, which were perfused for a total of 24 hours. MSC therapy led to a significant reduction in inflammatory cytokines and an increase in ATP concentration in comparison with the control group without MSC therapy.[23] In our study, we took the crucial step to transplant the MSC-treated grafts to evaluate retention of MSCs and possible functional effects prior to and after transplantation. All pigs transplanted with MSC treated kidneys survived the crucial first post-operative days, suggesting the safety of administration of 10 million MSCs to an isolated donor kidney. In addition, we measured GFR at day 14, as this is considered the gold standard to assess renal function. We found a significantly higher GFR in the NMP+vehicle group in comparison with the oxHMP group, which could be related to the tendency to lower body mass and significantly smaller kidneys in the oxHMP group, as also indicated by the slightly lower peak p-creatinine in the oxHMP group. MSC treatment during NMP did not have a beneficial effect on GFR when compared to NMP alone. Within the 14 days of follow up, both p-creatinine and p-NGAL

increased significantly in all groups within the first few days post-transplant. Neither NMP, nor the addition of MSCs during NMP improved renal injury or function in the initial post-transplant phase compared to oxHMP. These outcomes are in line with results published by Darius *et al.,* who demonstrated that the use of NMP after a period of HMP did not further improve early graft recovery. In their study, NMP even led to higher serum creatinine levels in comparison with oxHMP alone, but important to note is that NMP was only preceded by either SCS or non-oxygenated HMP and GFR was not reported.[28] The superiority of cold preservation methods was not supported in a study by Urbanellis *et al.,* who performed 16 hours of NMP and compared outcomes to both aSCS and non-oxygenated HMP groups.[29] Significantly lower levels of serum creatinine were measured in the NMP group, but only until day three (vs. HMP) and day four (vs. SCS). The NMP duration of four hours in our study, although longer than in the study by Darius *et al.,* may have been too short to demonstrate a significant effect on kidney function in comparison with cold storage preservation alone.[28] However, our objective was not to perform prolonged NMP as a possibly better organ preservation strategy, but only to utilise NMP as a platform to deliver potentially regenerative therapy to injured kidney grafts. Before these series of auto-transplantations, we investigated the feasibility of NMP as a cell therapy delivery method during seven hours of NMP. In those studies we showed that MSCs were retained in the renal cortex, mainly localising to glomeruli (Chapter 5). These results were similar during pig kidney NMP with lower doses of MSCs administration [22] and also confirmed our earlier *in vivo* study.[30]

In the present study, we were able to detect MSCs in the kidney after NMP with cells mainly present in the cortex as expected. More interestingly, we were able to demonstrate, that one hour *after* reperfusion, the majority of detected MSCs after NMP were still present. This confirms that NMP is a unique and effective method of delivery for cellular therapy to a kidney *ex vivo*, which will still be present in the kidney one hour *after* reperfusion *in vivo*. MSCs are generally short-lived[20] and, hence the smaller fraction

of viable cells detected on day 14 was expected. However, potentially beneficial effects exerted by MSCs do not solely depend on their long-term viability, as the interaction between MSC and host cells may initiate immunoregulatory and regenerative processes that continue after disappearance of MSC.[31,32]

In this study, kidneys were treated with 10 million MSCs. This dose was based on pre-clinical pig studies and a human study, where intra-arterial administration of 10 million MSCs improved kidney function.[33-35] Furthermore, in Chapter 5 it was demonstrated that MSCs could be found in the kidney after seven hours of NMP with administration of 10 million MSCs and without adversely affecting perfusion parameters. In addition, our group showed that intra-arterial administration of 10 million MSCs in contrast to a higher dose of 100 million MSCs, did not lead to any side effects (unpublished data). In the present study, the addition of MSCs during NMP proved to be safe and no macroscopic changes or significant alterations in *ex vivo* perfusion dynamics occurred. From previous research, where MSCs were added to ischaemically damaged kidneys, it was concluded that when adding lower doses, 100 000 and 1 million cells, tracing of MSCs became difficult, if not impossible.[22] Differences in flow between groups in the present study were not the results of MSC therapy as these differences were already present prior to MSCs infusion.

For our current study, we chose to have an experimental group with porcine MSCs and also one with human MSCs. This approach was inspired by the idea that, on the one hand, administration of porcine MSCs would constitute a same-species model and (exclusively male) pMSCs could be detected more easily through Y chromosome quantification in renal tissue from exclusively female pigs. On the other hand, with human MSCs we could actually test the behaviour of a realistic clinical product-to-be. Furthermore, there is evidence that human and porcine MSCs respond differently to suspension conditions and the perfusion fluid.[36] Evidence so far suggests that MSCs do

not elicit a substantial immunological allo- or xeno-response.[37-39] Hence, we felt it unlikely that introducing human MSCs into porcine kidneys would lead to relevant cross-species reactions. No macroscopic changes or adverse effects were seen in the kidneys treated with human MSCs. Therefore, we can conclude that this porcine model is also suitable for testing human cells. Nevertheless, the presence of a subtle xeno-effect in our data cannot be ruled out completely.

