CHAPTER

MnTMPyP, a selective superoxide dismutase mimetic, reduces oxidative stress in kidneys of brain-dead rats

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In preparation
CHAPTER 6

ABSTRACT

Introduction

Delayed graft function (DGF) is a common complication in renal transplant recipients receiving kidneys from brain-dead donors. DGF is associated with acute rejection, chronic allograft failure, and decreased renal function. Brain death (BD)-related oxidative stress, measured as malondialdehyde (MDA) levels, correlates well with DGF. Therefore, we aimed to decrease renal oxidative stress in brain-dead rats through pre-treatment with MnTMPyP, a superoxide dismutase mimic.

Methods

BD induction was performed on 32 mechanically ventilated male Fisher rats by inflating a 4.0 F Fogarty catheter in the epidural space. Rats were observed for 4 h and maintained hemodynamically stable through the administration of colloids and norepinephrine. Plasma, urine, and kidney tissue were collected for analysis. MnTMPyP (5.0 mg/kg) or saline vehicle was administered intraperitoneally 30 min prior to BD induction.

Results

BD resulted in increased levels of renal superoxide, renal and serum MDA levels, renal oxidized glutathione, and renal HO-1 expression compared to sham values. These changes were all attenuated by MnTMPyP pre-treatment. BD also led to increased plasma creatinine levels, urinary NAG activity, renal leukocyte influx, and renal IL-6, TNF-α, and E-selectin gene expression which were not reduced by MnTMPyP pre-treatment.

Conclusion

MnTMPyP pre-treatment of brain-dead rats leads to decreased oxidative stress in kidneys of brain-dead rats. Amongst others, BD-related MDA levels were decreased which correlate with DGF. Therefore, MnTMPyP treatment of brain-dead donors could lead to decreased incidence of DGF and thereby better renal transplantation outcomes.

INTRODUCTION

Delayed graft function (DGF) is a serious complication in renal transplant recipients. DGF is associated with acute rejection, chronic allograft failure, and decreased renal function. Kidneys from brain-dead donors, the most frequently transplanted kidneys, show DGF rates of 15-30%.

With the present study, we aimed to decrease renal oxidative stress and thereby serum creatinine levels, urinary NAG activity, renal leukocyte in

MATERIALS AND METHODS

Animal BD model

Male adult Fisher F344 rats (250-300 g) were anesthetized using isoflurane and subsequently intubated. The left femoral artery and vein were cannulated for blood pressure monitoring and administration of plasma expanders or norepinephrine. A 4.0 Fogarty catheter (Edwards Lifesciences Co., Irvine, CA, USA) was placed in the epidural space through a frontolateral burr-hole in the skull. The catheter was inflated (16 µl/min) with saline using a syringe pump (Terufusion, Termo Co., Tokyo, Japan). Inflation at this speed leads to increased levels of renal oxidative stress compared to faster inflation. The increase in intracranial pressure (ICP) leads to BD after approximately 30 min. Inflation of the balloon was stopped when the mean arterial pressure (MAP) reached 80 mmHg due to the catecholamine storm characteristic for BD. BD was confirmed by the absence of corneal reflexes. Anesthesia was stopped after BD confirmation and the animals remained ventilated with O2/air. MAP was kept between 80-120 mmHg by using 10% hydroxyethyl...
Glutathione measurements were conducted as described before. Glutathione measurements for amount of protein and expressed as µmol/g protein. measured at 480 nm excitation and 590 nm emission wavelengths. Samples were corrected for total amount of protein and expressed as µmol/g protein. To measure GSSG, 1-methyl-2-vinyl pyridinium triflate was added at a concentration of 3 mM to the supernatant to block reduced glutathione (GSH). GSH content was calculated by subtracting GSSG from the total glutathione values.

