Brain dead donor graft deterioration and attenuation with N-octanoyl dopamine preconditioning

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CHAPTER 9

Summary, Discussion and Future Perspectives

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Summary

After immunosuppression enabled successful transplantation, solid organ transplantation has become the treatment of choice for terminal chronic endorgan diseases. However, donor organs are susceptible to injury during the transplantation process initiating inflammation and subsequent graft deterioration. The latter cause a reduction in organ function. Lungs with substantial functional impairment, determined by the ratio of arterial partial oxygen pressure and fraction of inspired oxygen (PaO_2/FiO_2) below 300 mmHg, are considered not suitable for lung transplantation. These lungs are at a higher risk for primary graft dysfunction and impaired graft survival if used for transplantation. Findings in kidney transplantation are comparable. Nevertheless, lungs seem to be more susceptible to injury and at particular risk for limited graft survival, as illustrated in figure 1, in comparison to other solid organ transplantations.

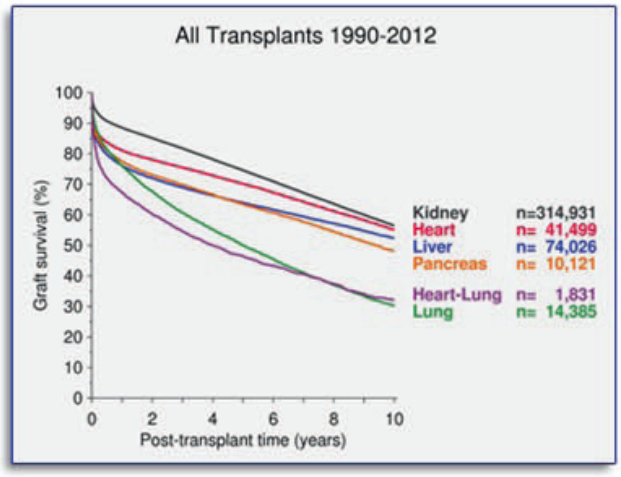

Figure 1. Graft survival of lung transplants is inferior to other solid organ transplants.
Source: www.ctstransplant.org (Accessed 06.01.2015)

In the *Introduction* of this thesis, initiation of the systemic immune response in patients with cerebral insult is described. This is followed by the onset of brain death and subsequent induction of hemodynamic, endocrinologic and metabolic changes which exacerbate the immune response in the lung and kidney allograft. The deleterious mechanisms of cold ischemia/reperfusion contribute synergistically to the injury. Inadequate ventilation strategies, if applied, enhance the local and systemic immune response. The extent of immune activation during these processes is considered to determine the graft survival. Brain dead donor preconditioning strategies are

introduced which limit the immune response during the transplantation process and improve graft survival. Finally, N-octanoyl dopamine, a recently developed preconditioning agent is introduced followed by the aims of this thesis.

In *Chapter 1*, the effect of dopamine and N-octanoyl dopamine on acute kidney injury was tested using an ischemia reperfusion injury model in rats. While saline (NaCl), dopamine and N-octanoyl dopamine treated animals showed no difference at day one after ischemia/reperfusion in kidney function, only the NOD treated animals had significantly improved kidney function at day 3 and 5 after induction of acute kidney injury. It seemed that NOD attenuated NFκB activation, however, the effect was less pronounced than in vitro. On a histological level, all groups showed at day 1 after induction of acute kidney injury cytolysis in the proximal tubule segment 3 of the pars recta. At day 5, NOD treated animals presented substantially less tubular epithelial deterioration; it even seemed that it initiated restoration of the cell integrity. Supplementary in vitro data showed that N-octanoyl dopamine inhibited dose dependent activation of NFκB and VCAM-1 expression, while higher dosages of dopamine were needed to show an effect. Independent of these findings, N-octanoyl dopamine but not dopamine was found to excite TRPV1 specific dorsal root ganglion neurons, also found in the kidney. TRPV1 has been implicated to attenuate or aggravate acute kidney injury. However, the direct involvement has not been tested in this setting.

Heart transplants are extremely susceptible to prolonged cold storage. Since dopamine preconditioning improved the clinical outcome after heart transplantation, in *Chapter 2*, it was tested if dopamine and its lipophilic derivative, N-octanoyl dopamine protect neonatal rat cardiomyocytes (NRCMs) from cold storage. After determining an adequate cold storage time, it has been shown that dopamine and NOD dose dependently prevented the release of LDH. Correlating to this, the two agents also decreased ATP depletion. In both circumstances, NOD was by 20 times more potent than dopamine on a molar basis, while there was no difference in their maximal achievable protection. After rewarming, only preconditioned NRCMs regenerated similar ATP values compared to cells not exposed to cold storage. In line with this, NOD also prevented LDH release in rat donor hearts during cold storage. However, not only the viability but also the functionality was restored by treatment. While only 5% of the untreated cells regained contractility 90% of the preconditioned cells regained spontaneous contractility which remained constant over 24 hours. Subsequently, also isoprenalin induced cAMP formation was only in the untreated NRCMs substantially impaired, while in the preconditioned cells cAMP levels were comparable to NRCMs not exposed to cold storage. The structural entities that mediate cryoprotection of N-octanoyl dopamine are redoxactivity and a certain degree of hydrophobicity.