Our study has several limitations. Firstly, the porcine and human MSCs that we utilised were not completely comparable as the human donors were of various ages and the human MSCs were isolated from perirenal adipose tissue, while the porcine MSCs were harvested from subcutaneous adipose tissue. This may have had an impact on cell surface marker expression and phenotype.[22] MSCs derived from different sources can have different therapeutic potential on different indications.[40-42] In this study we only used adipose tissue derived MSCs and we did not compare their effect to, for example, bone marrow derived MSCs. Adipose tissue derived MSCs were chosen as in clinical applications the harvesting approach is safe, less invasive and associated with a lower number of complications. Also, a significantly higher number of cells can be obtained from the same amount of tissue in comparison with MSCs harvested from other sources [43], while *in vitro* research has shown that the immunosuppressive potential of bone marrow MSCs may be inferior to that of adipose tissue-derived MSCs.[40, 41] Our study did utilise thawed cryopreserved MSCs, as opposed to other experimental studies that used fresh MSCs directly from culture.[20, 22, 30, 44] Although the latter approach may have advantages in terms of elevated MSC activity[36, 45-48], the method we chose is more in line with eventual clinical reality. Secondly, in this study, 14 hours of total storage in the oxHMP group was compared to a total storage of 18 hours in the NMP groups. This design was chosen from a clinical perspective based on offering the regenerative MSC therapy upon graft arrival at the transplantation centre after a set period of HMP. Our

setup was similar to that used in another study in which SCS was followed by various periods of NMP leading to a difference in preservation time of up to 16 hours.[49]

In summary, this study has shown that delivery of 10 million MSCs during normothermic *ex vivo* perfusion of ischaemically-injured porcine kidneys is feasible and likely to be safe without negatively affecting perfusion characteristics or early transplantation outcomes. In addition, our methods allowed us to detect MSCs in treated kidneys up to 14 days post-transplant. Our current dose and methodology of cell therapy during NMP did not improve early renal function in ischaemically damaged kidneys when compared to oxHMP+NMP alone or only oxHMP prior to transplantation. The present study has provided a proof of concept of *ex vivo* MSC therapy during kidney NMP and we have gained a number of valuable first insights on targeted cell therapy for donor kidneys that were subsequently successfully transplanted.

REFERENCES

1. National Health Service Blood and Transplant. Organ Donation and Transplantation: Activity Report 2018/2019. Statistics and Clinical Studies, NHS Blood and Transplant; 2019.

2. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2016 Annual Data Report: Kidney. *Am J Transplant*. 2018;18 Suppl 1:18-113.

3. Eurotransplant International Foundation. Eurotransplant Annual Report 2018. Eurotransplant International Foundation; 2018.

4. Moers C, Leuvenink HG, Ploeg RJ. Donation after cardiac death: evaluation of revisiting an important donor source. *Nephrol Dial Transplant*. 2010;25:666-673.

5. Heylen L, Jochmans I, Samuel U, et al. The duration of asystolic ischemia determines the risk of graft failure after circulatory-dead donor kidney transplantation: A Eurotransplant cohort study. *Am J Transplant*. 2018;18:881-889.

6. Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study Group. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med*. 2012;366:770-771.

7. Jochmans I, Moers C, Smits JM, et al. Machine perfusion versus cold storage for the preservation of kidneys donated after cardiac death: a multicenter, randomized, controlled trial. *Ann Surg*. 2010;252:756-764.

8. Brat A, van Heurn E, Huurman V, et al. Hypothermic Machine Perfusion as a National Standard Preservation Method for Deceased Donor Kidneys . *Transpl Int.* 2019;32.

9. Rijkse E, IJzermans JN, Minnee RC. Machine perfusion in abdominal organ transplantation: Current use in the Netherlands. *World J Transplant*. 2020;10:15-28.

10. Jochmans I, Hofker DS, Davies L, Knight S, Pirenne J, Ploeg RJ. Oxygenated Hypothermic Machine Perfusion of Kidneys Donated after Circulatory Death: An international Randomised Controlled Trial. *Am J Transplant.* 2019;19:(suppl 3).

11. Summers DM, Ahmad N, Randle LV, et al. Cold pulsatile machine perfusion versus static cold storage for kidneys donated after circulatory death: a multicenter randomized controlled trial. *Transplantation*. 2019.

12. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable

but Resuscitated by Ex Vivo Normothermic Machine Perfusion. *Am J Transplant*. 2016;16:3282-3285.

13. Hamar M, Urbanellis P, Kaths MJ, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. *Transplantation*. 2018;102:1262-1270.

14. Weissenbacher A, Lo Faro L, Boubriak O, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. *Am J Transplant*. 2019;19:178-192.

15. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation*. 2018;102:35-43.

16. Morigi M, Benigni A. Mesenchymal stem cells and kidney repair. *Nephrol Dial Transplant*. 2013;28:788-793.

17. Franquesa M, Hoogduijn MJ, Reinders ME, et al. Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: lessons learned from first clinical trials. *Transplantation*. 2013;96:234-238.

18. Lalu MM, McIntyre L, Pugliese C, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One*. 2012;7:e47559.

19. Thompson M, Mei SHJ, Wolfe D, et al. Cell therapy with intravascular administration of mesenchymal stromal cells continues to appear safe: An updated systematic review and meta-analysis. *EClinicalMedicine*. 2020;19:100249.

20. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol*. 2012;3:297.

21. de Witte, S. F. H., Luk F, Sierra Parraga JM, et al. Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. *Stem Cells*. 2018;36:602-615.

22. Pool M, Eertman T, Sierra Parraga J, et al. Infusing Mesenchymal Stromal Cells into Porcine Kidneys during Normothermic Machine Perfusion: Intact MSCs Can Be Traced and Localised to Glomeruli. *Int J Mol Sci*. 2019;20:10.3390/ijms20143607.

23. Brasile L, Henry N, Orlando G, Stubenitsky B. Potentiating Renal Regeneration Using Mesenchymal Stem Cells. *Transplantation*. 2019;103:307-313.

