Chapter 9

General discussion
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9.1 Summary

Solid organ transplantation, as a treatment modality for patients with end-stage organ disease, has become possible by adequate improvements in surgical techniques, organ preservation and immunosuppression. Organs procured from deceased donors have already suffered from damage as a consequence of brain death. Some of these donors are hemodynamic instable, which might lead to disturbances in organ perfusion (1, 2). Optimal donor management is therefore of critical importance to the success of transplantation. Apart from the deleterious event of brain death, organ allografts are further injured when in transit to the recipient. Static cold storage is the most widely used approach for organ preservation, yet allograft quality and function deteriorate with the increasing cold preservation time (3-5). The studies presented in this thesis were aimed to understand why prolonged cold preservation leads to endothelial cell injury, how this injury subsequently influences vascular function and might provoke inflammation, and finally to find strategies to prevent endothelial damage during static cold storage. With respect to the latter, we focussed on two different compounds, i.e. dopamine and related compounds and on carbon monoxide releasing molecules (CORMs).

Donor preconditioning might be a meaningful strategy to maintain organ allograft quality (7, 8). Most of the published experimental studies on donor preconditioning were aimed at reducing ischemia/reperfusion (IR) injury, a complex interrelated sequence of events that classically involves the vascular endothelium and activated leukocytes (6-9). Interestingly, donor dopamine usage is significantly associated with a reduced risk for delayed graft function, a reduced incidence of acute rejection episodes and an improvement of long-term graft survival (10-13). Usually, dopamine is given to the donor because of hemodynamic instability, but the beneficial effect of dopamine on transplantation outcome is independent of blood pressure stabilization. Recent studies from our group have demonstrated that dopamine can protect cells and tissues against cold preservation injury, which might explain, at least partially, the salutary effect of donor dopamine usage (14, 15).

Although different organs can withstand different time periods of cold ischemia before significant damage occurs, in general tissue damage increases, and hence organ quality deteriorates, with increasing cold ischemia time (3, 4, 14). As an example, cold ischemia time longer than 8-10 hrs for heart and lung allografts is considered to be undesirable while for renal allografts cold ischemia time is usually longer (3, 4, 16). One of the clinical complications of organ allografts that have been subjected to prolonged cold ischemia time is
the loss of the endothelial barrier function (17-20). *In vitro* dopamine can not completely prevent endothelial barrier dysfunction during cold preservation. Nevertheless, this treatment restores barrier function rapidly upon rewarming (15).

In **Chapter 2**, we employed a rat model to test the hypothesis that dopamine treatment ameliorates tissue damage associated with hypothermic preservation and reperfusion of lung allografts. As tissue oedema is a consequence of barrier dysfunction, resulting in an increase in lung weight and an increase in pulmonary arterial and inspiratory pressure (21), we used these parameters as read out for barrier dysfunction and assessed the effect of different time periods of cold storage followed by reperfusion in an isolated ventilated and perfused lung model. In line with our previous *in vitro* findings (15), this study showed that a cold preservation time longer than 6 hrs resulted in profound oedema formation. Dopamine pre-treatment significantly inhibited oedema formation as was reflected by a decrease in peak inspiratory pressure (PIP), pulmonary arterial pressure (PAP) and lung weight compared to lungs obtained from untreated rats. These findings have also been reported for human lung allografts that were not suitable for transplantation (22). In addition, dopamine pre-treatment significantly abrogated CINC-1 production, the upregulation of adhesion molecules during reperfusion was likewise inhibited. Since pulmonary oedema largely contributes to poor lung function after transplantation (23), implementation of donor dopamine in donor management might be considered even in hemodynamic stable donors. Our group finished recently a prospective randomized multi-centre study on the beneficial effect of donor dopamine usage in hemodynamic stable donors on delayed graft function after renal transplantation. Analysis of the data revealed that dopamine treatment significantly reduced the risk for delayed graft function, this effect was more pronounced when cold ischemia time was long. Whether other organ allografts also have been benefited from dopamine is currently being investigated in this study.

