Reduced Inflammatory Response of the Brain Dead Kidney Graft with Nyk pre-treatment

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Abstract

Introduction Kidney transplantation is ultimately needed for end-stage renal failure patients to maintain life. Short-term and long-term outcomes are higher with living kidney donation compared to heart-beating (HB) kidney donation. The discrepancy cannot be explained with differences in human leukocyte antigen difference or ischemia times. Comparisons between living kidney grafts and HB kidney grafts revealed that HB kidney grafts are inflammatory active at the time of donation. The inflammatory state induces histological damage, which enhanced ischemia/reperfusion injury in the recipient. Described here is a first attempt with pre-treatment of geranyl-geranyllactone (GGA)-derivate Nyk, to test if it can reduce the inflammatory active state in the kidney from HB donors via heat shock protein (HSP)-72 and heme oxygenase (HO)-1 expression.

Material and methods A graduate onset brain death model was used with male Fischer rats (N=36) to analyze the inflammatory and protective response of the kidney to brain death. Rats were divided in three groups: Nyk, GGA, and Saline. Inflammatory parameters were adhesion molecules E-selectin, Intracellular adhesion molecule (Icam)-1, and neutrophil and monocyte/macrophage infiltration.

Results A decreased up-regulation of E-selectin, Icam-1, and subsequent infiltration of neutrophil was seen after Nyk pre-treatment compared to GGA or Saline pre-treatment. HSP-72 showed broad expression with Nyk pre-treatment compared to low expression of GGA and Saline pre-treatment. HO-1 expression was low in all pre-treated HB animals.

Conclusion A reduced renal inflammatory response was seen with Nyk pre-treatment in brain dead rats compared to GGA or Saline pre-treatment. Nyk did not induce a significant higher expression of HSP-72 or HO-1. Other HSPs should therefore be analyzed to understand how Nyk pre-treatment reduces the renal inflammatory response. Short-term outcomes should be analyzed with ischemia/reperfusion experiments.
Samenvatting

Introductie Patiënten met eindstadium nierfalen hebben uiteindelijk een donornier nodig om te overleven. Er bestaan grote verschillen tussen de korte en lange termijn uitkomsten van levende en hersendode nierdonatie. Deze verschillen kunnen niet verklaard worden door verschillen in humaan leukocytenantigeen of ischemie tijden. Nieren van hersendode donoren blijken op het moment van donatie inflammatoir actief te zijn vergeleken met nieren van levende donoren. De inflammatie veroorzaakt histologische schade, wat de ischemie/reperfusie schade in de ontvanger versterkt. Dit onderzoek is een eerste poging met geranyl-geranylaceton (GGA)-derivaat Nyk voorbehandeling, om te onderzoeken of het de inflammatie in de nier van hersendode donoren kan verminderen via heat shock protein (HSP)-72 en heme oxygenaze (HO)-1 expressie.


Resultaten Een verminderde expressie van de adhesiemoleculen E-selectin en Icam-1 werd gemeten, tezamen met een verminderde infiltratie van neutrofielen na voorbehandeling met Nyk vergeleken met GGA of Saline voorbehandeling. HSP-72 liet een brede expressie zien, maar de expressie was hoger vergeleken met de andere groepen. HO-1 was in alle groepen laag.

Conclusie Een verminderde inflammatierespons was gemeten na voorbehandeling met Nyk vergeleken met GGA of Saline voorbehandeling. Nyk veroorzaakte geen significant hogere expressie van HSP-72 of HO-1. Andere HSPs zullen onderzocht moeten worden hoe een voorbehandeling met Nyk de inflammatierespons kan verminderen. Ischemie/reperfusie experimenten moeten de korte termijnuitkomsten duidelijk maken.
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**Introduction and rationale**

End-stage renal failure (ESRF) is the progressive end result of chronic kidney disease, and is defined as the need for permanent replacement therapy, i.e. dialysis or transplantation (1). The former however is not comparable with a functional kidney: it can only adopt some functions of the kidney and it is associated with a number of complications like bacterial peritonitis, cardiovascular disease, and sepsis (2). Therefore, renal transplantation is the treatment of choice for ESRF-patients as quality of life and long-term survival are increased compared to dialysis (3,4). Due to shortage of organ donors, hundreds of people are each year waiting for a kidney graft in The Netherlands (5).

**Kidney donation and transplantation**

There are two types of kidney donation: living and cadaveric. The cadaveric kidney donations are further subdivided in the heart-beating (HB) and non-heart-beating (NHB) donations, and this subdivision is related to the criteria of brain death. Brain death is defined as the irreversible loss of all function of the brain, including the brainstem (6). NHB donors do not meet the criteria for brain death, and therefore the donation is also called *donation after cardiac death*.