24. Thompson ER, Bates L, Ibrahim IK, et al. Novel delivery of cellular therapy to reduce ischemia reperfusion injury in kidney transplantation. *Am J Transplant*. 2020.

25. Singer E, Marko L, Paragas N, et al. Neutrophil gelatinase-associated lipocalin: pathophysiology and clinical applications. *Acta Physiol (Oxf)*. 2013;207:663-672.

26. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation*. 2019;103:2057-2064.

27. Gruessner RW, Levay-Young BK, Nakhleh RE, et al. Portal donor-specific blood transfusion and mycophenolate mofetil allow steroid avoidance and tacrolimus dose reduction with sustained levels of chimerism in a pig model of intestinal transplantation. *Transplantation*. 2004;77:1500-1506.

28. Darius T, Gianello P, Vergauwen M, et al. The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model. *Am J Transplant*. 2019;19:752-762.

29. Urbanellis P, Hamar M, Kaths JM, et al. Normothermic Ex Vivo Kidney Perfusion Improves Early DCD Graft Function Compared With Hypothermic Machine Perfusion and Static Cold Storage. *Transplantation*. 2020;104:947-955.

30. Sierra-Parraga JM, Munk A, Andersen C, et al. Mesenchymal Stromal Cells Are Retained in the Porcine Renal Cortex Independently of Their Metabolic State After Renal Intra-Arterial Infusion. *Stem Cells Dev*. 2019;28:1224-1235.

31. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol*. 2014;5:148.

32. Luk F, de Witte SF, Korevaar SS, et al. Inactivated Mesenchymal Stem Cells Maintain Immunomodulatory Capacity. *Stem Cells Dev*. 2016;25:1342-1354.

33. Saad A, Dietz AB, Herrmann SMS, et al. Autologous Mesenchymal Stem Cells Increase Cortical Perfusion in Renovascular Disease. *J Am Soc Nephrol*. 2017;28:2777-2785.

34. Eirin A, Zhu XY, Krier JD, et al. Adipose tissue-derived mesenchymal stem cells improve revascularization outcomes to restore renal function in swine atherosclerotic renal artery stenosis. *Stem Cells*. 2012;30:1030-1041.

35. Ebrahimi B, Eirin A, Li Z, et al. Mesenchymal stem cells improve medullary inflammation and fibrosis after revascularization of swine

atherosclerotic renal artery stenosis. *PLoS One*. 2013;8:e67474.

36. Sierra Parraga JM, Rozenberg K, Eijken M, et al. Effects of Normothermic Machine Perfusion Conditions on Mesenchymal Stromal Cells. *Front Immunol*. 2019;10:765.

37. Mansilla E, Marin GH, Sturla F, et al. Human mesenchymal stem cells are tolerized by mice and improve skin and spinal cord injuries. *Transplant Proc*. 2005;37:292-294.

38. Grinnemo KH, Mansson A, Dellgren G, et al. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *J Thorac Cardiovasc Surg*. 2004;127:1293-1300.

39. Li J, Ezzelarab MB, Cooper DK. Do mesenchymal stem cells function across species barriers? Relevance for xenotransplantation. *Xenotransplantation*. 2012;19:273-285.

40. Mattar P, Bieback K. Comparing the Immunomodulatory Properties of Bone Marrow, Adipose Tissue, and Birth-Associated Tissue Mesenchymal Stromal Cells. *Front Immunol*. 2015;6:560.

41. Najar M, Raicevic G, Fayyad-Kazan H, et al. Impact of different mesenchymal stromal cell types on T-cell activation, proliferation and migration. *Int Immunopharmacol*. 2013;15:693-702.

42. De Ugarte DA, Alfonso Z, Zuk PA, et al. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol Lett*. 2003;89:267-270.

43. Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev*. 2012;21:2724-2752.

44. Fischer UM, Harting MT, Jimenez F, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev*. 2009;18:683-692.

45. Hoogduijn MJ, de Witte SF, Luk F, et al. Effects of Freeze-Thawing and Intravenous Infusion on Mesenchymal Stromal Cell Gene Expression. *Stem Cells Dev*. 2016;25:586-597.

46. Chinnadurai R, Garcia MA, Sakurai Y, et al. Actin cytoskeletal disruption following cryopreservation alters the biodistribution of human mesenchymal stromal cells in vivo. *Stem Cell Reports*. 2014;3:60-72.

47. Gurgul A, Romanek J, Pawlina-Tyszko K, Szmatola T, Opiela J. Evaluation of changes arising in the pig mesenchymal stromal cells transcriptome following cryopreservation and

Trichostatin A treatment. *PLoS One*. 2018;13:e0192147.

48. Bahsoun S, Coopman K, Akam EC. The impact of cryopreservation on bone marrow-derived mesenchymal stem cells: a systematic review. *J Transl Med*. 2019;17:397-7.

49. Kaths JM, Echeverri J, Linares I, et al. Normothermic Ex Vivo Kidney Perfusion Following Static Cold Storage-Brief, Intermediate, or Prolonged Perfusion for Optimal Renal Graft Reconditioning? *Am J Transplant*. 2017;17:2580-2590.

