Towards ex vivo repair of damaged donor kidneys

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DOI:
10.33612/diss.130535652

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SUMMARY

Chapter 1 provides a general introduction on the subject of kidney transplantation. The process of ischaemic injury and techniques that are used to optimise preservation of donor kidneys are described. The concept of normothermic machine perfusion (NMP) is introduced and a possible intervention, the addition of mesenchymal stromal cells (MSCs), is discussed.

In **Chapter 2** an overview of what is known about the reparative and regenerative effect of MSCs in different fields such as orthopaedics, wound healing and acute kidney injury is provided. From studies in these fields it can be concluded that MSCs could potentially play an important role in the outcome of marginal quality kidney transplantations by releasing factors that increase angiogenesis, reduce fibrosis and inflammation, increase the number of regulatory T cells and improve renal function. Concerns regarding the safety of the administration of MSCs are also addressed and the optimal timing of their administration, just prior to the peak of inflammation, is suggested. In renal transplantation, a procedure which goes hand in hand with immunosuppression, most research has focussed solely on the immunomodulatory properties of MSCs. Pre-clinical studies are necessary to determine if MSCs could be administered to an isolated organ prior to transplantation during a period of normothermic machine perfusion.

The results in **Chapter 3** revealed that it is possible to administer MSCs to a porcine kidney during NMP in such a way that a portion of these cells remain detectable and structurally intact. Using immunohistochemistry and fluorescence microscopy it was established that the MSCs were retained in the kidney inside the lumen of the glomerular capillaries. Additional experiments with iron-labelled MSCs in an MRI scanner revealed that there was a very inhomogeneous distribution of the cells in the renal cortex.

In **Chapter 4** bone marrow and adipose tissue derived MSCs were infused during prolonged NMP of porcine kidneys. The addition of MSCs to an ischaemically damaged kidney during NMP led to a reduced expression of injury markers lactate dehydrogenase and neutrophil gelatinase-associated

lipocalin. Furthermore, the addition of MSCs resulted in an increased secretion of interleukin-6, interleukin-8 and hepatocyte growth factor into the perfusate.

The results in **Chapter 5** demonstrated that the delivery of ten million MSCs during normothermic ex-vivo perfusion of porcine kidneys is feasible and safe without negatively affecting perfusion haemodynamics or reperfusion after transplantation in a pig autotransplantation model. However, this dose of MSCs did not improve early renal function of an ischaemically damaged kidney during fourteen days of follow up, when compared to NMP alone prior to transplantation.

Four different perfusion solutions were evaluated during seven-hour NMP of ischaemically damaged porcine kidneys in **Chapter 6**. Three of the four solutions were pre-existent and one of the solutions was designed by our group to exert a physiological colloid osmotic pressure and contain electrolytes in physiological concentrations. Perfusion with all solutions proved feasible but the solution with physiological electrolyte concentrations yielded superior results. These results indicated that the composition of an NMP perfusion solution has a significant impact on many aspects of ex vivo normothermic perfusion and that a small change in the composition of the perfusate can lead to considerable differences in kidney function, tissue injury and perfusion dynamics.

Chapter 7 revealed that an isolated porcine kidney can also be perfused normothermically with allogeneic porcine red blood cells (RBCs) during seven hours without causing more injury than autologous RBCs. The use of human RBCs as an oxygen carrier did lead to significantly more damage of the kidney during NMP. This led to the conclusion that, to enable NMP of porcine kidneys with an RBC-based perfusion solution in an autotransplantation model, allogeneic porcine RBCs are the best choice.

In **Chapter 8** it was identified that an oxygen carrier is necessary during normothermic machine perfusion and that HBOC-201 can be used when solely assessing its oxygen carrying capacity. However, renal function is inferior to kidneys perfused with red blood cells and high methaemoglobin levels in the HBOC-201 kidneys indicate that it is probably less suitable for periods of prolonged NMP.