Short-term transplantation outcomes are defined as delayed graft function (DGF: the need for dialysis during the first week after transplantation) and primary non function (PNF: the non-functioning of the kidney graft during the first three months after transplantation). Long-term transplantation outcomes are defined as survival rates like one-year or five-year survival. Both outcomes vary among the donation types. Living has the best outcomes in both the short-term and the long-term (7-9). HB donation has better short-term outcomes than NHB donation, but the long-term are almost equal (10). The better rate in outcomes in living kidney grafts are above all due to the quality of the kidney grafts, but also due to the circumstances associated with living donation compared to cadaveric donations. A living donor is uniformly healthy; he is not brain dead, nor is he injured from trauma or cardiac failure. The donation has minimal warm ischemia time, and the cold ischemia time is very short as well because transplantation occurs right after donation.

In HB- and NHB donations, both the quality of the graft and the circumstances are less optimal. The inferior quality is due to donor conditions and due to the circumstances which are not optimal for donation. It is almost always in the nighttime, and a long cold ischemia time exists for transportation to the recipient’s hospital. Furthermore, a long warm ischemia time occurs with NHB donation. Besides the donation type, differences exist between recipients as well. Living donations are more often scheduled. The donor is often a relative, and the donor and recipient are prepared for the donation and transplantation procedure before the recipient is dialysis-dependent. Every recipient of a cadaveric kidney graft has been on the waiting list, the largest part for multiple years. In those years, these patients were dialyzing in the hope to survive the waiting period. Dialysis itself, and its complications drop the patient’s quality of life and the possibility to survive surgery (11). Half of the dialyzing patients will not survive a five year waiting time (12). These conditions associated with dialyzing recipients are also contributing to the lower outcomes after transplantation.
However, many differences between living and HB kidney donation do not explain the graft quality differences. Donors and recipients in humans are coupled with each other according to their human leukocyte antigen (HLA) match to prevent rejection. However, even fully HLA-mismatched living kidney grafts showed higher outcomes compared to reasonable matched HB kidney grafts (9). Furthermore, the differences in outcomes cannot be fully attributed to prolonged cold ischemia times for grafts procured from HB donors, because no significant effect of cold ischemia on kidney outcomes was seen (9). Studies with HB kidney donation have shown that the process of brain dead has a major contribution to the lower outcomes compared to living kidney donation (13-16). This effect was seen in histological differences in kidney grafts. Comparisons between living kidney grafts and HB kidney grafts revealed that HB kidney grafts are inflammatory active at the time of donation. That is, HB kidney grafts show a massive increase in leukocytes infiltration and subsequently histological damage as seen as pronounced expression of histocompatibility antigens, as will be described below.

After transplantation, these kidneys have a more severe ischemia/reperfusion injury and subsequently higher chance of DGF and PNF, hence decreasing short-term outcomes compared to the living kidney grafts (13,17,18). The link between ischemia/reperfusion injury and short-term outcomes can be explained with the hyperfiltration hypothesis. This postulates that kidneys with a reduced functioning nephron mass due to profound histological damage will progress toward failure due to hypertrophy of the remaining nephrons to meet the excess load, eventually leading to nephron exhaustion. The histological damage is furthermore a trigger for the recipient’s immune system to actively infiltrate the kidney graft, producing more damage after transplantation (19-21). The latter is called the reflow-paradox: reperfusion of the grafts result in severe injury by neutrophil infiltration and subsequent reactive oxygen substance production, followed by increasing numbers of infiltrating macrophages and T-cells (22-24). The net effect is a decreased functioning outcome and/or accelerated acute rejection (13,14).

**Brain death**

From the moment of a severe brain damaging insult, there is an active process initiated which may lead to brain death. The local cell death, inflammation, and edema from the insult will extend globally throughout the brain causing an increase in the intracranial pressure (ICP) (25,26). An increased ICP suppresses the arterial blood flow to the brain, causing enhanced brain damage and leads to brain death.