To evaluate the potentially anti-inflammatory effect of N-octanoyl dopamine and to elucidate the underlying mechanism, the effect of NOD on a TNF-α induced immune response in HUVECs was investigated in *Chapter 3*. A genome wide gene expression screening was performed. Downregulation of a wide range of κB regulated genes as chemokines and adhesion molecules was observed and was confirmed with qPCR. Inhibition of adhesion molecule expression was also observed on protein level, accompanied by the functional consequence that PBMCs adhesion to HUVECs was reduced. It has been also shown, that NOD can regulate the expression of chemokines and adhesion molecules through NFκB activation. This was independent of the cytosolic NFκB inhibitor IκBα. However, a general p65 reduction and the inhibition of its SER276 phosphorylation, needed for the recruitment of coactivators, was observed. HO-1, substantially induced by NOD on gene and protein level, has been described to inhibit NFκB activation in literature. However, in this study involvement of HO-1 or its transcription factor Nrf-2 on the inhibition NFκB were excluded by blockage with siRNA. These findings were confirmed by a de novo protein synthesis independent inhibition of NFκB. Structural requirement for the anti-inflammatory effect of NOD on VCAM-1 expression were the same as in *Chapter 2* for protection against cold ischemia induced injury.

N-octanoyl dopamine preconditioning improved immediate graft function in renal transplantation and prevented cell damage during heart transplantation in a rat model. In *Chapter 4*, a rat lung transplantation model was used to investigate the effect of N-octanoyl dopamine as preconditioning agent on brain dead donor lung transplants. Physiological parameters did not change significantly during the donor or recipient phase, in contrast to an increasing pulmonary inspiratory pressure for both brain death groups before and after transplantation. After brain death and warm ischemia in both donor groups, an induction in pro-inflammatory gene expression was found. After transplantation, ICAM-1 was lower in NOD preconditioned transplants after transplantation compared to the NaCl treated BD group. In general, a trend for decreasing gene expression in TNF-α, CINC-1 and VCAM-1 was observed. However, it was only significant in the NOD pretreated allografts, with the exception of VCAM-1. Histological analysis revealed a significant increase in total lung injury in both groups overtime, but there was no difference between the two groups.

Mechanical ventilation in cerebral injury and brain death has been suggested to exacerbate the immune response. However, the ideal mechanical ventilation strategy has so far not been defined nor the underlying mechanism that leads to acute respiratory dysfunction. For that reason, the effect of a considered to be lung-protective ventilator strategy by using LV_T/OLPEEP was compared to traditional

HV_T/LPEEP in the presence and absence of brain death in *Chapter 5*. LV_T/OLPEEP resulted in an improved survival compared to HV_T/LPEEP. In the non-brain dead animals no substantial physiological differences were found between the ventilation modalities. In contrast to this, in the presence of brain death, the oxygenation in the HV_T/LPEEP group was significantly impaired compared to the LV_T/OLPEEP group. Independent of ventilation strategy the mean arterial pressure (MAP) was substantially lower in the six hour brain dead animals than in the non-brain dead groups. The LV_T/OLPEEP groups received in general more fluids, but only reached significance in the brain dead group compared to HV_T/LPEEP non-brain dead. Histological examination showed a more pronounced inflammatory reaction in HV_T/LPEEP than LV_T/OLPEEP groups, and overinflation was only pronounced in HV_T/LPEEP brain death. Subsequently, total lung injury score was generally higher in the HV_T/LPEEP compared to LV_T/OLPEEP, but also only reached significance in the HV_T/LPEEP groups between non-/brain death. Genome wide gene expression screening revealed an increased gene expression in the HV_T/LPEEP groups compared to LV_T/OLPEEP, particularly in inflammatory genes. Comparing HV_T/LPEEP with LV_T/OLPEEP on RNA level showed increased IL-6, CINC-1 and Angiopoietin-4 expression in brain death but not in non-brain death groups.

In acute respiratory distress syndrome, comparable to transplantation process induced lung injury, it has been shown that mechanical ventilation not only has a local impact but may also cause distant organ injury in the kidney. For this reason, *Chapter 6* elucidates the effect of a lung-protective ventilator strategy by using LV_T/OLPEEP compared to HV_T/LPEEP in the presence and absence of brain death on the donor kidneys procured from the same animals used in *Chapter 5*. Irrespective of the ventilation strategy, blood pressure in the non-brain dead groups was higher than in the brain dead groups. In the presence of brain death, independent of the ventilation strategy, systemic TNF-α and IL-6 levels were increased. In line with this, clinical chemistry parameters were independent of ventilation. Brain death resulted in renal dysfunction as demonstrated by the two renal markers creatinine and urea that were substantially increased. This was accompanied by a significant decrease in total protein concentration, as well as urine osmolarity and potassium levels in these animals. In genome wide gene expression screening, it became evident that brain death had a more pronounced effect on gene expression than ventilation. Variance in gene expression was higher in HV_T/LPEEP groups. From a pathway analysis in the LV_T/OLPEEP it was shown that the inflammatory processes were most influenced. As a confirmation of the gene array data, 9 genes were chosen and confirmative RT-PCR was performed. With the exception for Caspase 1, we could show a clear upregulation in brain death. In line with this, neutrophil granulocyte infiltration