Heme oxygenase-1 immunohistological staining
Renal heme oxygenase-1 (HO-1) staining was performed on paraffin sections (4 µm). Sections were de-waxed, rehydrated, and subjected to heat-induced antigen retrieval by microwave heating in 1 mM EDTA (pH=8.0). Endogenous peroxidase was blocked with 0.03% H2O2 in PBS for 30 min. Incubation of the primary antibody lasted for 60 min at room temperature. Binding of the antibody was detected by incubation with appropriate peroxidase-labelled secondary and tertiary antibodies (Dako, Glostrup, Denmark) for 30 min at room temperature. Antibody dilutions were made in PBS supplemented with 1% bovine serum albumin (BSA) and 1% normal rat serum. Peroxidase activity was visualized using 3,3’-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin. Negative antibody controls were performed in which the primary antibody was omitted. HO-1 quantification was achieved by using the positive field algorithm provided Apoio ImageScope version 11.1.2.760. Results are expressed as the proportion of the total number of positive pixels and the total number of pixels.

Granulocyte immunological staining
Five-micrometer renal sections were fixed in acetone and stained with mouse monoclonal anti-rat granulocyte antibody (IQ products, Groningen, the Netherlands) which was dissolved in PBS at 37°C in dark conditions for 30 min. Subsequently, sections were washed twice with PBS and 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 superoxide production with dihydroethidium staining
Superoxide production was determined as described before 15. Four µm cryosections were mounted on slides and washed with Dulbecco’s phosphate-buffered saline (DPBS). Sections were incubated with 10 µM dihydroethidium (Sigma, St. Louis, MO, USA) dissolved in DPBS at 37°C in dark conditions for 30 min. Subsequently, sections were washed twice with DPBS and 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
Lipid peroxidation levels were determined as described before 15. MDA was measured fluorescently after binding to thiobarbituric acid. Twenty µl of kidney tissue homogenates or plasma were mixed with 2% SDS and 5mM butylated hydroxytoluene followed by 400 µl of 0.1 N HCl, 50 µl 10% phosphotungstic acid and 200 µl 0.7% TBA. The mixture was fluoroscently measured at 480 nm excitation and 590 nm emission wavelengths. Samples were corrected for amount of protein and expressed as µmol/g protein.

Glutathione measurements
Glutathione measurements were conducted as described before 15. Tissue was lysed in cell lysis buffer composed of 253 M HEPES, 5 mM MgCl2, 5 mM Ethylenediaminetetraacetic acid (EDTA), 2mM phenylmethylsulfonyl fluoride (PMSF) and 10 ng/µl pepstatin and leupeptin. The buffer was adjusted to a final pH of 7.5. Cell suspensions were centrifuged and the supernatant was analyzed. To measure total glutathione, 20 µl of the supernatant was added to buffer A (125 mM NaH2PO4, 0.0 and 6.3 mM NaEDTA adjusted to pH 7.5 with NaOH) to a total volume of 100 µl in a transparent flat bottom 96-well plate. Next, 20 µl of 6 mM 5,5'-dithiobis-2-nitrobenzoate, 42 µl of 0.3 mM nitocinamide adenine dinucleotide phosphate (NADPH), all dissolved in buffer A, were added to the wells. Finally, 38 µl of glutathione reductase, dissolved to an enzyme activity of 5 units/ml in buffer A, was added to the wells. The absorbance was measured at 430 nm for 15 min at 30 °C. The linear part of the kinetic curve was used for the rate estimation and compared with a standard curve of oxidized glutathione (GSSG). Samples were corrected for total amount of protein and expressed as µmol/g protein. To measure GSSG, 1-methyl-2-vinyl pyridinium triflate was added at a concentration of 3 mM to the supernatant to block reduced glutathione (GSH). GSH content was calculated by subtracting GSSG from the total glutathione values.

RNA isolation and quantitative polymerase chain reaction
Quantitative polymerase chain reaction (qPCR) was performed as described before 17. The SYBR Green Total RNA isolation kit (Promega, Leiden, the Netherlands) was used to isolate total RNA from rat kidneys per manufacturer’s protocol. RT-PCR reactions were performed to verify samples for the absence of genomic DNA contamination, 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, per supplier’s protocol (Invitrogen, Breda, The Netherlands). The ABI Prism 7900-HT Sequence Detection System (Applied Biosystems, Foster city, CA, USA) was used for amplification and detection using emission from SYBR green. All assays were performed in triplicate. PCR cycles consisted of an initial activation step at 50°C for 2 min and a hot start at 95°C for 10 min, after which the cycles consisted of 40 cycles of 95°C for 15 s and 60°C for 60 s. Specificity of qPCR products was assessed with 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 expressed as 2-ΔCT (CT threshold cycle). Amplification primers were designed with Express software and validated in a six-step 2-fold dilution series. The primer sequences and product sizes are given in table 1.
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MnTMPyP DECREASES RENAL OXIDATIVE STRESS DURING BRAIN DEATH

