The potential use of N-octanoyl-dopamine (NOD) in organ transplantation
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1.1 General introduction

Organ transplantation is nowadays the method of choice for the treatment of terminal organ failure and often the last or only option for patients to restore normal organ function. The number of patients with chronic organ failure is increasing as a consequence of an increased incidence of widespread diseases such as diabetes. While these patients are in need for organ transplantation, the number of post-mortem donor organs has not increased in parallel. In 2012 there were 7,919 dialysis patients on the waiting list (active) [1], yet only 1,820 post-mortem kidneys were available [2]. This exemplifies the current hurdle in transplantation medicine and warrants new strategies to increase the donor pool. In line with this, organs from living donors, in particular renal and liver allografts, are increasingly used to overcome this hurdle. Not only is the organ quality of such donors generally better, it also prevents the need for or diminishes the duration of dialysis for patients who will enter in end-stage renal failure. Yet, organs from post-mortem donors remain and will remain to constitute the largest part of the donor pool. Facing the increasing demand for donor organs, the transplantation community also agreed on the use of so called marginal donors, i.e. donors that do not fulfill the optimal but rather the extended donor criteria (ECD; “extended criteria donors”). Such donors are usually older and may have pre-existing diseases, e.g. diabetes. Clearly, organ quality of these donors is in general terms inferior to that of optimal donors and as such marginal organs have shorter graft survival expectancies after transplantation in recipients.

In keeping with this notion, the senior program of Eurotransplant ("old-for-old" program) was initiated to use donor organs from older donors particularly for older recipients and thus mitigating the already existing shortage of organs for young patients [3]. Also a local distribution of donor organs to minimize cold ischemia-induced injury has been discussed in the past. However, this represents a major ethical dilemma for a fair allocation of post-mortem donated organs. Since 1967 Eurotransplant allocates post-mortem organs according to a distribution algorithm as a function of blood group, human leukocyte antigen (HLA) compatibility, waiting time, urgency, potential cold ischemia time and if appropriate according to patient’s height and weight. This attempts to compensate for unfavorable conditions such as rare HLA types or regions with a low willingness to donate [4].
1.2 Graft loss and its causes

In addition to the limited availability of potential donors, also graft loss puts pressure on the donor organ pool as patients with graft loss are in need for re-transplantation. Graft rejection is the main cause for graft loss after successful transplantation [5]. Depending on the rate and time of occurrence, graft rejection can be classified into different categories:

*Hyperacute rejection*

Hyperacute rejection occurs immediately after reperfusion of the transplanted allograft. It is mediated by the presence of pre-existing anti-donor antibodies that bind to the graft’s vasculature and causes complement activation. Activated complement not only has the propensity to destroy the endothelium via formation of the membrane attack complex, it is also able to cleave heparan sulfate proteoglycans from the glycocalyx [6]. This changes the anti-thrombotic environment of the endothelium and favours initiation of intravascular coagulation. Because of improved pre-transplantation diagnostics, e.g. screening of recipients for the presence of anti-HLA antibodies and cross-matching, hyperacute rejection occurs only rarely. It should however be noted that anti-endothelial cell antibodies are not routinely tested. These antibodies may recognize polymorphic antigens that are exclusively present on endothelial cells [7].

*Acute rejection*

Acute rejection starts three to five days after transplantation and is divided into interstitial and vascular rejection. Acute interstitial rejection is characterized by infiltration of immune cells into the graft parenchyma and necrosis. This type of rejection can be successfully treated with immunosuppressive drugs, e.g. calcineurin inhibitors.

In acute vascular rejection both immune cells and IgG-antibodies are involved. Antibodies bind to the endothelium and activate the complement system resulting in necrosis of the endothelium. Acute vascular rejection is more difficult to treat and therefore has a poor long-term prognosis [8].

