Brain death and organ donation

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CHAPTER 7

MnTMPyP treatment of brain-dead rats leads to improved renal function during *ex vivo* reperfusion

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ABSTRACT

Introduction

Delayed graft function (DGF) is a common complication in renal transplant recipients receiving kidneys from brain-dead donors. Brain death (BD)-related lipid peroxidation, measured as maldondialdehyde (MDA) levels, correlate with DGF in renal transplant recipients. We aimed to assess the effects of MnTMPyP treatment of brain-dead rats on renal function in an ex vivo isolated perfused kidney (IPK) model.

Methods

BD induction was performed in 18 mechanically ventilated male Fisher rats by inflating a 4.0F Fogarty catheter in the epidural space. Rats were observed for 4 hrs following BD induction. Rats were maintained hemodynamically stable through the administration of colloids and norepinephrine. After 4 hrs, the left kidney was cannulated and reperfused in the IPK model for 90 min. The other organs, urine and blood were collected. Perfusate and urine samples were collected at different time points in the IPK model.

Results

BD resulted in increased levels of renal superoxide and MDA levels which were attenuated by MnTMPyP treatment. In the IPK model, MnTMPyP treatment resulted in increased renal blood flow, decreased perfusate creatinine levels, increased sodium absorption, increased urine output, and decreased edema.

Conclusion

MnTMPyP treatment in brain-dead rats leads to improved renal function ex vivo. MnTMPyP treatment could lead to improved transplantation outcomes.

INTRODUCTION

Delayed graft function (DGF) is a complication occurring in 20-35% of renal transplant recipients[1-3]. DGF is associated with acute rejection, chronic allograft failure, and decreased renal function[3-6]. Kidney grafts retrieved from brain-dead donors, the most frequently transplanted grafts, show DGF rates of 15-30%[7,8]. These findings cannot be solely explained by human leukocyte antigen (HLA) mismatches, longer cold ischemia times, or donor age[9]. Instead, brain death (BD) itself elicits detrimental effects in the donor.

BD pathophysiology comprises hemodynamic, hormonal, and inflammatory changes. Brain stem herniation results in a catecholamine storm and neurogenic shock through ischemia of the spinal cord[10]. Inflammatory changes are characterized by an increase in circulating cytokines such as interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-alpha (TNF-α)[11-13]. These cytokines trigger inflammatory responses in different organs through the influx of inflammatory cells. Further, a drop in hormonal levels is evident due to pituitary dysfunction[14].

BD pathophysiology results in increased systemic and renal lipid peroxidation which is measured as malondialdehyde (MDA) levels[15-18]. Possible causes of the increase in lipid peroxidation are the changes in hemodynamics, inflammation, and hormonal impairment[19-21]. Lipid peroxidation leads to membrane dysfunction and cell toxicity[22-24]. BD-associated MDA levels correlate with DGF, acute rejection, and immediate and long-term renal allograft survival[18]. Therefore, preventing lipid peroxidation in brain-dead donors could lead to improved renal transplantation outcomes.

Ischemia-reperfusion (I-R) injury poses a major threat to transplanted kidneys and has serious consequences[25]. Early I-R injury is characterized by apoptosis and is likely mediated by the generation of reactive oxygen species (ROS)[26-28]. ROS lead to damaged cellular components such as DNA, proteins, and lipids[29]. The ROS-related effects lead to the production of pro-inflammatory cytokines and signaling which contributes to increased damage and immunogenicity[30,31]. Consequently, many studies have focused on decreasing I-R injury through the administration of anti-oxidative molecules during reperfusion. However, these studies have showed differing clinical results.

BD pathophysiology activates donor organs and is associated with worse I-R injury[11]. Considering the correlation between MDA levels and renal function after transplantation, we hypothesize that decreasing lipid peroxidation in the brain-dead donor will lead to decreased I-R injury and result in improved renal function. In a previous study we showed that MnTMPyP, a selective superoxide dismutase mimetic, is effective in reducing renal and systemic MDA levels. Here, we test the effects of MnTMPyP treatment of brain-dead rats on kidney function during reperfusion in an isolated perfused kidney (IPK) system.