Table 1. qPCR primer sequences of the genes b-actin and IL-6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Bp</th>
</tr>
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<tbody>
<tr>
<td>b-actin</td>
<td>5’-GGAAAATCGTGCGTGACATTAAA-3’ 5’-GCCGACATGGCCACATC-3’</td>
<td>74</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-CCCACTTCAATGCTCTCTAAATG-3’ 5’-TCTCAAGTGCCCTTTCAAGAAGTGTTG-3’</td>
<td>89</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’-GGGTCTGCTGTTCAGTATG-3’ 5’-CAAGTGGAAGGCAAACCTACAGTT-3’</td>
<td>79</td>
</tr>
<tr>
<td>E-selectin</td>
<td>5’-GTCTGCAGATGCTCCTACCTG-3’ 5’-CTGCCACAGAAAGTGCCACTAG-3’</td>
<td>73</td>
</tr>
</tbody>
</table>

NAG injury biomarker

To estimate tubular damage, N-acetyl-β-D-glucosaminidase (NAG) activity in urine was measured using a method based on enzymatic hydrolysis of p-Nitrophenyl N-acetyl-β-D-glucosaminidase to p-Nitrophenyl and N-acetyl-β-D-glucosaminidase. Enzymatic activity was expressed as the amount of enzyme required to release 1 µmol of product per minute. NAG levels were corrected for urine creatinine levels and expressed as U/mmol UCr.

Statistical analyses

Data were analyzed using GraphPad Prism 5.04 (GraphPad, San Diego, USA). Groups were compared using the Kruskall-Wallis test with Dunns post-hoc correction. P < 0.05 was considered statistically significant. All data are expressed as the mean ± SD (standard deviation)

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 min as was described before15. All animals were kept at a mean arterial pressure higher than 80 mmHg during the experiment. No differences were observed in blood pressure profiles between groups (Figure 1). No statistical differences were observed in amount of HAES and NA administration between groups (Table 2).

Table 2. Total Noradrenaline (1 mg/ml) and HAES infusion requirements per group.

<table>
<thead>
<tr>
<th></th>
<th>BD + saline</th>
<th>BD + MnTMPyP</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenaline (ml)</td>
<td>0.31 ± 0.1</td>
<td>0.28 ± 0.3</td>
<td>0.54</td>
</tr>
<tr>
<td>HAES (ml)</td>
<td>3.5 ± 0.4</td>
<td>4.1 ± 2.5</td>
<td>0.28</td>
</tr>
</tbody>
</table>

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

Figure 1. Blood pressure course during the induction and brain-dead phase. 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.

Superoxide production with dihydroethidium staining

BD led to significantly increased levels of superoxide compared to sham values (p < 0.05, respectively, Figure 2). Superoxide levels were significantly reduced in brain-dead rats pretreated with MnTMPyP (p < 0.01).
Lipid peroxidation

Renal and serum MDA levels were increased significantly by BD compared to sham values ($p < 0.01$, Figure 3). Both renal and serum MDA levels were significantly decreased by MnTMPyP pre-treatment ($p < 0.01$ and $p < 0.05$, respectively).

![Figure 3](image)

GSH, GSSG, GSH + GSSG, and the GSSG:GSH ratio

BD did not lead to decreased GSH levels compared to sham-operated rats. However, brain-dead rats pre-treated with MnTMPyP resulted in significantly increased GSH levels compared to saline-treated brain-dead rats ($p < 0.01$, Figure 4). BD did lead to significantly increased levels of GSSG which were significantly attenuated by MnTMPyP pre-treatment ($p < 0.01$). Also, BD led to an increased GSSG:GSH ratio which was significantly attenuated by MnTMPyP pre-treatment ($p < 0.05$).