In recent years the introduction of new immunosuppressive drugs has resulted in a significant reduction of acute interstitial rejection episodes, thereby significantly increasing the median overall graft survival (Figure 1). This success is mainly attributed to an improved 1-year graft survival yet the rate of organ loss did not
change significantly in the past decades [9,10]. Thus chronic graft loss remains the major cause for re-entering onto the waiting list [5].

**Chronic rejection**

Chronic rejection describes a long-term decline in graft function. Although organ specific forms of chronic rejection are known, *e.g.* chronic allograft nephropathy in renal allografts or bronchiolitis obliterans in lung-transplanted patients, transplant vasculopathy (TV) may occur in most solid grafts with significant impact on heart and kidney allograft long-term survival. Histologically, it is defined by intimal thickening (neointima), increased proliferation of vascular smooth muscle in the intima and medial apoptosis [11]. Occlusion of the vessel leads downstream to ischemic events resulting in decline in graft function. Until now, no adequate therapeutic options are available in the clinic [12]. Although the exact pathophysiological mechanisms are not yet elucidated it is believed that both immunological and non-immunological factors are implicated in the pathogenesis of TV [13]. The risk factors for chronic rejection are shown in Figure 2 and discussed in more detail in the following section.

![Figure 1: Graft survival in the first or following four years after cadaveric kidney transplantation.](image)

Changes in graft survival over the last 25 years of cadaveric kidney transplantation in relation to the first or the following four years after transplantation. After the introduction of calcineurin inhibitors in the 80s graft survival has significantly improved in the first year (left), however chronic graft loss (after the first year) remained unchanged (right). *Graph adapted from [5].*
**Risk factors for chronic transplant loss**

**Organ quality**

In the early years of transplantation medicine very strict criteria for organ donors were established which had to be conditionally extended due to donor organ shortage. As mentioned earlier, organ quality of the latter donor is inferior as compared to that of organs from optimal donors because of age-dependent factors and medical history of marginal donors. In addition, it also seems that organs from marginal donors are more prone to culminate tissue damage as a consequence of brain death, ischemia, organ preservation and reperfusion. Particularly brain death is to date genuinely considered as an important factor for damaging donor organs and therefore represents a potential therapeutic target to improve organ quality.

After the onset of brain death a brief parasympathetic-dominated phase with bradycardia is followed by an “autonomic storm”, a massive sympathetic activation with release of catecholamines [14]. The combination of tachycardia, hypertension, and massively increased peripheral resistance leads to impaired organ perfusion with relative ischemia [15]. Once the release of sympathetic hormones is exhausted, a hypotensive phase follows leading to hypoperfusion and consequently organ damage [16]. Also the endocrine system undergoes dramatic changes. With the onset of brain death the hypothalamic-pituitary axis is impaired with far-reaching hormonal changes that can lead to neurogenic diabetes insipidus with electrolyte imbalance as well as a shift from an aerobic to an anaerobic metabolism or coagulopathies [17-19].

![Figure 2: Risk factors for the development of transplant vasculopathy.](image)
In the serum of brain dead patients increased levels of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6 are present [20]. The release of cytokines leads amongst others to activation of the endothelium followed by recruitment of immune cells. In the donor this is reflected by an increase in mononuclear cells in end-organs. After transplantation these mononuclear cells can migrate to peripheral lymph nodes in the recipient to initiate an adaptive immune response. The activated endothelium of the donor graft may also facilitate the recruitment of recipient immune cells to the graft after transplantation [21,22]. Hence activation of the graft’s endothelium could be associated with an increased risk for graft rejection and shorter graft survival [23].