Chapter 7

Summary

General discussion

Future perspectives

7.1 SUMMARY

Patients suffering from renal diseases may finally develop end-stage renal failure. To date, kidney transplantation is the primary choice of treatment for suitable patients with end-stage renal disease, as it offers better life expectancy and improved quality of life compared to dialysis. Marginal organs, from older and higher risk brain death donors and donation after circulatory death (DCD) donors, are increasingly accepted due to the high discrepancy between organ supply and demand, despite having inferior quality.[1] As a result, clinicians are uncertain about organ quality and may discard higher risk organs.[2] Improvement of previously discarded and injured organs will not only allow us to use them for transplantation and benefit patients in many kidney transplant centres but also enhance our insight into how acute organ injury, in general, can be monitored and repaired. Normothermic machine perfusion (NMP) of injured kidneys provides an opportunity to assess organ quality of marginal grafts and improve organ viability prior to transplantation. In addition, NMP offers a unique opportunity for active cellular interventions to an isolated organ *ex vivo* under near-physiological conditions prior to transplantation.[3-5] Mesenchymal stromal cells (MSCs) may be able to ameliorate ischaemia reperfusion injury (IRI), as they possess potent anti-inflammatory and regenerative properties.[6-9] Phase I clinical trials showed that intravenous infusion of MSCs to transplant recipients is safe. However, this mode of systemic administration of MSC is associated with off-target distribution.[10-12] This thesis focused on the optimisation of donor kidney NMP conditions and studied the optimal duration of NMP needed to allow homing and initiation of biological effects of infused MSCs on the donor kidney during NMP.

In **Chapter 3**, we developed and optimised a reproducible slaughterhouse model. We have demonstrated that NMP of pig kidneys can be performed both on an open or closed circuit and without the addition of a vasodilator. The use of sodium nitroprusside (SNP)

as a vasodilator caused worsening lactic acidosis, potentially by the accumulation of its toxic metabolite cyanide and it should be avoided. Furthermore, stable kidney NMP with a red blood cell-albumin based solution can be performed for a minimum of seven hours using a porcine slaughterhouse model, maintaining an appropriate acid-base balance, concentration of electrolytes as well as normal metabolic parameters.

In **Chapter 4**, we focussed more on the necessity of an oxygen carrier during NMP. The results in this chapter demonstrated that an oxygen carrier is necessary during NMP of healthy porcine kidneys. NMP of kidneys without an oxygen carrier led to significantly impaired renal function and high lactate and aspartate aminotransferase levels, indicating injury and anaerobic metabolism. Even though HBOC-201 showed similar oxygen oxygen-carrying capacity and allowed for comparable oxygen consumption as allogenic concentrated red blood cells, significant impaired renal function and high methaemoglobin levels during NMP indicate that it is less suitable for prolonged periods of perfusion.

In **Chapter 5**, we performed a dose study to determine the optimum dose of MSCs that can be administered during NMP without detriment to perfusion and to identify the location and distribution of MSCs in the kidney during NMP. Results showed that administration of 2, 10 and 50 million MSCs to the donor kidneys during NMP is safe and feasible as there were no adverse effects on perfusion characteristics and injury markers. After seven hours of NMP, MSCs were mainly localised in the glomeruli and detected in all three doses administered. Furthermore, the percentage of detected cells after administration of 10 and 50 million MSCs during NMP were similar. Flow cytometry and quantitative polymerase chain reaction showed that the distribution of MSCs in the tissue was heterogeneous. In addition, there were only low amounts of MSCs found in the perfusate after administration of MSCs. Furthermore, results in Chapter 5 revealed that perfusate prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF)-A and

angiopoietin 1 increased over time during NMP in the presence or absence of MSCs, whereas perfusate interleukin (IL)-10 only increased in the perfusate with the addition of MSCs.

In **Chapter 6**, we evaluated the safety and feasibility of MSC therapy during kidney NMP while also evaluating any functional effect of the MSCs therapy after 14 days of transplant in a DCD pig auto-transplantation model. The results demonstrated that the delivery of 10 million MSCs during normothermic *ex vivo* perfusion of porcine kidneys is feasible and safe without negatively affecting perfusion characteristics or early transplantation outcomes. However, the administration of 10 million MSCs during NMP did not improve early renal function of ischaemically-damaged kidneys compared to NMP alone.

7.2 GENERAL DISCUSSION

There is an increasing number of studies by different groups focusing on using NMP technology in kidney transplantation. However, the application into clinical practice has been limited and it is primarily used as a research tool.[13-15] There is currently no consensus on the best NMP technique, perfusion duration or the optimal perfusate composition. **Chapter 3** describes the evolution of the NMP protocol adopted in this thesis to achieve prolonged kidney perfusion in a porcine slaughterhouse model to allow successful administration of porcine MSCs. The literature served as an inspiration to develop the red blood cell-albumin-based perfusion solution and alterations of the colloid concentration, electrolytes, vasodilator and amoxicillin-clavulanate concentrations were made before the definite perfusion composition was established. We demonstrated that NMP could be performed on open and closed circuits without using a vasodilator for a minimum of seven hours. However, the use of SNP as vasodilator caused worsening lactic acidosis, potentially by the accumulation of its toxic metabolite cyanide[16, 17] and it should probably be avoided. Results in this chapter also showed that kidneys could be perfused with bolus doses of amoxicillin-clavulanate given throughout perfusion as perfusion is compromised when kidneys are perfused with a lower concentration of amoxicillin-clavulanate administered at the beginning of perfusion. Furthermore, data on the extent of bacterial growth showed that the minimal inhibitory concentration of kidney perfused with a bolus dose of amoxicillin-clavulanate was reached for all cultured micro-organisms, except for one species of pseudomonas that continued to proliferate, which is likely to be due to resistance to amoxicillin-clavulanate.[18]

Most NMP protocols include red blood cells (RBCs) as oxygen carriers to deliver oxygen to the tissues.[4, 5, 19, 20] Although the use of RBCs resembles normal physiology, it has several limitations. RBCs need to be retrieved from the deceased donor's own blood or from human donors via a hospital blood bank and they have a relatively short shelf life.