![Figure 4](image)
**Figure 4.** Renal levels of reduced glutathione (GSH), oxidized glutathione (GSSG), total glutathione (GSH+GSSG) and the ratio of oxidized to reduced glutathione (GSSG:GSH) in sham-operated and brain-dead rats. MnTMPyP pre-treatment of brain-dead rats resulted in significantly increased GSH levels compared to saline-treated brain-dead rats. BD resulted in significantly increased GSSG levels and GSSG:GSH ratio which were reduced significantly by MnTMPyP pre-treatment. * and ** indicate p < 0.05 and 0.01, respectively, compared to vehicle-treated brain-dead rats. # indicates p < 0.05 compared to sham-operated rats.

**HO-1 protein expression**

BD led to a significant increase in renal HO-1 expression compared to sham-operated rats (p < 0.01, figure 5). This increase was significantly attenuated in rats pre-treated with MnTMPyP (p < 0.05).

**Figure 5.** Renal expression of the cytoprotective protein heme oxygenase-1 (HO-1) in sham-operated and brain-dead rats. BD resulted in significantly increased HO-1 expression compared to sham-operated rats which was markedly decreased by MnTMPyP pre-treatment. ## indicates p < 0.01 compared to sham-operated rats. * indicates p < 0.05 compared to saline-treated brain-dead rats. 100x magnification.
Granulocyte protein expression

BD led to a significant increase in renal cortical granulocyte expression compared to sham-operated rats (p < 0.01, figure 6). This increase was not decreased by MnTMPyP pre-treatment.

Figure 6. Renal influx of polymorphonuclear (PMN) leukocytes in sham-operated and brain-dead rats. BD resulted in significantly increased PMN influx compared to sham-operated rats which was not attenuated by MnTMPyP treatment. ** indicates p < 0.01 compared to sham-operated rats. 20x magnification.

Renal gene expression levels

Renal mRNA levels of interleukin-6 (IL-6), TNF-α, and E-selectin were significantly increased in brain-dead rats compared to sham-operated animals (Figure 7) which was not attenuated by MnTMPyP pre-treatment.

Figure 7. Renal expression of the pro-inflammatory genes IL-6, TNF-α, and E-selectin in sham-operated and brain-dead rats. BD led to significant increase in renal IL-6, TNF-α, and E-selectin expression. MnTMPyP pre-treatment did not result in decreased expression of these genes. # indicates p < 0.05 compared to sham-operated rats. 20x magnification.

Biochemical determinations

BD resulted in increased plasma creatinine levels and decreased creatinine clearance compared to sham-operated rats (table 3). Also, BD resulted in increased fractional sodium excretion. These effects were not affected by MnTMPyP pre-treatment.

Table 3. Biochemical parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham + saline</th>
<th>Sham + MnTMPyP</th>
<th>BD + saline</th>
<th>BD + MnTMPyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crs, μmol/L</td>
<td>34.86 ± 7.20</td>
<td>34.29 ± 6.45</td>
<td>36.00 ± 22.62 #</td>
<td>102.71 ± 27.80</td>
</tr>
<tr>
<td>Cr/C, ml/min</td>
<td>0.38 ± 0.04</td>
<td>0.37 ± 0.03</td>
<td>0.24 ± 0.08 #</td>
<td>0.28 ± 0.09</td>
</tr>
<tr>
<td>FeNa, %</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.7 ± 0.3 #</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Urinary NAG, U/mmol UCr</td>
<td>0.04 ± 0.03</td>
<td>0.03 ± 0.27</td>
<td>0.10 ± 0.36 ##</td>
<td>0.08 ± 0.13</td>
</tr>
</tbody>
</table>

Variables measured 4 hrs after BD or immediately after sham procedure
# indicates p < 0.05 compared to sham-operated rats
## significant at p < 0.05 compared to sham-operated rats

Urinary NAG activity

After 4 hours of BD, urinary NAG activity was significantly increased compared to sham-operated rats (p < 0.01, table 3). The increase in NAG activity was not decreased by MnTMPyP pre-treatment.
postulate that the observed lipid peroxidation in brain-dead donors renders cells more susceptible to I-R injury which leads to increased mitochondrial damage after reperfusion and thereby leads to increased risk of DGF.

The compound MnTMPyP was very effective in our model as renal and plasma MDA levels were reduced to sham values. However, in our study, as a proof of concept, MnTMPyP was administered before BD induction and not during BD. In future studies, the effect of MnTMPyP administration during BD should be evaluated to assess the clinical feasibility of this compound. Since MDA levels only become evident after 2 hours of BD, we hypothesize that the administration of MnTMPyP after BD confirmation will lead to the same effects as observed with pre-treatment.