**Cold ischemia time / reperfusion injury**

The success of organ transplantation is undoubtedly linked to the availability of organ preservation modalities, which is the prerequisite for nation-wide donor organ allocation. Static cold organ preservation was developed by Belzer *et al.* in the beginning of the 70s [24] and further optimized over the years. This method is still the most commonly used organ preservation modality and is based on the principle of hypometabolism, induced by cooling of the graft to 4°C in a cytoprotective preservation solution [25] (approximately every 10°C, adenosine triphosphate (ATP) consumption is lowered by a factor 1.5 to 2 [26]). Nevertheless, also static cold organ preservation has its limitations and can damage organs depending on the duration of preservation. Even though tolerance for cold ischemia time is different for various organ systems, for all organs cold preservation time correlates with a decreased median graft survival. Although at 4°C ATP consumption is greatly reduced as compared to 37°C, also ATP synthesis is inhibited, and therefore static cold preservation will ultimately result in ATP depletion [27]. Inasmuch as ATP-dependent ion channels are no longer able to maintain the necessary ion gradients, the imbalance in ion homeostasis can lead to cell swelling. ATP depletion also causes an increased intracellular calcium-concentration resulting in activation of a series of tissue degrading enzymes (phospholipases, proteases and endonucleases) and of enzymes that increase cytotoxic free radical levels (xanthine oxidase) [14,28]. Accumulation of degradation products during ischemia leads to an increased production of toxic metabolites after reperfusion, which is characteristic for ischemia/reperfusion (I/R)-injury [29].
In 1935 the first attempt to perfuse organs with oxygenated blood at 37°C and to maintain the entire organ system over a period of several days was described [30]. About 30 years later, the first liver transplant with machine perfusion was performed [31]. Today, there are two strategies for machine perfusion, i.e. normothermic and hypothermic perfusion. While the former is aiming to maintain physiological processes, the latter can be considered as a compromise between hypometabolism and physiological perfusion. Through continuous circulation machine perfusion allows provision of substrates and elimination of waste products. Machine perfusion can thereby reduce ATP loss after ischemia and induce cytoprotective proteins such as HO-1 under normothermic conditions [32,33]. It has been successfully used for various organs, but it requires a high level of expertise, good organization and might be more expensive as compared to the static cooling [34]. The effect of machine perfusion on transplant outcome varies from organ to organ. For kidney transplantation, a systematic review recently identified a lower incidence of delayed graft function (DGF), with no change in 1-year graft survival or 1-year mortality [35]. Data with regard to long-term transplant outcome are thus far not available.

After implantation organ perfusion is restored and causes additional damage, also referred to as I/R-injury. Under ischemic conditions xanthine oxidase is activated, enabling the oxidation of hypoxanthine to xanthine upon reperfusion with formation of free oxygen radicals [36]. Increased reactive oxygen species (ROS) lead to lipid peroxidation of the cell membrane and to endothelial activation with expression of adhesion molecules [37,38]. I/R-injury is therefore associated with a strong inflammatory response in which neutrophils and their activation products, cytokines, leukotrienes and platelet-activating factor are involved. Through the chemotactic activity of a variety of such inflammatory mediators, tissue inflammation is perpetuated [39] and may cause capillary leakage, edema and secondary ischemia, finally culminating in organ failure [40].

**Calcineurin inhibitors**

In addition to the availability of organ preservation modalities, the success of organ transplantation is also attributed to the availability of strong immune suppressive drugs. The use of calcineurin inhibitors (CNI) has tremendously improved long-term graft survival, particularly by inhibition of the adaptive immune system which plays a pivotal role in acute interstitial rejection. Paradoxically, CNI are also a risk factor for chronic graft loss because of their nephrotoxic [41] and
neurotoxic \cite{42} side effects. Attempts to reduce or even completely withdraw CNI from immune suppressive regimen in the experimental setting have shown that renal function can be stabilized in patients with signs of CNI-mediated renal impairment. However, this occurred at the expense of an increased incidence of alloimmune reactions \cite{43}. Also the classical cardiovascular risk factors, \textit{e.g.} arterial hypertension and hyperlipidemia, especially increased low density lipoprotein (LDL) levels with increased LDL oxidation, are adversely affected by CNI \cite{44}. CNI may also display toxic effect on endothelial cells by increasing ROS production \cite{45}, endothelin \cite{46}, thromboxane B\(_1\) expression \cite{47} and decreasing nitric oxide (NO) and prostaglandin I\(_2\) levels \cite{47,48}. Endothelial dysfunction is now regarded as the initial event in the development of transplant vasculopathy (TV) \cite{49}. For these reasons, the development of a calcineurin inhibitor-based long-term maintenance immunosuppressive drug regimen with improved long-term tolerability is a highly desirable endeavor.