Prolonged storage of RBCs at 2-6°C leads to reduced intracellular ATP and 2,3-diphosphoglycerate, leading to a left shift in the oxygen dissociation curve, resulting in a higher oxygen affinity, and when these RBCs are used for transfusion or during NMP they are less capable of releasing oxygen, and therefore less effective.[21] The use of HBOC-201, a polymerised bovine-derived haemoglobin-based oxygen carrier (HBOC) has been studied in clinical and pre-clinical liver NMP as an alternative to RBCs[22-24], but studies in kidney NMP have been limited.[25] **Chapter 4** demonstrated that healthy porcine donor kidneys perfused with no oxygen carrier or HBOC-201 as oxygen carrier have significantly impaired renal function during six hours of NMP, compared with kidneys perfused using allogenic concentrated RBCs. Although injury marker levels are relatively low in the HBOC-201 group, high methaemoglobin levels, potentially due to the absence of nicotinamide adenine dinucleotide hydrogen-dependent enzyme methaemoglobin reductase, indicate that HBOC-201 might be less suitable as an oxygen carrier for prolonged periods of perfusion *ex vivo*. Furthermore, the mechanism of HBOC and free haemoglobin toxicity is still not fully elucidated.[26] Kidneys perfused with allogenic concentrated RBCs showed increased levels of the oxidative stress marker MDA compared to HBOC-201 and this can be potentially explained by the fact that a significant level of haemolysis was detected in several kidneys in this group. The haemolysis seen in these experiments is most likely related to each individual RBC unit storage time, as more haemolysis is to be expected from RBCs that are older and have prolonged storage.[21] This finding is relevant for clinical perfusions since RBC unit storage times for NMP vary considerably in the clinical setting.

Studies indicate that beside immunomodulatory properties, MSCs also possess regenerative properties with different small and large animal studies reporting a beneficial effect on IRI with the possibility of acute repair in the kidney.[6, 7, 27] Intravenous infusion (IV) of MSCs to kidney transplant recipients is safe but is associated with inefficient delivery to the kidneys. In **Chapter 5**, we delivered different doses of MSCs to

the kidney during NMP. The NMP *ex vivo* circuit offers the opportunity to deliver lower doses of MSCs to the kidneys and ensures direct contact with the kidney vasculature, avoiding systemic effects of the patients. Administration of 2, 10 and 50 million MSCs during NMP proved to be safe and feasible and MSCs were found mainly located in the glomeruli after seven hours of perfusion. There were notably higher amounts of cells traced in the kidney when higher doses of MSCs were delivered and similar percentages of identified MSCs were found when 10 and 50 million MSCs were administered during NMP. Although a small percentage of viable MSCs were found in the kidney cortex after administration during NMP, they may still have had a protective effect. MSCs are generally short-lived after IV administration[11, 12, 28, 29]; however, there is evidence that the potential beneficial effects of MSCs do not necessarily depend on their viability since inactivated MSCs might initiate immunoregulatory and regenerative processes after the disappearance of MSC.[12, 30] The mechanism of the reparative and regenerative effects of MSCs is still not fully understood. However, it is believed that MSCs effects do not rely only on cell-to-cell interaction, but also on the paracrine interaction of MSCs with the injured tissue, driven by their produced soluble factors (secretome) leading to anti-inflammatory, anti-fibrotic, anti-apoptotic and pro-angiogenic effects.[27, 31-33] Therefore, we also characterised cytokines and growth factors released by MSCs in the perfusion fluid during slaughterhouse pig kidney NMP in Chapter 5.

Complete regeneration cannot be achieved during a few hours of machine perfusion, however, regenerative processes can be initiated *ex vivo* while the kidney is being perfused on NMP and might further improve kidney quality and post-transplant outcomes.[34, 35] To determine if the addition of MSCs was safe and feasible during NMP, with beneficial effects on post-transplant graft function, a transplantation study was necessary. **Chapter 6** demonstrated that administration of MSCs during *ex vivo* NMP proved to be safe and feasible without a negative effect on perfusion characteristics or graft survival. Even though only a low amount of MSCs were identified in the kidney

after 14 days of transplantation, MSC therapy during NMP did not improve early renal function. It is possible that the MSC dose was too low and could be further optimised, that the follow-up time was too short or that MSC do not directly impact kidney function. Brasile *et al.* showed reduced inflammation and increased ATP synthesis with administration of 100 million MSCs during NMP of human discarded kidneys. Furthermore, there was evidence of regeneration with restoration of the cytoskeleton and mitosis.[36] Longer follow-up post-transplant would give us more insight in terms of fibrosis and other molecular changes in the kidney tissue. Nevertheless, the present study has provided a proof of concept for *ex vivo* MSC therapy during kidney NMP and we have gained a number of valuable first insights on targeted cell therapy for donor kidneys that were subsequently successfully transplanted.