into the glomeruli was more pronounced in brain death. However, no difference was observed in the infiltration of the proximal tubule segment 3 of the pars recta.
Clinical data is inconclusive on the influence of etiology of brain death on lung transplantation outcome. I*n Chapter 7,* two commonly used rat brain death induction models, slow and fast, were compared for half an hour to four hours of brain death period. For both brain death models a distinct hemodynamic pattern was observed during BD induction. The slow BD induction model had a significantly lower MAP curve during induction than the fast model, while there was no difference 4 hours after brain death induction. However, in the fast model this was accompanied by a respective higher need of noradrenalin. Immediately after fast brain death induction six animals were lost due to a failure of hemodynamic resuscitation and presented fulminant lung edema at dissection. Gene expression of proinflammatory cytokines (TNF-α, IL-6, VCAM-1 and MCP-1) changed over time but did not differ between the models. On a histological level a higher histological lung injury was found in the fast brain death induction model. The increased total lung injury score was the result of a more pronounced hemorrhagic infarction, edema and pleura infarction. The lung injury correlated with the release of the heart injury markers CK-MB and troponin.
In contrast to lung transplantation, in renal transplantation not only brain death (BD) but also its etiology has been identified as a risk factor for inferior immediate transplantation outcome. However, this is considered to be the result of other contributing risk factors. Therefore, it was investigated in *Chapter 8* whether the duration for intracranial pressure increase, mimicking differences in brain death etiology, has a differential effect on the donor kidney and liver. In the slow model severe hypotension occurred for approximately 10 minutes during BD induction, in contrast to a brief hypertensive period in the fast model. Hemodynamics did not differ after BD induction except for a higher need of hemodynamic support in the fast BD induction model. Slow BD induction led at the investigated time points to a decrease in kidney function and an initial systemic rise of IL-6 compared to the fast ICP increase. While in the kidney, IL-6 and MDA levels were increased at 4 hrs after slow BD induction compared to the fast model, in the liver the same accounted for TNF-α, BAX/BCL-2 ratio and protein caspase 3 expression. However, the liver showed no decrease in function. The pronounced deleterious effect of the slow BD induction model could be the result of a double hit. The hypotension during BD induction in the slow model is considered to be the first hit inducing acute kidney injury, exacerbated by the second hit, brain death. Subsequently, kidney and liver injury are amplified compared to the fast BD induction model.

Discussion

In the past, the potentially beneficial effect of dopamine observed in organ transplantation has been investigated [1-6]. The studies presented in the first part of this thesis focus rather on the recently developed and more potent lipophilic derivative N-octanoyl dopamine (NOD) [7]. It is questionable whether the in vivo studies represent the full potential of N-octanoyl dopamine, since the given dosage of N-octanoyl dopamine was not chosen relying on pharmacokinetic studies but was given as equimolar dosage to dopamine [8]. Dopamine dosage was limited to a low-dose treatment (<10 μmol/kg of body weight) in vivo due to its hemodynamic action [9, 10], which N-octanoyl dopamine does not exert [7]. Therefore, administration of higher N-octanoyl dopamine dosages might be beneficial in vivo. In vitro, the maximal protective effect against cold ischemic injury is comparable between both agents, dopamine and NOD. However, at lower concentrations the equimolar dosage of N-octanoyl dopamine was superior to dopamine in its salutary effect [11]. The same was observed for the anti-inflammatory potential of both agents [12]. Thus, N-octanoyl dopamine is the favorable agent, especially since only NOD was protective in the setting of acute kidney injury (AKI) [7, 11, 12], correlating to clinical findings that showed no benefit for dopamine treatment in acute kidney injury [13-15]. On the other hand, dopamine and NOD were given as bolus injections in the setting of acute kidney injury (AKI) while it has been shown that the time of dopamine donor preconditioning in humans positively correlated with independency of dialysis and recovery of kidney function by day 7 after kidney transplantation [8]. Also, dopamine might have been quickly degraded by its degrading enzymes [11]. N-octanoyl dopamine, in contrast might have been degraded less due to the greater mitochondrial uptake and the cytoplasmic localization of the dopamine degrading enzymes, which possibly also degrade NOD [7, 16]. This would also explain why even after removal of NOD endothelial cells were protected against cold ischemia and low ATP levels were sustained [17]. On the other hand, for capsaicin, with which NOD shares some properties (Figure 2), it has been reported that it may not be removed by repeated washing steps. The effect of capsaicin could only be reversed by addition of BSA suggesting a strong mitochondrial binding [18], which was not investigated for NOD, yet.