It also remains to be addressed if the effect of dopamine in the isolated and ventilated lung model was receptor mediated or not. Several studies have indicated that the Na/K ATPase plays an important role in fluid handling in lungs. Stimulation of β-adrenergic receptors activates the Na/K ATPase (24). Therefore, unlike in the *in vitro* studies, the effect of dopamine might be receptor-mediated. Further studies are required to elucidate this issue.

In order to understand the beneficial effect of dopamine on cold preservation injury, in **Chapter 3**, we employed an *in vitro* model and investigated the changes in intracellular ATP concentration, redox balance and Ca^{2+} homeostasis during hypothermic preservation and how
this was influenced by dopamine pre-treatment. Our study demonstrated that increased oxidative stress, reflected by the occurrence of a redox imbalance, changes in Ca\(^{2+}\) homeostasis and depletion of intracellular ATP are major cellular events that are associated with hypothermic preservation. As demonstrated previously, dopamine pre-treatment renders endothelial cells transient resistance to hypothermia-induced damage. Dopamine pre-treatment furthermore prevents the occurrence of a redox imbalance and mitochondrial [Ca\(^{2+}\)] overload. Also ATP depletion is retarded during cold preservation in dopamine treated endothelial cells. Based on the results, we postulate that mitochondrial [Ca\(^{2+}\)] overload is a key event in cold preservation injury and that perhaps prevention of this by dopamine might explain the protective effect of dopamine. As the protective effect of dopamine is strictly redox-dependent, we must assume that the increase in intracellular [Ca\(^{2+}\)] during hypothermic preservation is initiated by a redox imbalance. It has been demonstrated in other studies that depletion of glutathione (GSH) activates ryanodin receptors in the endoplasmic reticulum and consequently Ca\(^{2+}\) is released from intracellular stores (25, 26). Subsequently activation of store-operated calcium channels will result in an influx of Ca\(^{2+}\) from the extracellular milieu (27-29). This sequel of events is compatible with two important observations described in Chapter 3: firstly dopamine prevents depletion of SH reduction equivalents, and secondly depletion of Ca\(^{2+}\) from the intracellular stores by thapsigargin treatment can overcome the protective effect of dopamine. However, the release of Ca\(^{2+}\) from the intracellular stores is not sufficient to drive cell death, because thapsigargin treatment can not overcome the protective effect of EDTA. The reason why ATP is depleted much faster in untreated endothelial cells might be the consequence of ATP exhaustion to maintain Ca\(^{2+}\) homeostasis or might be the result of cell leakage.

This study did not address why depletion of SH reduction equivalents occurred during cold preservation. However, as deferoxamine (30, 31) or HO-1 over-expression (32) have shown to be also protective during cold preservation, we postulate that an increase in the chelatable intracellular iron pool (30) might give rise to hydroxyl radicals by Fenton chemistry. These radicals, in turn might convert GSH to GSSG. In the presence of intracellular dopamine, the hydroxyl groups of the catechol moiety of dopamine can scavenge hydroxyl radicals and thus prevent oxidation of GSH.

Inasmuch as our study indicates that prevention of a Ca\(^{2+}\) influx during cold preservation might be beneficial, it does not imply that Ca\(^{2+}\) should be completely omitted from
preservation solutions. Omission of Ca\(^{2+}\) from the extracellular milieu can lead to opening of unselective cation channels and hence to membrane depolarization (33, 34). In fact, addition of small amounts of Ca\(^{2+}\) to preservation solutions has proven to be more protective in experimental liver transplantation (33, 34).