MATERIALS AND METHODS

Animal BD model

For this experiment, male adult Fisher F344 rats (250-300 g) were used. Animals were anesthetized using isoflurane and subsequently intubated. Cannulae were brought into the left femoral artery and vein for blood pressure monitoring and administrating plasma expanders or norepinephrine. Brain death was induced as described previously. A no. 4 Fogarty catheter (Edwards Lifesciences Co., Irvine, CA) was placed in the epidural space through a frontolateral hole drilled in the skull and slowly inflated (16µl/min) with saline

using a syringe pump (Terufusion, Termo Co., Tokyo, Japan). The increase in intracranial pressure results in brain death after approximately 30 minutes. Inflation of the balloon was stopped when the mean arterial pressure (MAP) reached 80 mmHg due to the catecholamine storm characteristic for brain death. Anesthesia was stopped after brain dead induction and the animals remained ventilated with O_2/air. BD was confirmed by the absence of corneal reflexes and an apnoea test. MAP was kept between 80-120 mmHg by using 10% hydroxyethyl starch (Fresenius Kabi AG, Bad Homburg, Germany), and if needed norepinephrine. 4 hours after BD induction blood was collected through the abdominal artery after which the organs were flushed with saline. Centrifuged blood samples and urine from the bladder were snap frozen. Kidneys were harvested and sections stored in formalin as well as snap frozen. Rats were randomly divided, each group consisting of eight animals. Sham-operated rats, which were ventilated for half an hour under anaesthesia before scarification, served as controls. MnTMPyP (5mg/kg) or saline was administered intraperitoneally, 30 min before the start of the operation. MnTMPyP was purchased from Merck Millipore (Darmstadt, Germany).

The following experimental groups can be distinguished:

Group 1: Brain dead rats receiving saline vehicle
Group 2: brain dead rats receiving MnTMPyP

Isolated perfused kidney system

To assess renal function after brain death the left kidney was evaluated in an IPK model as described before[32]. The renal artery and ureter are cannulated and placed in a chamber in which the kidney is perfused with DMEM supplemented medium. Supplements included L-glutamine and pH was adjusted to 7.4. Perfusate and urine samples were collected to estimate renal function. Perfusion medium was maintained at 37°C and oxygenated with 95% O_2 and 5% CO_2. Kidneys were perfused at a perfusion pressure of 100 mm HG during 90 mins. Samples were stored at -80°C.

Determination of superoxide production with dihydroethidium staining

Four µm cryosections were mounted on slides and washed with Dulbecco's PBS (DPBS). Sections were incubated with 10 µM dihydroethidium (Sigma, St. Louis, MO) dissolved in DPBS at 37°C in the dark for 30 min. Sections were washed twice with DPBS and immediately scanned for superoxide with a Leica inverted fluorescence microscope equipped with rhodamine filter settings. Images were acquired at 40X magnification and analyzed using NCBI ImageJ.

Determination of lipid peroxidation with thiobarbituric acid reactive substances

MDA was measured as described previously[17]. MDA is measured fluorescently after binding to thiobarbituric acid. 20µL plasma samples were mixed with 2% SDS and 5mM butylated hydroxytoluene followed by 400µL 0.1 N HCL, 50µL 10% phosphotungstic acid and 200µL 0.7% TBA. The mixture was incubated for 30 min at 97°C. 800µL 1-butanol was added to the samples and the centrifuged at 960 *g*. 200 µL of the 1-butanol supernatant was fluorescently measured at 480 nm excitation and 590 nm emission wavelengths.

RNA isolation and qPCR

qPCR experiments were conducted as described before[15]. Total RNA was isolated from rat kidneys using the SV Total RNA isolation kit (Promega, Leiden, the Netherlands) according to the manufacturer's protocol. RNA samples were verified for the absence of genomic DNA contamination by performing RT-PCR reactions, in which the addition of reverse transcriptase was omitted, using GADPH primers. cDNA synthesis was performed from 1 µg total RNA using T11VN oligo's and M-MLV reverse transcriptase, according to suppliers's protocol (Invitrogen, Breda, The Netherlands). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems, Foster city) using emission from SYBR green (SYBR green master mix, Applied biosystems). All assays were performed in triplicate. After an initial activation step at 50°C for 2 min and a hot start at 95°C for 10 min, PCR cycles consisted of 40 cycles of 95°C for 15 s and 60°C for 60 s. Specificity of qPCR products was routinely assessed by performing a dissociation curve at the end of the amplification program and by gel electrophoresis.