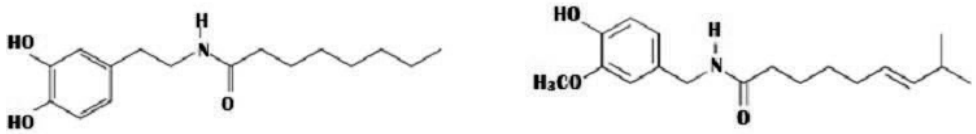

Figure 2. N-octanoyl dopamine (left structural formula) and capsaicin (right structural formula)

Another difference between dopamine and NOD is that only NOD has the ability to activate transient receptor potential vanilloid 1 (TRPV1), though to a lesser extent than the TRPV1 agonist capsaicin [12]. The literature is controversial regarding the effect of TRPV1 activation in proinflammatory processes. However, in different organ systems including the lung, it is protective against ischemia induced injury [19-21]. In TRPV1 gene knockout animals, the absence of the ion channel of nociceptive neurons resulted in impaired recovery after the ischemic insult and abrogated the protective effect of ischemic preconditioning [22, 23]. While they do not elucidate the exact mechanism, this is at least partially the result of a TRPV1 induced vasodilatation, accompanied by an increased organ function [24, 25]. This was not tested in the setting of ischemia induced acute kidney injury [25], but vasoconstriction was induced pharmacologically [24]. In contrast to the TRPV1 mediated protection, it has been shown that dopamine may even increase vasoconstriction in patients with acute renal failure [26]. This explains the finding that dopamine treatment does not improve AKI [14, 15]. Nevertheless, in the AKI model described in this thesis, changes in perfusion were not investigated. Also the question of whether or not TRPV1 activation is part of the protective mechanism was not studied. Interestingly, the beneficial effect of NOD in AKI was not found immediately but delayed [12]. In contrast to this, NOD brain dead donor preconditioning resulted in improved kidney function one day after kidney transplantation [27], possibly as a result of the difference in bolus and continuous application. These forms of application probably differ in the ratios between subcellular uptake and degradation. It is possible that this is why the efficacy of dopamine preconditioning correlates with the time of preconditioning in human kidney transplantation, as mentioned above [8].

Nonetheless, while dopamine fails to be protective in AKI it has been shown to be protective in heart and kidney transplantation [8, 14, 15, 28]. This suggests that the protective effect of NOD is not solely TRPV1 mediated [27]. In kidney transplantation, especially renal grafts with prolonged cold storage benefited from the donor preconditioning with an improved graft survival after kidney transplantation [8]. Hearts procured from the dopamine preconditioned donors led to superior 3 year survival in the recipients [28]. The success of heart transplantations depends on short cold ischemia periods [29]. It was concluded from these results that dopamine may exert its protective effect by preventing cold ischemia/reperfusion induced

injury, as previously suggested [2, 3, 30]. When this hypothesis was tested, both dopamine and NOD prevented cold ischemia induced cell injury and ATP depletion in cardiomyocytes, and led to the regeneration of ATP levels after rewarming [11]. Although the direct effect of NOD and dopamine on cell death was not investigated one may assume that the reduced release of LDH is the result of reduced cell death [11]. Turning point of cell survival and cell death are mitochondria [31]. The extent of cell injury and death during organ preservation and reperfusion depends on the preservation of mitochondrial integrity [32, 33]. One dysfunctional mitochondrium may initiate the oscillation of all mitochondria of one network and result in organ dysfunction [34, 35]. For dopamine it was already found that it may prevent the loss of mitochondrial membrane potential, decrease the depletion of -SH reducing equivalents, prevent calcium influx into the mitochondria and retard ATP depletion, all processes which are associated with reduced cell injury [2, 36]. Except for the retarded ATP depletion, it has not yet been investigated whether NOD has the same effects as dopamine [11]. However, reduced cell injury and restoration of ATP levels after rewarming suggest that both agents may prevent injury to the electron transport chain and preserve the oxidative phosphorylation (Figure 3), possibly as a result of limited oxidative stress at rewarming/reperfusion [11, 27, 37].