If we appreciate that dopamine can prevent intracellular Ca\(^{2+}\) accumulation in a redox-dependent manner, the next question to be addressed is then what are the structural requirements for dopamine to be protective. Since we already have demonstrated that the protective effect of dopamine is not receptor-mediated, occurs relatively fast, is not specific for dopamine but also holds true for other dihydroxyphenolic compounds and does not require de novo protein synthesis (14, 15), we postulated that structural entities within these compounds mediate protection. Further studies to elucidate the structure-function relationship with respect to protection against cold preservation damage are described in Chapter 4. In this study we analysed the protective potency of a series of related compounds that either differ in their relative hydrophobicity or in the position of the dihydroxyl groups. We could demonstrate that the relative hydrophobicity, expressed as LogP value, tightly correlates with the efficacy of protection. The importance of the relative hydrophobicity is that it facilitates cellular uptake. However, hydrophobicity alone is not sufficient to mediate protection, because protection also requires reducing substituents on the benzene ring. This is emphasized by the fact that only ortho- or para- positioned hydroxyl groups on the benzene ring will yield protective compounds. Meta-dihydroxybenzene or benzoic acids do not convey protection even when the hydrophobicity was increased by covalent coupling of a fatty acid. Ortho- and para-dihydroxybenzenes are known to be strong reducing agents due to the ease of quinone formation, while the meta-dihydroxyl derivatives have no radical scavenging potential since oxidation of the hydroxyl groups does not occur under normal conditions.

These two structural entities found in our study together with the data presented in Chapter 3 may promote the understanding of the mechanisms involved in preservation injury of organ allografts and might eventually lead to the use of more effective compounds for organ preservation that are devoid of hemodynamic action. Since acylated dopamine derivatives and alkyl dihydroxybenzamides have been demonstrated to be anti-inflammatory agents (35, 36), clinical application of these compounds seems to be a promising strategy not only for prevention of pre-transplantation injury in donor organs but also to ameliorate inflammation after transplantation.
During cold preservation degradation of cytosolic proteins has been reported (15, 37). Proteolysis is generally mediated by activation of a variety of enzymes. This might occur as a consequence of ATP depletion and an increase in cytosolic calcium. In particular, activation of the Ca\(^{2+}\)-dependent proteases, i.e. calpains, seems to be corollary to the imbalance in intracellular calcium homeostasis during cold storage (38, 39). The ubiquitin proteasome system (UPS) (40) and caspases (41) might be equally involved in proteolysis during cold preservation. In Chapter 5, we investigated to what extent cold storage changes the cellular proteome and if activation of these proteolytic pathways occurred in HUVEC during cold storage. Our findings demonstrated that indeed activation of the calpain pathway and the ubiquitin proteasome system occurs under cold preservation conditions. In dopamine pre-treated cells activation of the calpain pathway did not occur, however, ubiquitination was not influenced by dopamine. Neither proteasome inhibitors nor an inhibitor of calpain 1 were able to prevent cell death during hypothermic preservation. These data thus suggest that although proteolysis might occur during cold preservation, inhibition of single proteolytic pathways is not effective to prevent cell death during cold storage. It must be noted, that inhibition of multiple proteolytic pathways was not tested in this study.

According to danger hypothesis, tissue injury of allografts is not only recognized in the recipient by the immune system, but also evokes immune activation (42). Hypothermic preservation induces cell necrosis and tissue damage in a time-dependent fashion (14, 43). Nevertheless it is not yet clear how hypothermic preservation might cause immune activation. In Chapter 6, we therefore investigated if soluble factors were released upon hypothermic preservation that might signal to the innate immune system. We focussed on HMGB1 and adenosine, two important molecules known to modulate innate immunity (44-47). We showed that expression of HMGB1 is completely lost in endothelial cells subjected to cold preservation, but not when the cells were rendered resistant by dopamine pre-treatment. HMGB1 was bioactive, indicated by the findings that supernatants of damage cells were able to upregulate the expression of adhesion molecules and the production of IL-8 in an HMGB1-dependent fashion. However, the unexpected finding that the same supernatants were also able to downregulate TNF-\(\alpha\) production in LPS stimulated whole blood assays suggested that other factors were likely present. Subsequent experiments revealed that adenosine is also released during hypothermic preservation and that adenosine was responsible for the inhibitory effect on TNF-\(\alpha\) production. While the release of HMGB1 only occurred in damaged cells, the release of adenosine was independent of cell damage. Because adenosine
not only modulates innate immunity but also improves endothelial barrier function (48, 49), adequate donor treatment might thus prevent the release of HMGB1 but might preserve the action of adenosine on the endothelium.