Gene expression was normalized with the mean of β-actin mRNA content and calculated relative to controls using the relative standard curve method. Results were finally expressed as $2^{-}\Delta ct$ (CT threshold cycle). Amplification primers were designed with Primer Express software (Applied Biosystems) and validated in a six-step 2-fold dilution series. The primer sequences and product sizes are given in table 1.

Table 1. qPCR primer sequences of the genes b-actin and iNOS.

Gene	Primer Sequences	Bp
b-actin	5'-GGAAATCGTGCGTGACATTAAA-3' 5'-GCGGCAGTGGCCATCTC-3'	74
iNOS	5'-GAGGAGCCCAAAGGCACAAG-3' 5'-CCAAACCCCTCACTGTCATTTTATT-3'	81

Table 2. Total Noradrenaline (1 mg/ml) and HAES infusion requirements and number of rats which required Noradrenaline.

	BD + saline	BD + MnTMPyP	P value
Noradrenaline (ml)	0.31 ± 0.1	0.28 ± 0.3	0.54
HAES (ml)	3.5 ± 0.4	4.1 ± 2.5	0.28

* indicates a significant difference between MnTMPyP- and saline-treated brain-dead rats.

RESULTS

Hemodynamic changes and donor management during BD

BD induction showed the characteristic drop and subsequent increase in blood pressure over a mean of 30.5 minutes (Figure 1). All 16 animals (n=8 per group) were kept at a mean arterial pressure higher than 80 mmHg during the experiment. No significant differences were observed between groups in terms of HAES and NA administration. In saline treated brain-dead rats, infusion of 1.5[0.0-4.0] ml HAES 10% was necessary to maintain stable blood pressure. In MnTMPyP treated brain-dead rats, infusion of 3.0[2.0-3.5] ml HAES 10% was needed to maintain stable blood pressure. Saline treated brain-dead rats required 0.7[0.0-2.4] mg NA and MnTMPyP treated brain-dead 0.0[0.0-5.1] mg NA.

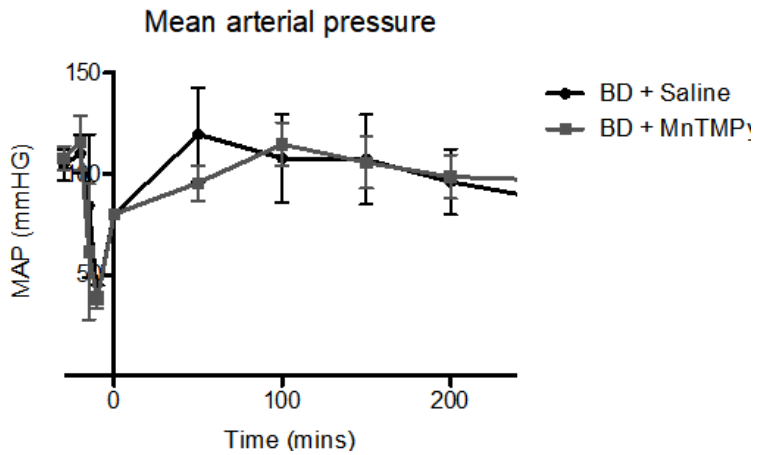

Figure 1: Mean arterial pressure (MAP) course during brain-death (BD)- induction and BD. The induction phase showed a characteristic drop in blood pressure. No differences were observed in blood pressure levels between saline and MnTMPyP-treated brain-dead groups

Renal superoxide production in the brain-dead rat

After 4 hrs, superoxide levels were significantly reduced in brain-dead rats pre-treated with MnTMPyP compared to non-treated rats ($p < 0.05$, Figure 2).