Glutathione is regarded as a major anti-oxidative molecule because of its high cellular concentrations and the central role it fulfills in redox reactions. Glutathione can serve as a cofactor for anti-oxidative enzymes but can also react directly with oxidants such as superoxide. The glutathione disulfide (GSSG)/glutathione (GSH) ratio is a traditional marker for reflecting the level of cellular oxidative stress. In our study, BD led to an increased GSSG/GSH ratio which was decreased by MnTMPyP treatment. Interestingly, increased GSSG levels did not coincide with decreased GSH levels which indicates GSH levels are replenished during BD. In a previous study, we showed that renal GSH levels decreased after 2 hours of BD but returned to reference levels after 4 hours. Renal glutathione synthesis is totally dependent on the uptake of glutathione conjugates from the circulation. Therefore, the unchanged renal GSH levels could be the result of the increased plasma glutathione levels we observed during BD in the aforementioned study. Increased plasma glutathione is likely the result of glutathione release into the circulation through hepatic apoptosis which has been shown previously. Since renal GSH levels do not remain decreased during BD, increasing GSH levels in brain-dead donors could be futile. Indeed, it has been shown that the administration of n-acetylcysteine to brain-dead donors does not lead to decreased incidence of DGF in renal transplant recipients.

HO-1 may be among the most critical cytoprotective mechanisms that are activated during times of cellular stress such as inflammation, ischemia, hypoxia, hyperoxia, hyperthermia, or radiation. HO-1 has found to be increasingly expressed during BD which reflects the need of cytoprotection under these conditions. In this light, it is understandable that transplanting kidneys from brain-dead donors in which HO-1 was induced, led to better graft survival. However, in our study, renal HO-1 expression was significantly reduced by MnTMPyP treatment indicating a decrease of the trigger for HO-1 expression and a cardinal role of superoxide in the development of BD-related renal cellular stress. Transplanting kidneys from brain-dead donors treated with MnTMPyP could therefore lead to similar beneficial results compared to studies in which HO-1 was induced.

Through its antioxidant properties, MnTMPyP has shown to improve markers of inflammation, damage, and renal function in several models. In rat models of renal I-R injury, MnTMPyP treatment leads to decreased damage markers, renal levels of inflammatory markers, infiltrating granulocytes and plasma creatinine levels. However, MnTMPyP pre-treatment did not lead to the above-mentioned effects in the present study. The explanation for this observation could be that in I-R injury, anoxia is the primary insult which results in damage, an inflammatory process, and decreased renal function, whereas in BD, the relative ischemia is not severe enough to cause similar effects. Moreover, in BD, inflammatory processes are likely also initiated through other mechanisms than solely ischemia, such as the dying brain.

Figure 8. Proposed mechanism of decreased lipid peroxidation in brain-dead donors by MnTMPyP treatment and subsequent decreased risk of IR injury and DGF (simplified). Brain death leads to increased superoxide production through hemodynamic instability, inflammation, and altered metabolism. MnTMPyP treatment decreases superoxide levels and thereby lipid peroxidation. Lipid peroxidation levels in the brain-dead donor correlate positively with DGF in renal transplant recipients. Therefore, we postulate that decreased lipid peroxidation in the brain-dead donor leads to less susceptibility to IR injury and DGF.

Lipid peroxidation leads to membrane dysfunction and subsequent cell toxicity. It has been shown previously that lipid peroxidation related to aging leads to increased I-R injury upon reperfusion and results in damaged mitochondria. Recently, post-transplant mitochondrial damage was shown to cause a metabolic deficit resulting in the development of DGF. Since BD-related lipid peroxidation is associated with DGF, we
In conclusion, MnTMPyP pre-treatment of brain-dead rats leads to decreased renal oxidative stress during BD. Amongst others, the oxidative membrane damage marker MDA, which correlates with DGF, acute rejection, and post-transplant renal function in renal transplant recipients, was reduced by MnTMPyP pre-treatment. Therefore, treatment of brain-dead donors with MnTMPyP could lead to decreased incidences of above mentioned processes and thereby improved transplantation outcomes.

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REFERENCES