\textit{Transplant vasculopathy}

Despite considerable efforts in contemporary transplantation research, transplant vasculopathy (TV) remains a major threat for all vascularized organs and is a leading cause for chronic graft loss. Even though the mechanism is not completely delineated, it is believed that both immunologic and non-immunologic factors are instrumental for the development of TV (Figure 3). Damage of the endothelium seems to be a starting point for the development of TV. Endothelial cell injury causes expression of adhesion molecules, \textit{e.g.} VCAM-1, ICAM-1 or E-selectin, which subsequently support sequestration of leukocytes into the vessel wall \cite{50}. In this process the release of pro-coagulation factors perpetuate vascular damage as a consequence of pro-thrombotic coagulation \cite{51}. Damage may also occur following deposition of alloreactive antibodies on the endothelium with subsequent complement and platelet activation. This in turn further activates the endothelium and further facilitates leukocyte invasion via release of cytokines \cite{52-54}.

Neointima formation is a hallmark for TV. Distinct lines of evidence have favored pathogenic roles for either T cell infiltration and IFN-\(\gamma\) secretion \cite{55,56} or development of donor specific antibodies and activation of complement in TV \cite{57,58}. Specifically, the pathogenic role of T cells has been investigated using analysis of human tissues and by experiments with various murine knockout models or humanized mice \cite{59}. It is believed that recruited alloreactive T cells
Figure 3: Model of transplant vasculopathy development.

1. Proinflammatory stimulus (e.g. cold preservation, ischemia/reperfusion-injury, virus infection)
2. Endothelium activation (VCAM-1↑, ICAM-1↑, E-selectin↑) → monocyte recruitment
3. Leukocyte rolling
4. Diapedesis
5. Leukocyte activation
6. CD8+ T-cell expansion
7. Endothelium activation
8. Stem cell recruitment from bone marrow
9. Dedifferentiation to SMLC
10. Apoptosis of intimal and medial cells
11. B-cell stimulation
12. Th1 differentiation → intimal SMLC-proliferation
13. Alloreactive antibodies
14. Complement activation
15. Platelet activation
16. Thrombus formation
17. Ischemia → graft loss
18. Neointima formation
19. Proliferation
20. Secretory phenotype (ECM-production)
produce INF-γ and TNF-α, leading to overstimulation and subsequent apoptosis of medial vascular smooth muscle cells (SMC) respectively chemokine-induced migration into the intima [60-63]. Apoptosis and endothelial denudation thus create a vicious circle of inflammation and cell death [64]. Apart from migration by medial SMC also recruitment of blood-borne progenitor cells from the bone marrow of the recipient occurs [65,66]. Progenitor cells differentiate, and to a lesser extent medial derived (neo)intimal SMC dedifferentiate, into smooth muscle-like cells (SMLC) which differ from normal SMC by an increased proliferative activity, loss of their contractile phenotype [67-70] and increased production of extracellular matrix (ECM) [71]. In many \textit{in vivo} studies, inhibition of SMC proliferation, and in particular of SMLC, was associated with reduced neointima formation [72]. The inhibition of endothelial cell activation or anti-apoptotic treatments have also shown a beneficial effect on TV [73]. Yet, none of these experimentally successful therapeutic approaches have been translated into effective strategies for prevention of TV in human organ transplantation.
1.3 Donor dopamine-treatment