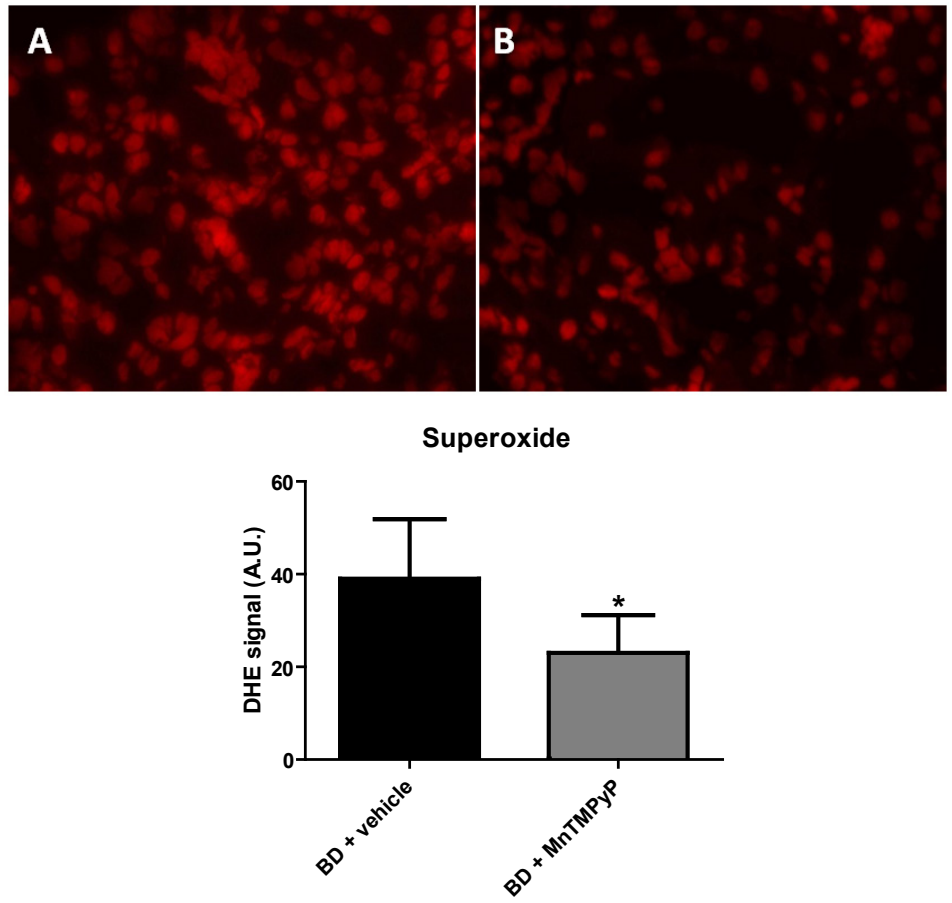

Figure 2: DHE staining for superoxide in brain-dead rats treated with vehicle or MnTMPyP. A, BD + vehicle. B, BD + MnTMPyP. MnTMPyP treatment resulted in decreased superoxide production compared to treatment with vehicle. * indicates $p < 0.05$. 40X magnification

Renal lipid peroxidation in the brain-dead rat

MDA levels were significantly reduced after 4 hrs of BD in brain-dead rats pre-treated with MnTMPyP compared to non-treated rats ($p < 0.01$, Figure 3).

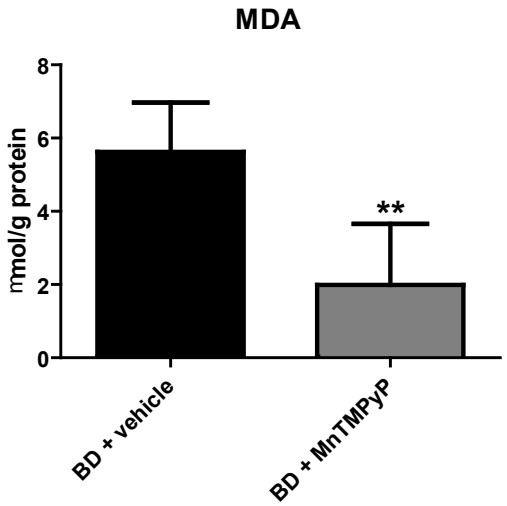

Figure 3: Renal levels of lipid peroxidation in brain-dead rats treated with vehicle or MnTMPyP. MnTMPyP treatment resulted in decreased renal MDA levels compared to treatment with vehicle. ** indicates $p < 0.01$ compared to saline-treated brain-dead rats.