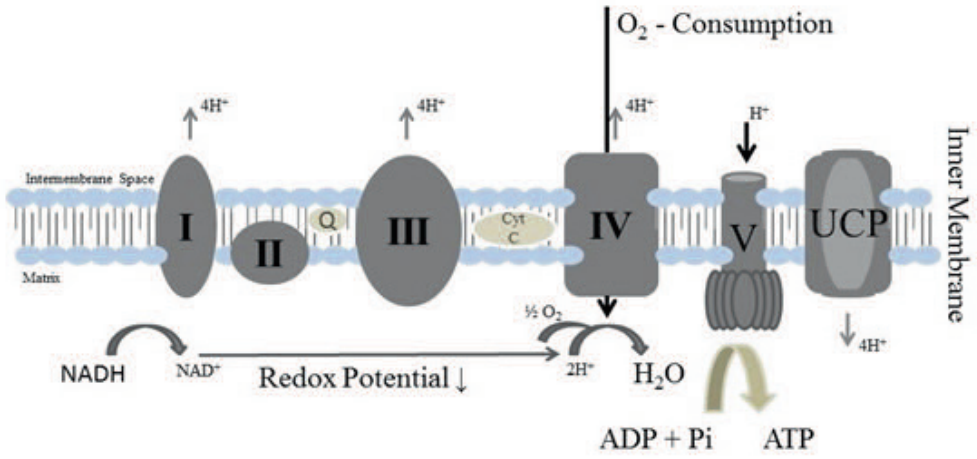

Figure 3. Electron transport chain (ETC) in a physiological coupled state - Oxidative Phosphorylation: During the passage of electrons down the electron transport chain, from a high redox potential (NADH) to a low redox potential (O_2) hydrogen ions are pumped from the mitochondrial matrix into the intermembrane space by complex I, III and IV. The electrochemical proton gradient across the inner mitochondrial membrane facilitates passive proton diffusion through complex V (ATP synthase) and the energy is bound to ADP by phosphorylation [38]. Passing on of unpaired electrons can result in superoxide production at complexes I and III. In an uncoupled state protons may also leak through uncoupling proteins (UCP) and other proteins, the energy is released by thermogenesis [38].

In independent preliminary experiments it was observed that the addition of 100μM N-octanoyl dopamine to isolated kidney mitochondria from five healthy Dark Agouti rats, in the presence of saturated ADP concentrations, resulted in a reduced oxygen consumption. This led also to a significant change in respiratory control ratio (RCR = state 3/oligomycin induced state 4) (Figure 4), while the oxygen consumption rate was not changed when substrates for other complexes of the electron transport chain were applied.

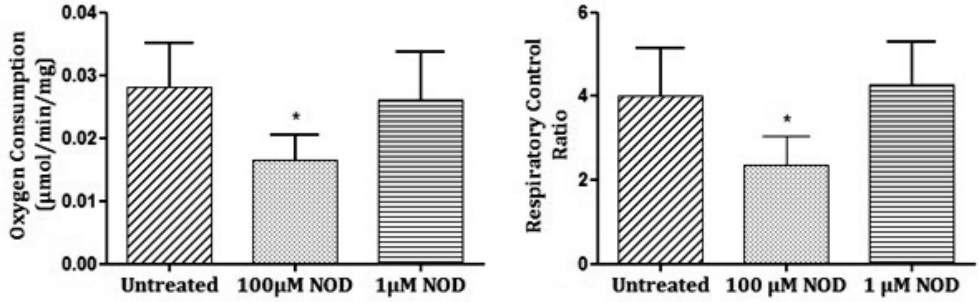

Figure 4. N-octanoyl dopamine reduces the oxygen consumption of mitochondria in the presences of ADP. Kidneys for mitochondria isolation were procured after harvest of the abdominal aorta. The oxygen consumption of the isolated mitochondria was measured using a Clark-Electrode. Typical substrates were used to investigate each complex and the coupling state. No significant differences other than the ones presented were found. Bars represent five independent experiments ± standard deviation. Statistics were performed using unpaired t-test compared to untreated mitochondria (* $p < 0.05$).

In this experimental setting ATP levels in the isolated mitochondria were not measured. However, these findings and the reduced ATP levels found in long term NOD stimulation suggest a reduced activity of the ATP-synthase [17]. Nonetheless, oxidative phosphorylation and electron transport chain are closely coupled [39], thus an inhibition of the ATP-synthase would result in a general reduction of oxygen consumption by each complex of the electron transport chain (Figure 3). Unchanged oxygen consumption by the electron transport chain and reduced ATP production in the presences of NOD are therefore probably the result of uncoupling [40, 41]. The opinion on uncoupling is rather controversial [38, 42]. Mild uncoupling is considered to be beneficial and may by a reduction of mitochondrial membrane potential reduce mitochondrial calcium uptake, as previously observed for dopamine [2, 43, 44]. Furthermore, it has been associated with a reduced formation of reactive oxygen species (ROS) formation at reperfusion [38, 45]. Thus, many effects found for dopamine or NOD could be explained by this mechanism. On the other hand, for capsaicin (Figure 2) it has been shown that there is a striking difference between its effects on mitochondrial respiration in isolated liver mitochondria dependent on the

present substrates. In the presence of the NAD-linked substrates the mitochondrial respiration for state 3 respiration (ADP), uncoupling (DNP) and $CaCl_2$ was dose dependently inhibited by capsaicin. Furthermore, capsaicin limited in uncoupled mitochondria the reverse ATPase activity. In contrast to this, capsaicin led in the presences of succinate to mitochondrial uncoupling and only slight inhibition of oxidative phosphorylation [18]. The authors suggest that there might be the potential for energy-conservation, as *Chapter 2* suggests for Dopamine and NOD [11, 18]. The uncoupling, which we found in the absence of succinate does not correlate however this may either be due to the fact that the organs were exposed to about 20 minutes of cold storage for transport before start of isolation. Or since the here presented data were recorded in isolated kidney mitochondria.