GENERAL DISCUSSION

To date, renal transplantation is regarded as the best treatment for patients with end stage kidney failure. However, the availability of kidney transplantations is limited as there is a persistent shortage of organ donors. In an attempt to shorten the waiting time by enlarging the deceased donor organ pool, the use of donor organs from donation after circulatory death donors and extended criteria donors plays a significant role [1]. However, the majority of these organs are of suboptimal quality, making preservation of function, and preferably also repair of these kidney grafts of vital importance for an effective transplantation. Pre-transplant normothermic machine perfusion at 37°C allows for metabolic activity and provides a platform for therapeutic interventions to an isolated organ prior to transplantation in the absence of an allogeneic immune system [2]. Normothermic ex vivo kidney perfusion has not been implemented into clinical practice but the first clinical trials have started. However, a standardised perfusion solution has not been established, nor is there a consensus regarding the optimal perfusion conditions. This thesis focuses on optimising the perfusion solution used during NMP and investigates MSCs as a possible regenerative and reparative therapy during NMP.

As discussed in **Chapter 2** no studies had focussed on administering MSCs to the kidney ex-vivo, prior to transplantation. From the few published studies in humans it became evident that intravenous infusions of MSCs could have a beneficial effect not only on immunomodulatory aspects but potentially also on renal function [3,4]. These intravenously infused MSCs will most likely never reach the kidney, but infusing them during NMP could lead to these cells being physically present in the kidney [5]. Also, this would require lower doses of MSCs which is favourable from a safety perspective [6]. As this cellular intervention during NMP had never been performed before, we chose to determine to which renal structures these cells localise and what would be an appropriate dose in **Chapter 3**. The MSCs were localised in the lumen of glomerular capillaries, but only when infused numbers were as high as ten million MSCs. A multiplex analysis on the samples from the experiments with unlabelled MSCs (n=3) was also performed and revealed a significantly higher concentration of interleukin-6 (IL-6) and interleukin-8 (IL-8) in the kidneys treated with MSCs versus the control group. As cytokine release in the additional experiments with pre-labelled MSCs

(n=5) could not be detected, the exact implication of this finding remained unclear *(data not shown in this thesis)*. Therefore, the choice was made to perform a new series of experiments in **Chapter 4** with unlabelled MSCs and this confirmed our findings regarding IL-6 and IL-8 in the earlier experiments. The fact that cytokines could not be detected when using labelled MSCs, is most likely the result of cytotoxic effects of fluorescent cell labelling [7]. IL-8 is regarded as an inflammatory cytokine with pro-angiogenic properties which could facilitate revascularisation [8]. IL-6 is predominantly regarded as a pro-inflammatory cytokine but it also possesses ant-inflammatory properties. It can suppress the secretion of several pro-inflammatory cytokines and there is evidence that it could also play a regenerative role [9,10]. Hepatocyte growth factor levels were also higher in the experiments in which kidneys were treated with MSCs and high levels are associated with improved late graft function [11]. Furthermore, damage markers were lower indicating that MSC treated kidneys sustained less injury. However, to determine if the administration was also safe posttransplant and if these ex vivo findings were associated with beneficial effects on short and longer term graft function a transplant study was necessary, described in **Chapter 5**. Administering MSCs during NMP proved to be feasible and did not negatively affect perfusion characteristics nor reperfusion after transplantation. Although a small number of MSCs remained traceable up to 14 days posttransplant, no effect on early renal function as a result of this cell therapy could be detected. This could be the result of a too low dose of MSCs or a relatively short follow up time. Further analyses will be performed to analyse possible differences in gene expression and cytokines release. Nonetheless, these first results in pigs in vivo indicate that the use of non-preconditioned MSCs during NMP will most likely not lead to the desired reparative and regenerative effects posttransplant.