Dopamine is an endogenous catecholamine and important neurotransmitter. In the central nervous system dopaminergic neurons are found mainly in the mesostriatal, mesolimbic, mesocortical and tuberoinfundibular system. Disturbances in central dopamine metabolism may lead to hypokinetic symptoms such as Parkinson’s disease, anhedonia or schizophrenic psychoses. In peripheral organs dopamine has an important role in the regulation of organ perfusion by increasing systolic blood pressure and heart rate [74]. In healthy subjects, in low dosages dopamine dilates renal arteries, increases renal plasma flow and reduces serum levels of creatinine and urea [75]. It was therefore believed that dopamine-treatment may prevent or mitigate acute renal failure in critically ill patients. In late 2000, the ‘Australian and New Zealand Intensive Care Society Clinical Trial Group’ launched a randomized placebo-controlled clinical study to assess the influence of dopamine-treatment on acute kidney injury [76]. Low-dose dopamine failed to improve renal function or diuresis in patients with systemic inflammatory response syndrome (SIRS) and did not reduce mortality of these patients. In fact, in a separate study it was even shown that dopamine-treatment of patients with septic shock was associated with a significantly higher 28-day mortality as compared to norepinephrine-treatment [77]. Dopamine-treatment as a mean to improve renal function in critically ill patients has therefore almost completely been abandoned from the intensive care unit.

More recently the use of dopamine on potential donors has been revisited by Schnuelle et al. showing in a prospective randomized multicenter study that low-dose dopamine-treatment of brain dead donors has a salutary effect on early renal graft function in the recipient [78]. A subgroup analysis showed that especially patients that received an organ with longer cold ischemia time benefit from dopamine-treatment, which was translated into a better graft survival. Also heart transplant recipients seem to benefit from donor dopamine-treatment [79], while it has no effect on liver transplantation outcome [78]. Based on numerous in vitro experiments, it was postulated that the protective effect of dopamine is independent of its hemodynamic effect and relies on the redox active catechol structure [80]. Interestingly, conjugation of octanoic acid to the amine side chain of dopamine results in a far more protective compound than dopamine, which no longer had hemodynamic activity [81]. These N-acyl-dopamine derivates (NADD) are hence promising drug candidates for the treatment of potential donors.
In Chapter 2 the available data of NADD and related compounds for their potential use in transplantation medicine will be reviewed.

1.4 Aim of the thesis

The general aim of this thesis is to investigate the potential beneficial effects of NOD on transplantation relevant processes, e.g. organ preservation, ischemia/reperfusion, inhibition of T cell activation and chronic rejection. We also attempted to identify the structural entities that govern NOD’s protective properties as well as how NOD protects in mechanistic terms.

In Chapter 2 we evaluated the potential use of N-acyl-dopamine derivates (NADD) and related compounds in transplantation medicine. Therefore we summarized the available data on biological activities of NADD in transplantation relevant entities, e.g. cold inflicted injury, I/R-injury, immune-modulation and inflammation.

In Chapter 3 we aimed to investigate the effect of NOD on ischemia-induced acute kidney injury (AKI), an in vivo model for I/R-injury. After i.v. administration of NOD the renal artery was clipped and blood flow restored after 30 min ischemia time. Kidney function in the hemi-nephrectomized rats was monitored in vivo by creatinine and urea levels. Tubular necrosis and macrophage infiltration were evaluated using (immuno-)histochemistry. We further looked into molecular mechanism in vitro by analyzing receptor- and transcription factor activation. TRPV1-receptor activation was measured using a fluorescence based calcium imaging technique and anti-inflammatory properties of NOD were investigated by Western blot. Transcription factor activation was assessed by its DNA binding capacity in electrophoretic mobility shift assays (EMSA).