Parameters and criteria to assess kidney quality during NMP are limited. The amount of urine production, together with renal blood flow and macroscopic appearance have previously been used to reflect a more viable graft and have only been validated for use after one hour of NMP in human discarded kidneys.[25] Slaughterhouse kidneys perfused on NMP in **Chapter 3** and **Chapter 5** had minimal urine production. Urine production in itself should not be considered a synonym of kidney function in an NMP setting as it may be influenced by the composition of the colloid in the perfusion solution,[37] and the variable nature of slaughterhouse kidneys makes it difficult to be conclusive. In **Chapter 6** no correlation between the amount of urine produced during NMP and graft function after auto-transplantation (data not shown in this thesis) were observed and this was also supported in a study by Kaths *et al.*[33] Furthermore, Ferdinand *et al*. assessed transcriptomic data from 33 DCD kidneys, undergoing one-hour of NMP as part of a randomized clinical trial (ISRCTN15821205), and showed that a high amount of urine production was associated with higher expression of immune pathway genes and that delayed graft function was more prevalent in kidneys that produced higher amounts of urine during NMP.[34] Standardisation of the NMP protocol and future pre-clinical and

clinical studies will bring further insight into assessing organs during NMP and their impact on early and long term graft function.

7.3 FUTURE PERSPECTIVES

The transplant community has implemented different strategies, all aiming to reduce IRI and increase and optimise utilisation of marginal donor organs for transplantation. Despite no improvement on early renal function of MSC therapy during NMP, MSC administration during NMP was successful, proving the feasibility of NMP as a method to deliver cellular therapy. However, challenges remain: in-depth investigation of mechanisms underlying the protective effects of MSCs is necessary to understand the beneficial effects of MSCs. Inconsistencies in MSC source *and in vitro* MSC isolation and expansion conditions also significantly limit the therapeutic values of MSCs.[38, 39] Standardisation of these factors is crucial to minimise differences caused by microenvironments and achieve reproducible results in upcoming pre-clinical and clinical research.[40, 41] Optimising the MSC survival rate after administration and enhancing their paracrine ability by pre-conditioning during cell culture can also further improve their regenerative potential and, consequently, achieve better results.[42] Furthermore, NMP with MSC-derived secretome can avoid any side effects of MSC-based therapy, including poor survival rate of the cells. MSC-derived secretome comprises growth factors, cytokines, chemokines, angiogenic factors and extracellular vesicles.[39, 43] These extracellular vesicles are loaded with proteins and genetic material and may themselves be used as a form of cell-derived therapy. [44, 45] On-going work beyond the scope of this thesis is planned. Proteomics of laser capture microdissection biopsies identified with and without MSCs from kidneys perfused on NMP will give us a better understanding how MSCs affect the kidney tissue during NMP and adjacent cells after homing.

Nevertheless, refinement of the NMP technique is necessary before the translation of NMP and targeted cell therapy during NMP into clinical practice. NMP technology is complex and still evolving, and to date there is no consensus on the optimal strategy and conditions for kidney NMP. The duration of NMP, perfusion pressure, and the exact

components of the perfusion solution are amongst the conditions that need to be agreed on and standardised. Future pre-clinical and clinical NMP studies will help provide a set of parameters to define kidney quality during NMP and their prediction on early and long-term graft function after transplantation.

In conclusion, optimisation of the NMP technique and a better understanding of the underlying protective effects of MSC is necessary to optimise and increase utilisation of marginal donor organs for transplantation, following cell-therapy.

REFERENCES

1. Heylen L, Jochmans I, Samuel U, et al. The duration of asystolic ischemia determines the risk of graft failure after circulatory-dead donor kidney transplantation: A Eurotransplant cohort study. *Am J Transplant*. 2018;18:881-889.

2. National Health Service Blood and Transplant. Organ Donation and Transplantation: Activity Report 2018/2019. Statistics and Clinical Studies, NHS Blood and Transplant; 2019.

3. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable but Resuscitated by Ex Vivo Normothermic Machine Perfusion. *Am J Transplant*. 2016;16:3282-3285.

4. Hamar M, Urbanellis P, Kaths MJ, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. *Transplantation*. 2018;102:1262-1270.

5. Weissenbacher A, Lo Faro L, Boubriak O, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. *Am J Transplant*. 2019;19:178-192.

6. Furuichi K, Shintani H, Sakai Y, et al. Effects of adipose-derived mesenchymal cells on ischemia-reperfusion injury in kidney. *Clin Exp Nephrol*. 2012;16:679-689.

7. Togel F, Zhang P, Hu Z, Westenfelder C. VEGF is a mediator of the renoprotective effects of multipotent marrow stromal cells in acute kidney injury. *J Cell Mol Med*. 2009;13:2109-2114.

8. Morigi M, Benigni A. Mesenchymal stem cells and kidney repair. *Nephrol Dial Transplant*. 2013;28:788-793.

9. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation*. 2018;102:35-43.

10. Thompson M, Mei SHJ, Wolfe D, et al. Cell therapy with intravascular administration of mesenchymal stromal cells continues to appear safe: An updated systematic review and meta-analysis. *EClinicalMedicine*. 2020;19:100249.

11. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol*. 2012;3:297.

12. de Witte, S. F. H., Luk F, Sierra Parraga JM, et al. Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. *Stem Cells*. 2018;36:602-615.

13. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? *Transpl Int*. 2015;28:657-664.

14. Kaths JM, Paul A, Robinson LA, Selzner M. Ex vivo machine perfusion for renal graft preservation. *Transplant Rev (Orlando)*. 2018;32:1-9.

15. Yong C, Hosgood SA, Nicholson ML. Ex-vivo normothermic perfusion in renal transplantation: past, present and future. *Curr Opin Organ Transplant*. 2016;21:301-307.

16. Schulz V. Clinical pharmacokinetics of nitroprusside, cyanide, thiosulphate and thiocyanate. *Clin Pharmacokinet*. 1984;9:239-251.