Renal blood flow and perfusate creatinine levels during reperfusion in the IPK model

During reperfusion in the IPK, renal blood flow increased significantly of kidneys from brain-dead rats treated with MntMPyP during BD. Renal blood flow was significantly increased compared to vehicle treated rats at 30, 60 and 90 minutes of reperfusion ($p < 0.05$, Figure 4). Perfusate creatinine levels were significantly reduced during reperfusion of kidneys from brain-dead rats treated with MnTMPyP compared to vehicle treatment at 60 and 90 minutes ($p < 0.05$).

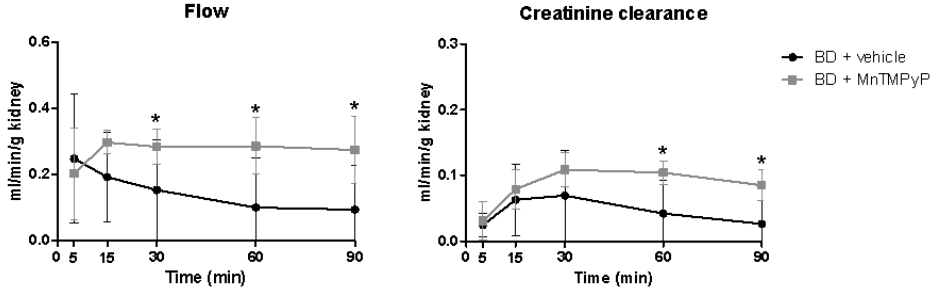

Figure 4: Assessment of renal flow and creatinine clearance during reperfusion in an isolated perfused kidney (IPK) system of kidneys from brain-dead rats pre-treated with vehicle or MnTMPyP. MnTMPyP treatment led to increased flow and creatinine clearance compared to non-treated rats. * indicates $p < 0.05$ between groups.

Renal sodium excretion and urine production during reperfusion in the IPK model

During reperfusion in the IPK, renal sodium excretion decreased significantly of kidneys from brain-dead rats treated with MnTMPyP. Renal sodium excretion was decreased significantly compared to vehicle treated rats at 60 and 90 minutes of reperfusion ($p < 0.05$, Figure 5). Urine output was increased significantly during reperfusion of kidneys from brain-dead rats treated with MnTMPyP compared to vehicle treatment at 60 and 90 minutes ($p < 0.05$).

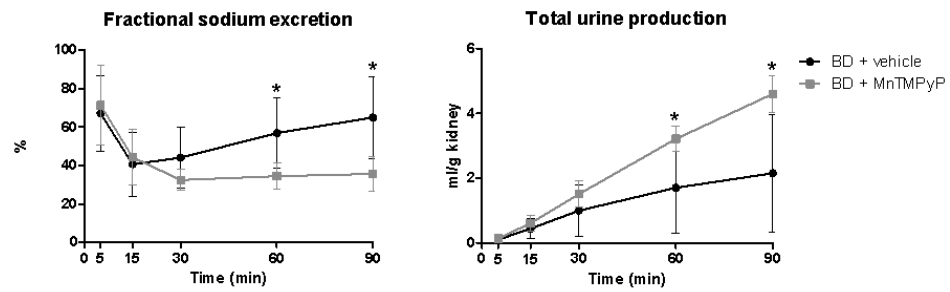

Figure 5: Assessment of fractional sodium excretion and urine production during reperfusion in an isolated perfused kidney (IPK) system of kidneys from brain-dead rats pre-treated with vehicle or MnTMPyP. MnTMPyP pre-treatment led to decreased sodium excretion and increased urine production compared to non-treated rats. * indicates $p < 0.05$ between groups.