Nevertheless, in a preliminary study on cytokine induced mitochondrial changes under the influence of NOD it was observed that NOD reduced the cytokine induced mitochondrial membrane potential in normal rat kidney epithelial cells (NRK). This was accompanied by a reduction in cytokine induced superoxide production at 100 μM and 10 μM NOD compared to cytokines only (Figure 5). While there was no influence on the general ROS production (CellROX probe; data not shown), suggesting a mitochondria specific reduction in ROS formation.

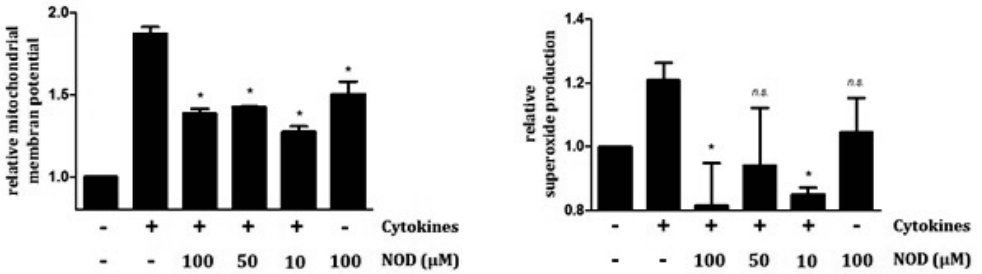

Figure 5. Differences in relative mitochondrial membrane potential (left Figure) and relative superoxide production (right Figure) in normal kidney cells under the influence of cytokines and/or NOD. For this cells were incubated either with JC-1 or MitoSOX probe. Subsequently cells were stimulated with a cytokine mix (IL-1β, IL-6 and TNF-α, each of a final concentration of 100ng/ml) and/or indicated concentration of NOD. Dead cells were excluded using SYTOX green and nuclei were visualized by Hoechst. Analysis were performed using a fluorescence microscope and analyzed using TissueFAXS-Tissue Quest analysis software (Tissue Gnostic). Bars represent four independent experiments ± standard deviation. Statistics were performed using unpaired t-test compared to cytokine only stimulation (n.s. = non-significant, * $p < 0.05$).

Protecting mitochondrial integrity and preventing cold ischemia reperfusion induced cell death could be the origin of the reduced allograft immunogenicity and the cause of reduced LDH levels after transplantation using N-octanoyl dopamine preconditioned brain dead donor grafts from rats [27, 33, 46].

Additionally, N-octanoyl dopamine also mediates an NFκB dependent anti-inflammatory effect in vitro [47]. Recently, it was suggested that this is mediated by the induction of the unfolding protein response (UPR) [17, 48]. Whether this in vitro effect is part of the reduced immune response in renal grafts of brain dead donors found after NOD preconditioning still needs to be elucidated [27, 49, 50].

The immune response during brain death and ischemia reperfusion determines the outcome after lung transplantation [51-53]. Unfortunately, in *Chapter 4*, data are not convincing that NOD inhibits the induction of the immune response after lung transplantation. This could be the result of the chosen time point for the investigation. To investigate the efficacy of NOD in the study described in C*hapter 4* the time point was chosen based on in vitro data [47]. At an earlier time point, we could have tested another potentially beneficial effect of NOD, due to some shared properties with Capsaicin. Capsaicin has been shown to reduce oxidative stress in lung transplantation and to improve the outcome [12, 21]. This is of importance, since ischemia in the lung is different compared to other organ systems. The presence of alveolar oxygen leads to hypoxia, and not anoxia, with increased risk for production of high reactive oxygen species levels [52]. Therefore, oxidative stress is yet another key risk factor for limited graft survival in lung transplantation. Thus, limiting oxidative stress could prevent the occurrence of primary graft dysfunction [52, 54]

Another difference to other organ transplantations is mechanical ventilation. Though sometimes clinically essential, mechanical ventilation can significantly contribute to lung injury and even death of the patient [55, 56]. In the process of transplantation, or in the comparable acute respiratory distress syndrome (ARDS), the utilization of mechanical ventilation with low tidal volume and PEEP, considered as lung protective strategy, has significantly prevented the progression of lung deterioration [56-58]. For this reason, studies in this thesis were performed using previously described successful protective ventilation strategies [58-60]. *Chapter 5* demonstrates that the effect of mechanical ventilation in brain death conditions on the lung is comparable to what was previously found in acute lung injury and acute respiratory distress syndrome [56].

However, mechanical ventilation can not only influence the quality of the lung itself. It has been shown that mechanical ventilation with a tidal volume of ~10ml/kg of body weight contributes to the development of multi-organ dysfunction compared to lung protective ventilation with a tidal volume of ~6ml/kg of body weight [61-63]. The multi-organ dysfunction is expected to be the result of a mechanical ventilation induced injury and subsequently induced organ cross talk, most commonly described between the lung and kidney [64-66]. Therefore, it was surprising that the influence of brain death on kidney graft deterioration in *Chapter 6* seems to have

been greater than the influence of the two different ventilation strategies, in contrast to the findings in ARDS [62]. An explanation for this could be that there was no difference in cytokine spill over as described by other studies comparing ventilation strategies [58, 63]. But, in *Chapter 6*, it was also not investigated if the used mechanical ventilation strategies lead to hormonal or local hemodynamic differences which may harm the kidneys [65, 66]. On the other hand, hemodynamic instability was observed for both BD groups, independently of the ventilation strategy, in *Chapter 5* and 6, which may have interfered with the immune response [67-70].