17. Baud FJ, Haidar MK, Jouffroy R, Raphalen JH, Lamhaut L, Carli P. Determinants of Lactic Acidosis in Acute Cyanide Poisonings. *Crit Care Med*. 2018;46:e523-e529.

18. Gad GF, el-Domany RA, Ashour HM. Antimicrobial susceptibility profile of Pseudomonas aeruginosa isolates in Egypt. *J Urol*. 2008;180:176-181.

19. Hosgood SA, Nicholson ML. First in man renal transplantation after ex vivo normothermic perfusion. *Transplantation*. 2011;92:735-738.

20. Harper S, Hosgood S, Kay M, Nicholson M. Leucocyte depletion improves renal function during reperfusion using an experimental isolated haemoperfused organ preservation system. *Br J Surg*. 2006;93:623-629.

21. Yoshida T, Prudent M, D'alessandro A. Red blood cell storage lesion: causes and potential clinical consequences. *Blood Transfus*. 2019;17:27-52.

22. Laing RW, Bhogal RH, Wallace L, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. *Transplantation*. 2017;101:2746-2756.

23. Matton APM, Burlage LC, van Rijn R, et al. Normothermic machine perfusion of donor livers without the need for human blood products. *Liver Transpl*. 2018;24:528-538.

24. de Vries Y, van Leeuwen OB, Matton APM, Fujiyoshi M, de Meijer VE, Porte RJ. Ex situ normothermic machine perfusion of donor livers using a haemoglobin-based oxygen carrier: a viable alternative to red blood cells. *Transpl Int*. 2018;31:1281-1282.

25. Aburawi MM, Fontan FM, Karimian N, et al. Synthetic hemoglobin-based oxygen carriers are an acceptable alternative for packed red blood cells in normothermic kidney perfusion. *Am J Transplant*. 2019;19:2814-2824.

26. Chen JY, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics (Sao Paulo)*. 2009;64:803-813.

27. Reinders ME, Leuning DG, de Fijter JW, Hoogduijn MJ, Rabelink TJ. Mesenchymal stromal cell therapy for cardio renal disorders. *Curr Pharm Des*. 2014;20:2412-2429.

28. Saat TC, van den Engel S, Bijman-Lachger W, et al. Fate and Effect of Intravenously Infused Mesenchymal Stem Cells in a Mouse Model of Hepatic Ischemia Reperfusion Injury and Resection. *Stem Cells Int*. 2016;2016:5761487.

29. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, et al. Mesenchymal stem cells induce an inflammatory response after intravenous infusion. *Stem Cells Dev*. 2013;22:2825-2835.

30. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol*. 2014;5:148.

31. Ninichuk V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int*. 2006;70:121-129.

32. Ueno T, Nakashima A, Doi S, et al. Mesenchymal stem cells ameliorate experimental peritoneal fibrosis by suppressing inflammation and inhibiting TGF-beta1 signaling. *Kidney Int*. 2013;84:297-307.

33. Chen YT, Sun CK, Lin YC, et al. Adipose-derived mesenchymal stem cell protects kidneys against ischemia-reperfusion injury through suppressing oxidative stress and inflammatory reaction. *J Transl Med*. 2011;9:51-51.

34. Hosgood SA, Hoff M, Nicholson ML. Treatment of transplant kidneys during machine perfusion. *Transpl Int*. 2020.

35. Hoogduijn MJ, Montserrat N, van der Laan, L. J. W., et al. The emergence of regenerative medicine in organ transplantation: 1st European Cell Therapy and Organ Regeneration Section meeting. *Transpl Int*. 2020;33:833-840.

36. Brasile L, Henry N, Orlando G, Stubenitsky B. Potentiating Renal Regeneration Using Mesenchymal Stem Cells. *Transplantation*. 2019;103:307-313.

37. Pool M. *Towards ex vivo repair of damaged donor kidneys.* University of Groningen; 2020.

38. Kusuma GD, Carthew J, Lim R, Frith JE. Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect. *Stem Cells Dev*. 2017;26:617-631.

39. Tsuji K, Kitamura S, Wada J. Secretomes from Mesenchymal Stem Cells against Acute Kidney Injury: Possible Heterogeneity. *Stem Cells Int*. 2018;2018:8693137.

40. Lukomska B, Stanaszek L, Zuba-Surma E, Legosz P, Sarzynska S, Drela K. Challenges and Controversies in Human Mesenchymal Stem Cell Therapy. *Stem Cells Int*. 2019;2019:9628536.

41. Mastrolia I, Foppiani EM, Murgia A, et al. Challenges in Clinical Development of Mesenchymal Stromal/Stem Cells: Concise Review. *Stem Cells Transl Med*. 2019;8:1135-1148.

42. Zhao L, Hu C, Zhang P, Jiang H, Chen J. Preconditioning strategies for improving the survival rate and paracrine ability of mesenchymal stem cells in acute kidney injury. *J Cell Mol Med*. 2019;23:720-730.

43. Vizoso FJ, Eiro N, Cid S, Schneider J, Perez-Fernandez R. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. *Int J Mol Sci*. 2017;18:1852. doi: 10.3390/ijms18091852.

44. Park KS, Bandeira E, Shelke GV, Lässer C, Lötvall J. Enhancement of therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. *Stem Cell Res Ther*. 2019;10:288-3.

45. Bruno S, Tapparo M, Collino F, et al. Renal Regenerative Potential of Different Extracellular Vesicle Populations Derived from Bone Marrow Mesenchymal Stromal Cells. *Tissue Eng Part A*. 2017;23:1262-1273.