For the autotransplantations in **Chapter 5** pigs had to be sacrificed purely for their blood in order to be able to use porcine red blood cells during NMP. This made us aware of the fact that not much NMP research had focussed on the composition of the perfusion solution nor on the use of different oxygen carriers. In **Chapter 6** the comparison between different perfusion solutions led to the conclusion that the composition of the perfusion solution, specifically the use of a colloid or vasodilator had a significant impact on perfusion characteristics and injury markers during NMP. Kidney function also partly relies on a balance

in electrolytes [12,13]; therefore it is important to specify the purpose and duration of NMP beforehand as it could call for a different need of additions during perfusion. These findings had a large impact on the perfusion solution used during the autotransplantations in **Chapter 5**. This particular solution was tested extensively and alterations were made before the definitive composition was established. As it is important to try to reduce the number of experimental animals, **Chapter 7** and **Chapter 8** focussed on using alternatives to allogeneic porcine red blood cells during NMP. It proved feasible to perfuse porcine kidneys with readily available human RBCs, but kidneys sustained significantly more damage, possibly as a result of a xeno-reactive response [14]. Hemopure (HBOC-201), a synthetic haemoglobin based oxygen carrier, has been tested extensively in liver NMP but not in renal machine perfusion [15,16]. Kidneys perfused with HBOC-201 during six hours showed significantly impaired renal function in comparison with kidneys perfused with allogeneic RBCs. Although injury marker levels were relatively low in the HBOC-201 group, high methaemoglobin levels as a result of the absence of nicotinamide adenine dinucleotide hydrogen (NADH)-dependent enzyme, indicated that HBOC-201 is less suitable for periods of prolonged machine perfusion. Furthermore, concerns regarding nephrotoxicity of synthetic haemoglobin based oxygen carriers have not fully been alleviated [17].

FUTURE PERSPECTIVES

Although therapy with non-preconditioned MSCs did not yield the desired effects, NMP as a delivery method for a cellular therapy was successful. From research in other fields we can conclude that there are several factors that limit (pre)clinical efficacy of MSC based therapy, the most important one being a poor survival rate of the administered MSCs [18]. Major causes of MSC death are inflammation, generation of reactive oxygen species and ischaemia. In our experiments we established that the number of MSCs declined rapidly during NMP. Heat shock pre-treatment of MSCs reduces the apoptosis rate and enhances reparative effects of MSCs in harsh environments such as that exists during machine perfusion [19]. Also, incubation with chemical compounds or cytokines has been proven to promote MSC survival. Finally, there is also the possibility of genetically modifying MSCs to overexpress certain (antiapoptotic) genes to improve the survival rate. Another possibility to bypass the problem of poor MSC survival rates is to solely administer the secretory proteins of MSCs during NMP, the so-called secretome. This secretome consists of cytokines, chemokines, growth factors as well as extracellular vesicles [20]. These extracellular vesicles appear to be responsible for the therapeutic effects of MSCs. When using the current markers to define MSCs, the cells exhibit inter-population heterogeneity. Using single-cell RNA sequencing one could possibly determine a certain MSC subtype or a cocktail of a defined population of subtypes, which demonstrate effectiveness during cellular therapy. Using these aforementioned strategies to limit death of MSCs or solely use its secretome, might still present us with an optimistic future regarding MSC therapy during NMP prior to kidney transplantation.

However, many aspects of normothermic machine perfusion remain under debate. To date it remains unclear to what extent ex vivo renal physiology resembles in vivo physiology and what markers are most important. If NMP is to be implemented clinically, several issues such as the duration of NMP, the perfusion pressure and the exact composition of the perfusion solution need to be resolved. In order to perform a standardised perfusion and representative quality assessment of the metabolically active kidney it is of vital importance to agree on these relatively basic aspects of perfusion. Only after the development of a standardised protocol, might we be able to unravel markers during NMP that

predict kidney function posttransplant. Potentially, we could then specifically target these markers with cell or drug therapeutic strategies.

Combining machine perfusion with imaging techniques such as magnetic resonance imaging (MRI) provides us with the opportunity to monitor perfusion real-time. When adding MRI-active compounds, oxygenation and cellular metabolism during ex vivo perfusion could be assessed, thereby enabling prediction of quality and function of an organ prior to transplantation. The ultimate goal would be to assess, target repair and improve the quality and function of every kidney that is to be transplanted.

In conclusion, normothermic machine perfusion remains a promising technique although many aspects still need to be refined in order for it to reach its full potential. Hopefully, in combination with the implementation of the new organ donation law in the Netherlands, this will result in the transplantation of more and better quality kidneys in the nearby future.

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