The cross-talk in BD could be of clinical importance since in critically ill patients the combined occurrence of acute lung injury (ALI) and acute kidney injury (AKI) leads to a mortality of 80% [71]. This is in contrast to the about 40% mortality in AKI patients and about 30% mortality in ALI patients [72-74]. A study from the acute respiratory distress network revealed that the development of AKI (defined as a rise in serum creatinine of >50% from baseline over the first four study days) in patients with ALI resulted in a 180-day mortality rate of 58% compared to a mortality of 28% in those ALI patients who did not develop AKI [75]. The organ cross-talk but also the increased mortality are probably a result of an increase in systemic cytokines, dysfunctional coagulation system, coupled with decreased kidney clearance and migration of immune competent cells [75, 76], which can also be found in brain death.

In C*hapter 8*, it was concluded that the hypotensive period during slow BD induction resulted in the onset of AKI. The AKI in the slow BD induction model probably induced a distant organ injury, as discussed for the liver in C*hapter 8*. However, it could also be the reason that there was no difference between the lungs of the two brain death models in *Chapter* 7. It was expected that the sudden hypertensive period during fast brain death induction, with a more pronounced catecholamine storm, would lead to a more pronounced immune response compared to the slow BD induction model [77, 78]. While the structural injury was as expected more pronounced in the fast BD induction model, the investigated gene expression was comparable between the two models. It has been suggested that there may be a distinct pathophysiology leading to ALI with preceding AKI [79], but this was not investigated in *Chapter 7* and *8*. Partly, because the resemblance of AKI induced lung injury is similar to the resemblance of brain death induce lung injury. In both, vascular permeability, cytokine gene expression, cellular infiltration, structural changes and gas exchange are impaired [49, 53, 70, 78-86].

Therefore, it is not surprising that Oto et al. [87] showed a correlation between transplantation outcome for organs procured from one multi-organ donor. However, it also questions the practice of procuring a single organ from a donor when other

organs do not fulfill transplantation criteria. Thus, it would be interesting if a comparable donor to the slow brain death induction model could not benefit from an AKI focused preconditioning. Early treatment could maybe not only limit AKI but also the distant organ injury [88, 89], preventing a potential additional hit. Particularly, since hypotension in the organ donor before onset of brain death is not a rare phenomenon due to development of shock and medication [90-93]. Thus, a future preconditioning strategy could exist of identifying donor specific risk factors and treating the donor specifically for these risk factors.

Future Perspectives

Donor preconditioning strategies in transplantation currently solely aim at brain dead donor conditioning [13]. However, particularly in the field of lung transplantation, donors after cardiac arrest are increasingly being used to meet the demand in donor organs [94]. Initially, ex vivo lung systems were developed with the aim to assess the lung function of cardiac arrest donors before transplantation, without placing the recipient at risk [95]. Nevertheless, it became an important tool to treat brain dead donor lungs not meeting the criteria for lung transplantation [96]. In particular, since the lung in the absence of the recipient's immune system can restore its cellular integrity even without additional intervention [96, 97] and the usage of hyperosmolar perfusion solution supports alveolar fluid clearance [98]. After ex vivo lung perfusion (EVLP) of carefully selected lungs, the majority of previously not eligible lungs becomes available for transplantation [99], with comparable outcome to recipients receiving standard donor lungs [99, 100]. In donors after cardiac death it particularly creates an opportunity to remove pulmonary emboli which may affect transplantation outcome substantially [96].

Currently, only glucocorticoids are added to the system as anti-inflammatory treatment [101], even though inflammation before transplantation greatly contributes to ischemia reperfusion injury in the transplant [102, 103]. Thus, EVLP represents a future opportunity for anti-inflammatory lung conditioning especially for lungs of donors after cardiac arrest (DCD), extending the time of treatment. Most importantly, since treatment can be delivered target specific and dependent on the reason for treatment, at higher dosage without putting other organs at risk [96]. Secondly, lungs that are resistant to the treatment may be identified and excluded without harm to the recipient [96].

N-octanoyl dopamine could be one of the pharmacological opportunities for intervention. The protective effect of N-octanoyl dopamine against ischemic injury and its NFκB inhibiting property may be a useful agent to treat lungs during allocation and in the EVLP system [104]. Importantly, because dopamine already protected lung grafts from prolonged cold storage, one would expect that NOD with its supremacy might even extend this time period [1]. On the other hand, the postconditioning effect of N-octanoyl dopamine has not been tested yet. Especially the combination of NFkB inhibition and its potential to activate TRPV1 suggest it to be a potential agent in lung ischemia reperfusion injury [21, 104].