Appendix

COVID-19 impact statement
Chapter involvement
List of publications
About the author

COVID-19 IMPACT STATEMENT

The COVID-19 pandemic had minimum impact on the work described in this thesis. Due to the restriction to the laboratory caused by COVID-19, I was unable to complete all the planned analyses for Chapter 5. The aim of chapter 5 was to determine the optimum MSC dose following administration to the kidney during NMP and to track the location of MSCs throughout organ perfusion. In addition, this chapter had the goal to study the 'secretome' released by MSCs during kidney NMP. When the laboratory re-opened, I finished the Y-chromosome PCR experiments and ELISA of the secreted factors PGE2, IL-10, VEGF-A and angiopoietin-1. However, due to delivery delays and limited access to the appropriate Luminex instrument, I was not able to complete a Luminex assay of NMP perfusate to analyse the secretion of analytes IL-6, IL-8, IL-1ß, TNFα and IFN-γ.

CHAPTER INVOLVEMENT

Chapter 3

- Design of the study;
- Performance of all of the experiments (retrieval, back-table, HMP, NMP);
- Collection of the data (sample collection, sample preparation for biochemistry analysis and microbiology);
- Analysis and interpretation of the data (tables, figure and statistics).

Chapter 4:

- Design of the study;
- Performance of the experiments (back-table and NMP);
- Collection of the data (sample collection);
- Analysis and interpretation of the data (tables, figure and statistics).

Chapter 5:

- Design of the study;
- Performance of all of the experiments (MSC culture and labelling, retrieval, back-table, HMP, NMP);
- Collection of all the data except for confocal microscopy (sample collection, sample preparation for biochemistry analysis, flow cytometry, PCR including DNA isolation, ELISAs);
- Analysis and interpretation of the data (tables, figure and statistics).

Chapter 6:

- Design of the study;
- Performance of the experiments (back-table procedure, HMP, NMP);

- Collection of the data (sample collection, sample preperation for biochemistry analysis);
- Analysis and interpretation of the data (tables, figure and statistics).

LIST OF PUBLICATIONS

Sierra Parraga JM, **Rozenberg K**, Eijken M, Leuvenink HG, Hunter J, Merino A, Moers C, Møller BK, Ploeg RJ, Baan CC, Jespersen B, Hoogduijn MJ. Effects of Normothermic Machine Perfusion Conditions on Mesenchymal Stromal Cells. Published in Frontiers in Immunology on April 10, 2019. DOI:10.3389/fimmu.2019.00765.

Lohmann S*, Pool M*, **Rozenberg KM**, Keller AK, Moers C, Møldrup U, Møller BK, Lignell S, Krag S, Sierra-Parraga JM, Lo Faro ML, Hunter J, Hoogduijn MJ, Baan CC, Leuvenink H, Ploeg RJ, Eijken M, Jespersen B. Mesenchymal stromal cell treatment of donor kidneys during ex-vivo normothermic machine perfusion: A porcine renal autotransplantation study. Published in American Journal of Transplantation on December 31, 2020. DOI: 10.1111/ajt.16473. * Authors contributed equally to the manuscript.

Lignell S, Lohmann S, **Rozenberg KM**, Leuvenink HGD, Pool MBF, Lewis KR, Moers C, Hunter JP, Ploeg RJ, Eijken M, Møldrup U, Krag S, Baan CC, Møller BK, Keller AK, Jespersen B. Improved Normothermic Machine Perfusion After Short Oxygenated Hypothermic Machine Perfusion of Ischemically Injured Porcine Kidneys. Published in Transplant Direct on January 15, 2021. DOI: 10.1097/TXD.0000000000001108.

Ogbemudia AE, Hakim G, Dengu F, El-Gilani F, Dumbill R, Mulvey J, Sayal K, Prudhomme T, Mesnard B, **Rozenberg K**, Lo Faro L, James T, Oliver J, Sharples E, Mittal S, Johnson P, Friend PJ, Ploeg R, Hunter J, Branchereau J. Development of ex situ normothermic reperfusion as an innovative method to assess pancreases after preservation. Published in Transplant International on August 26, 2021. DOI: 10.1111/tri.13990

ABOUT THE AUTHOR

The author of this thesis, Kaithlyn Mirugia Rozenberg was born on 18 January 1992 in ’s-Hertogenbosch, the Netherlands. In 2011 she finished her secondary school studies from Colegio Arubano, school for H.A.V.O. and V.W.O., in Oranjestad, Aruba. In the same year, she moved back to the Netherlands to follow the bachelor degree Biology and Medical Laboratory Research at Hanze University of Groningen. In 2016 she followed an internship at the Surgical Research Laboratory of the University Medical Center Groningen on an elective project. Here she investigated the activation of the complement system during kidney perfusion of pigs under the supervision of Professor H.G.D Leuvenink. During her internship, she discovered her interest in scientific research of organ transplantation. After that, she investigated the biological mechanisms contributing to delayed graft function and to different lengths of recovery after deceased donor kidney transplantation for her undergraduate project under the supervision of Professor R.J Ploeg and Dr L. Lo Faro at the Nuffield Department of Surgical Sciences at the University of Oxford, United Kingdom. She graduated with a bachelor with distinction in 2017. This has led to her interest in kidney transplantation and in the same year she was accepted to follow a DPhil trajectory at the Nuffield Department of Surgical Sciences at the University of Oxford under the supervision of Professor R.J. Ploeg and Mr J.P. Hunter.