Renal weight increase during reperfusion in the IPK model

Renal weight increase was significantly more of kidneys of brain-dead rats treated with vehicle compared to MnTMPyP treatment ($p < 0.05$, Figure 6)

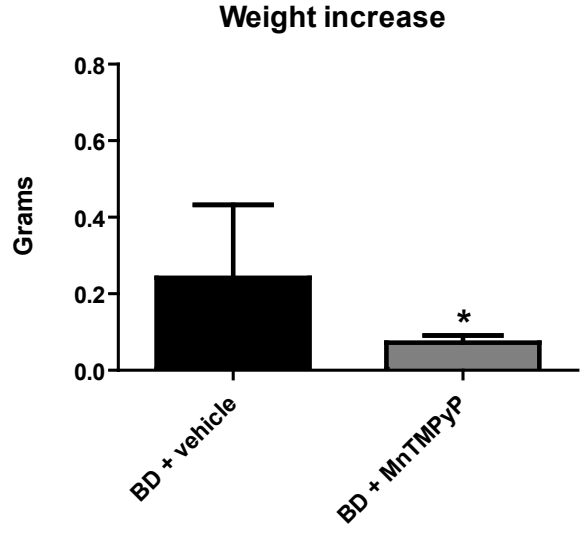

Figure 6: Kidney weight change after reperfusion in an isolated perfused kidney (IPK) model of kidneys from brain-dead rats treated with vehicle or MnTMPyP. MnTMPyP treatment led to less weight increase compared to non-treated rats. * indicates $p < 0.05$ between groups.

DISCUSSION

The role of antioxidants has been studied extensively in the context of I-R injury[33-35]. In these studies, antioxidants are administered to counteract the detrimental effects of oxidants produced during reperfusion. In our study, we counteracted oxidant production in the brain-dead donor rat as we hypothesized that oxidative damage in the donor predisposes kidneys to worse I-R injury. Our main findings are that MnTMPyP treatment led to increased renal blood flow and function which was assessed during the reintroduction of oxygen in an IPK model. This shows that decreasing lipid peroxidation in brain-dead could influence rates of DGF, acute rejection, and short and long-term allograft survival since MDA levels correlate with these processes[18].

In a previous study, we showed that MnTMPyP treatment of brain-dead rats decreases renal lipid peroxidation but does not lead to improved renal function in the rat. However, in models of sepsis, MnTMPyP treatment leads to improved renal function in the animal[36,37]. The improved renal function in these studies is attributed to the increased availability of nitric oxide though the decreased reaction with superoxide. Even though renal function decreases during BD, it could be that sepsis elicits more hemodynamic instability leading to longer phases of renal ischemia and thereby increased superoxide production. Therefore, reducing superoxide levels in sepsis could have an effect on kidney function within the rat. The present study shows that decreasing superoxide levels in the brain-dead rat leads to improved renal function after the kidneys have been subjected to I-R injury. We believe that the decrease in superoxide levels and thereby the decreased lipid peroxidation in the brain-dead rat results in less susceptibility to I-R injury. This idea has been shown before in the sense that BD primes organs to worse I-R injury[11]. This could lead to decreased sodium pump dysfunction and apoptosis of proximal tubular cells which could explain the increased sodium reabsorption, increased urine output, and decreased perfusate creatinine levels we observed. The decreased creatinine levels in the perfusate could also be influenced by the increased renal blood flow we observed. This increase in renal blood flow could be related to effects of manganese which forms the core of MnTMPyP. Manganese increases renal blood flow and GFR by acting as a calcium entry blocker[38]. Another explanation for the increased renal blood flow could be the effect of superoxide scavenging on renal resistance. Superoxide oxidizes membrane lipids which causes loss of membrane barriers[39]. Furthermore, mitochondrial membranes are affected which results in less ATP production for Na^+/K^+ pumps and leads to cellular swelling causing obstruction of the microvasculature and tubules.

Using the IPK model, we tested renal function during the reintroduction of oxygen. In this manner, I-R injury is mimicked in the sense that organs experienced a period of ischemia during organ harvest and the subsequent cold flush after which they were subjected to the reintroduction of oxygen. However, this model does not resemble all aspects of clinical I-R injury as it does not incorporate certain elements such as the presence of leukocytes in the perfusion medium. Future research should study longer term effects of MnTMPyP treatment on I-R injury. Nevertheless, our aim was to test the early effects of MnTMPyP treatment on kidney function with minimal external influences. Therefore, our research question could be addressed appropriately with the use of this model.

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