Because HMGB1 and adenosine are released during cold preservation and organ allografts are flushed before implantation, it can be argued that these in vitro findings have no relevance for organ transplantation. Endothelial cells express the receptor for advanced glycation end-products RAGE (50), a putative receptor for HMGB1, which might already bind its ligand during cold preservation. Subsequent warm reperfusion then can cause receptor activation leading to phenotypic and functional changes in endothelial cells. The relevance of adenosine release during hypothermia is indeed questionable as adenosine is added to some preservation solutions, e.g. UW.

Leukocyte extravasation, as occurring during inflammation, is a highly regulated process in which adhesion molecules expressed on the endothelial cell-surface interact with their ligands on leukocytes (51). In addition, leukocytes are recruited to the sites of inflammation via endothelial production of chemokines. Up-regulation of these inflammatory mediators facilitates leukocyte migration and subsequently amplifies inflammation (52). Understanding of the mechanisms that control inflammation are of utmost importance to identify putative targets for the development of anti-inflammatory drugs. In recent years, a new class of molecules, termed carbon monoxide releasing molecules (CORM), has been described that are capable of liberating CO under appropriate conditions. In particular, CORM-3 [tricarbonylchloro(glyconato)ruthenium(II)] and CORM-A1 (sodium boranocarbonate), which both are fully water-soluble, rapidly liberate CO when dissolved in physiological solutions (53). In Chapter 7, we studied how CORM-3 modulates the expression of adhesion molecules on endothelial cells and if HO-1 mediated-perpetuation was involved. Our results showed that CORM-3 consistently inhibited the upregulation of VCAM-1 and E-selectin on TNF-α stimulated HUVEC, partly due to the deactivation of NFκB. Interestingly, downregulation of VCAM-1 and E-selectin expression by CORM-3 even occurred when CORM-3 was added 24 hrs after TNF-α stimulation. Sustained expression of VCAM-1 required the continuous presence of TNF-α. TNF-α removal was more effective in the reduction of VCAM-1 mRNA level, but VCAM-1 protein was down-regulated more rapidly when CORM-3 was added compared to TNF-α removal. This suggests that the modulation of VCAM-1 by CORM-3 most likely also occurred post-transcriptionally. CORM-3 itself up-regulated HO-1
in an Nrf2 dependent fashion, however, HO-1 expression did not significantly contribute to the effect by CORM-3. Neither in HO-1- nor in Nrf2-siRNA treated HUVEC the efficacy of CORM-3 to down-regulate VCAM-1 expression was lost.

As discussed already, prolonged cold ischemia is significantly associated with initial organ non-function and late transplant loss (54, 55). Chronic transplant vasculopathy remains a leading cause for chronic organ loss after transplantation (54, 56). Intimal hyperplasia and arterial obliteration are prominent features of chronic vasculopathy. In Chapter 8, we investigated the beneficial effect of CORM-3 on hypothermia-induced injury in HUVECs and its influence on vascular remodelling and vascular function in syngeneic rat aorta transplantation model. Our data showed that CORM-3 protected endothelial cells against hypothermia-mediated injury via liberation of CO. Cold storage induced endothelial denudation and intercellular gap formation in isolated rat abdomen aortas, while this was prevented when CORM-3 was added to the preservation solution. Similarly, vascular function was significantly impaired after 24 hrs of cold storage. This was largely due to impairment of endothelia-mediated NO production, since the relaxation response by addition of SNP was not affected after cold storage. In line with the observation that CORM-3 prevented endothelial denudation, we could demonstrate that addition of CORM-3 during cold storage also better preserved vascular function. Two months after aorta transplantation in syngeneic rats, neo-intima was significantly increased in aortas that were subjected to 24 hrs of cold preservation. When CORM-3 was added during preservation, neo-intima formation was significantly abrogated.