In Chapter 4 and Chapter 5 we focused on the exact molecular mechanism of anti-inflammatory effects observed in the AKI-model. In human umbilical vein endothelial cells (HUVEC) anti-inflammatory properties were confirmed using different assays (Western blot, adhesion assays, flow cytometry). We performed gene expressing profiling to identify potential molecular targets of NOD. Array results were confirmed by qPCR. Activation of transcription factors that were found to be impaired by NOD were investigated by EMSA and lentiviral reporter assays. We further analyzed their regulation by Western blot and knock-down experiments using siRNA respectively blocking de novo protein synthesis. We monitored proliferation, cell cycle and cytotoxicity by \(^{3}\)H-thymidine incorporation, MTT-assays and flow cytometry respectively. Different NOD derivatives were tested to investigate the molecular entities required for NOD’s action.
During reperfusion of the graft, platelets are known to have an important role in recruitment of inflammatory cells to the endothelium [53,82,83]. In clinical studies, platelet activation could be linked to worse transplant outcome [54,84]. Therefore we aimed in Chapter 6 to investigate if NOD can influence platelet activation. To this end, we assessed fibrinogen receptor activation and platelet degranulation by flow cytometry and intracellular cAMP production by ELISA. Platelet aggregation was measured by whole blood impedance aggregometry. To investigate potential inhibitory effects of NOD on platelet activation, we activated platelets by ADP and using a thromboxane A₂ analogue.

The main reason for graft loss after transplantation is graft rejection. Since the introduction of calcineurin inhibitors (CNI) as immunosuppressive drugs the incidence of acute rejections has drastically lowered [9,10]. However, beside this beneficial effect, CNI have severe side effects causing amongst other chronic rejection [41,42]. Therefore the development of new potent immunosuppressive drugs is still necessary. As shown in Chapter 3 and 4 NOD is a potent NF-κB inhibitor, an essential transcription factor for T cell activation. We therefore hypothesized in Chapter 7 that NOD can influence T cell activation. We quantified mitogen-activated T cell proliferation by ³H-thymidine. Cell cycle distributions, cytotoxicity, switch in T cell subsets, T cell activation and generation of activation associated intracellular reactive oxygen species (ROS) were assessed by flow cytometry respectively Western blot. We performed EMSA to analyze the effect of NOD on transcription factor activation. To confirm that redox activity of NOD is required for its action we used the redox inactive N-octanoyl-tyramine (NOT) in proliferation assays.

Chronic rejection is nowadays the main reason for late graft loss and no adequate treatment is available [85]. Endothelial dysfunction and vascular inflammation are regarded as key events in the development of transplant vasculopathy (TV). Since NOD shows cytoprotective and anti-inflammatory properties on both, endothelial and T cells, we investigated the effect of NOD on TV. To this end, we developed a long-lasting i.v. delivery system for rats in Chapter 8. Two osmotic pumps were linked via a T-tube to a jugular vein catheter. Single-pump implanted rats served as control. Impact on animal welfare was monitored by its weight gain, visual control of animal’s activity and signs of discomfort. This i.v. delivery system was used in Chapter 9 to investigate the influence of NOD on TV development. We performed allogeneic orthotopic aorta transplantation from Dark Agouti (DA) to Brown Norway (BN) rats. Recipients were followed-up.
for 2 weeks to assess inflammatory infiltration and 4 weeks for analysis of neointima formation and neointimal composition respectively smooth muscle cell proliferation by (immuno-)histochemistry. We investigated NOD’s mechanism of action in vitro by quantifying human aortic smooth muscle cell (haSMC) proliferation, cell cycle distribution and cytokine-induced apoptosis by WST-1 proliferation assays, PCR analysis and flow cytometry.

We used the same animal model to investigate the effect of vanin inhibition on TV development. RR6 is a small molecule that inhibits the pantetheinase vanin. Because Dammanahalli et al. showed in a mechanical vascular injury model that $Vnn1^{-/-}$ prevents neointima formation [86] we aimed to investigate in Chapter 10 the effect of vanin inhibition by RR6 on the development of TV. RR6 was administered orally via the drinking water and daily water intake and serum vanin activity was monitored. T cell and macrophage infiltration was assessed 2 weeks after transplantation and neointima formation, composition, neointimal SMC proliferation respectively PPARγ expression were evaluated by (immuno-)histochemistry after 4 weeks. In vitro, we further analyzed the effect of RR6-treatment on haSMC PPARγ expression, cytokine-induced apoptosis and proliferation induced by different cytokines and hormones.
References