Another very promising and multimodal approach, and clinically already used in the treatment of myocardial infarction [105], are mesenchymal stem cells (MSCs). MSCs have moved into the focus as therapeutic for a wide variety of inflammatory

diseases [106-108]. Their potent immune-modulatory effect is on one hand based on shifting the immune response toward tolerant, anti-inflammatory phenotypes [108] and on the other hand supporting the parenchyma regeneration [106, 109, 110]. This broad therapeutically range is probably the origin of the great potential that has already been shown for mesenchymal stem cell treatment in the EVLP system in the setting of experimental acute lung injury [107, 111], commonly found before and/or after transplantation. In the EVLP system it might be a unique opportunity to treat passenger macrophages [112] and prevent further injury possibly by the induction of IL-10 expression [113].

Since brain death was identified as a major risk factor for inferior transplantation outcome in renal transplantation, strict donor management protocols have been implemented into the clinic to prevent organ deterioration [114, 115]. In some centers supplementary preconditioning strategies have been added [8, 28, 116, 117], as well as lung protective mechanical ventilation strategies [58]. While these strategies resulted in an increased number of organs available for transplantation and improved immediate graft survival [58, 116, 117], long term graft survival remains unchanged [118, 119]. This is probably the result of on one hand not reversing the immune response but solely inhibiting downstream proinflammatory mediators during preconditioning [47], on the other hand is the immune response simply tempered with immunosuppression in the recipient [120-122] instead of inhibiting or even better reversing inflammatory processes [123]. Moreover, the pre-injured donor organ, considerably susceptible to injury [124], is placed into the pro-inflammatory environment of the recipient [125]. The pro-inflammatory environment may amplify the systemic effect of the local and systemic immune response during reperfusion [52, 126], with subsequent progression of graft deterioration and comorbidities in the recipient [127-129].

According to the 'Danger Hypothesis' by Polly Matzinger [130] graft deterioration is the result of the immune system responding to injury and danger molecules rather than foreign or not-self [130]. It explains the finding that in a brain death model upon injury, in the absence of additional proinflammatory stimuli, a pronounced inflammatory reaction is observed and that serum or cross-circulation from these animals causes a similar response in healthy animals [86]. By now, a number of these danger molecules have been defined and are summarized as Danger Associated Molecular Patterns (DAMPs) [46, 131]. It has been shown that DAMPs are released upon injury and cell death [33, 131]. Both occur during cerebral injury [132], which all donors except living donors sustain, probably exaggerated at the onset of brain death [133]. Comorbidities and transplantation associated factors as ventilation

[134] and ischemia reperfusion amplify release of DAMPs [135, 136]. For that reason, necrosis and not apoptosis, results in an impairment of the lung graft after transplantation [137]. The lung seems to be an organ that is particularly susceptible to secondary injury upon DAMP release and neutrophil leukocyte migration [131].
In the field of transplantation it was shown that only in brain dead donors the DAMP HMGB1 is expressed compared to living organ donors. In both, kidney and lung transplantation, HMGB1 expression and activation of pattern recognition receptors were correlated to graft dysfunction [133, 138]. However, it has been shown that HMGB1 has only a weak proinflammatory activity [139], thus the correlation might be rather the result of parallel to HMGB1 released DAMPs. Mitochondrial DAMPs are considered to be the source of the strong proinflammatory response. Since mitochondria still share many properties with their prokaryotic precursors [140], they are being recognized as foreign. The proinflammatory potential and the recruitment of leukocytes [141, 142] is considered to be the missing link between tissue injury and sterile inflammation [143]. In systemic inflammatory response syndrome the extent of mitochondrial DNA release can be correlated to outcome and risk for multi organ dysfunction [141].
DAMPs induce the immune response by being recognized by pathogen recognizing receptors (PRR), which upon binding activate the downstream mediators NFκB, mitogen activated protein kinase and type I interferon pathways. Subsequently, gene expression of cytokines and chemokines, regulated by these mediators, is induced and causes the migration of immune cells [144]. Stimulation of Toll-like receptors, a group of PRR, has been shown to prevent tolerance towards the transplant while inhibition resulted in improved allograft acceptance [133, 145]. Preventing PRR activation in the donor and recipient might therefor improve long term outcome. Interestingly, DAMPs can abrogate tolerance and promote rejection in a PRR dependent mechanism resulting in the activation of the adaptive and innate immune system [146].
However, it has also been suggested that chronic inflammation, considered to lead to chronic rejection in the recipient [147], is associated with persistent release of DAMPS and redox alterations [148]. This might be the result of an increased oxidative stress after impairment of the electron transport chain. Particularly the mitochondrial DNA in comparison to nuclear DNA is susceptible to oxidative stress induced break down and release [37, 140, 149, 150], possibly inducing a vicious circle. Thus, in the future, focusing on mitochondria specific treatment and prevention of the release of subcellular fragments could become a great tool to improve graft survival.

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