9.2 Conclusions and future perspectives

Based on the finding that kidney transplants from unrelated living donors are performing exceedingly well despite poor HLA compatibility (57), the concept of pre-transplantation tissue injury as an important risk factor for long-term allograft survival has been more appreciated in recent years. Since cold preservation tissue damage is a major cause of pre-transplantation injury, prevention of this type of damage is regarded as an effective approach to improve transplantation outcome (3-5). Damage of the vascular endothelium is a prominent feature of cold ischemia (58). This may facilitate inflammation through the release of HMGB1 and through impairment of the endothelial barrier function.
To meet the growing demand of organ allografts and relative stable supply of cadaveric organ donors, the use of organs from so called “marginal” donors has increased dramatically. Yet, organs obtained from these donors might be more susceptible to cold preservation injury, resulting in a higher rate of delayed graft function (59) and possibly in a decrease in allograft-survival (60). Therefore better ways to preserve organs, or as suggested from the data presented in this thesis, new strategies to prevent cold preservation injury are warranted.

Although in the past dopamine has been frequently used in hemodynamic instable donors, in more recent years dopamine has been largely abandoned at the ICU because the beneficial effect of dopamine on renal function could not be demonstrated (61). However, our recently finished prospective randomized multi-centre study, together with the data presented in this thesis strongly favors the use of dopamine in donor management. Nevertheless, the question whether we should use hemodynamic active compounds to achieve a better organ quality after prolonged preservation is genuine and deserves more attention. Since the beneficial effect of dopamine on cold preservation injury is independent of its hemodynamic action, the use of dopamine-like compounds that are devoid of blood pressure stabilizing effects, e.g. n-octanoyl-doapmine (NOD) would be more appropriate in hemodynamic stable donors. Also in non heart beating donors, the use of positive inotropic agents is not desirable. Renal allografts from these donors are more prone to develop delayed graft function after transplantation.

Inasmuch as our in vitro data suggest that the use of NOD as donor pre-conditioning strategy might be more favorable than dopamine, the hydrophobic character of NOD makes its application more difficult. Ways to deliver NOD to donor organs are therefore of equal importance in future research. One possible strategy to overcome this problem is the use of so-called semifluorinate alkanes (SFA) emulsions. SFA’s are clinically used in eye surgery as temporal tamponade. Interestingly they have the propensity to act as solvent for a number of hydrophobic agents (62). Preliminary data from our group indicate that NOD can be dissolved in SFA without losing its protective effect on cold preservation injury in vitro (unpublished data). Moreover SFA emulsions have anti-inflammatory properties as they inhibit TNF-α production in whole blood assays. We could also demonstrate in a model of warm ischemia-mediated acute renal failure that intravenous application of these emulsions significantly inhibited the rise in serum creatinine and that recovery of renal function was much faster when SFA’s were applied prior to ischemia (unpublished data).
Maintaining good organ quality during cold preservation can also be achieved by addition of CORM to the preservation solution. We have not studied the efficacy of this approach in vascularized organs, and thus the conclusions reach in this study must be interpreted with some pre-caution.

In conclusion, this thesis demonstrates the detrimental influence of cold preservation and unveils some of the processes that might be involved in cell damage. Moreover this thesis provides experimental evidence on how to prevent this type of injury. In the light of the increasing shortage of cadaveric donors, attempts should be made in future research to keep transplant recipients from re-entering the waiting list as long as possible. Because chronic transplant loss is partly attributable to prolonged cold ischemia time, either logistical change to reduce cold ischemia time, i.e. local donors for local patients, or donor pre-conditioning to adapt organ allografts to cold ischemia changes should be considered.
References

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Chapter 9: General discussion