The period of brain damage till brain death is not restricted to the brain. Damage extends early to the blood brain barrier (BBB) cells as well, resulting in leakage of the produced pro-inflammatory cytokines into the systemic circulation (Figure 1) (27,28). The leakage into the systemic circulation will activate peripheral organs, including the kidneys. Pro-inflammatory cytokines cause endothelial activation (27,29,30), leading to the activation of mitogen-activated protein (MAP)-kinases: the cell’s regulatory proteins (Figure 6 in attachments). MAP-kinases include p38, nuclear factor kappa Beta (NFκβ), and extracellular signal-regulated kinases (ERK). These will eventually lead to up-regulation and expression of the adhesion molecules, and further up-regulation of pro-inflammatory cytokines like IL-6 (27,30-38). E-selectin and Icam-1 are important for diapedesis: The release of pro-inflammatory cytokines activate peripheral tissues like the kidney. This results, besides adhesion molecules up-regulation and further up-regulation of pro-inflammatory cytokines, in up-regulation of chemo attractants. Circulating leukocytes like neutrophils and monocytes are localized to the tissue which produces chemo attractants. E-selectin expressions on the vascular wall slows the circulating leukocytes down, and initiate leukocyte-rolling over the
vessel wall. Icam-1 expression then binds to rolling leukocytes, and assist with tissue-migration. The final result of these events is neutrophil and monocyte/macrophage infiltration in the kidney and histological damage, confirming the image of an inflammatory active kidney graft (16,27,31,33,35,36,39).

**Figure 1 Brain damage initiate a systemic inflammation response due to blood-brain barrier dysfunction and results in inflammatory active kidney grafts.** Activation of pro-inflammatory cytokines due to brain death and leakage of them into the systemic circulation result in peripheral endothelial activation, and subsequently in kidney inflammation via activation of mitogen-activated protein (MAP) kinase pathways. As well as the kidney, intestinal inflammation occurs, and results in bacterial translocation leading to endotoxemia. This induces a systemic inflammation response via bacterial toxins leading to inflammatory active kidneys. Ischemia is the results of hypothalamus necrosis. Massive catecholamines release induces the Cushing response to maintain adequate blood pressures to the brain, using severe vasoconstriction. This leads to hypoperfusion or ischemia to the peripheral organs. Ischemia leads to kidney inflammation via activation MAP kinase pathways.

IL-6 release in systemic circulation has been doubled to show the impact of endotoxemia to the kidney. ICP= intracranial pressure; MAP= mean arterial pressure; IL-6= interleukin-6

**Intestinal permeability hits the kidney**
As well as the kidney, intestinal inflammatory activation is seen in HB donors. As a consequence of intestinal inflammation, the permeability of the intestine increases, resulting in bacterial translocation, leading to endotoxemia (39-41). This activates endothelial cells, followed by the same reaction cascade with leukocytes infiltration and histological damage in the kidney as pro-inflammatory cytokines (**Figure 1**) (31,40,41).
Hemodynamic changes alters kidney perfusion

The damage due to brain death include the cells which control the sympathetic nervous system in the brainstem (25,42). Experiments with brain death induction in rodents showed a classical blood pressure pattern, with a hypotensive period during brain death induction, and a hypertensive period when brain dead is reached (Figure 1) (42,43). The net result of the hemodynamic changes is a reduced perfusion of the peripheral organs, including the kidney, leading to ischemic periods for the peripheral organs (44).

Besides changes in blood pressure, haemodynamic changes are furthermore related to inflammatory and hormonal alterations:

- The described events of inflammatory activation and impaired perfusion for the kidney are also happening to the heart, and induce a diminished cardiac output, thereby enhances the ischemia in the kidney (45,46).
- Among the damaged brain areas is the hypothalamic-pituitary-axis, which produces low amount of anti-diuretic hormone (ADH) in brain death donors. This is seen as diabetes insipidus (DI), thereby reducing the organ perfusion even more (45). DI can also be caused by the down-regulation of specific proteins, like aquaporin-2, a protein for urine concentration in the collecting duct cells (36,45).

The resulting ischemia of these effects injures the kidney graft. Ischemia leads to the disruption of the binding between NFκβ and its inhibitor, Inhibitor kappa B (I-κB) in renal cells. Activated NFκβ then induces the same inflammatory actions as explained above (47,49). Furthermore, ischemia leads to a metabolism shift from aerobic to anaerobic, reducing the energy levels in the cells, and induces either apoptosis or necrosis (45,50). Both leads to a decreased functioning mass and necrosis, and furthermore increases the inflammatory state of the kidney.

Protection mechanisms in the HB donor

The severe cellular stress associated with brain death initiates a protection response in the kidney, called the stress response, with up-regulation of heat shock proteins (HSPs) (35,51-53). HSPs were first discovered as a result of heat shock treatment on drosophila, which induced expression of a specific group of genes afterwards called the heat shock protein genes (54). Nowadays, it is known that these genes are inducible by a variety of stresses and drugs but are also conservatively expressed (55,56). Conservative HSPs function in assisting the folding of newly synthesized polypeptides, the assembly of multiprotein complexes and the transport of proteins across cellular membranes.

HSPs are divided in families, according to their approximate molecular mass, in which the HSP-70 family is the most studied and best known family. Within the family, HSP-72 is the best known stress-inducible variant. HSP-72 has been often studied, and results showed that it is up-regulated in brain dead animals, and humans (51,52). HSP-72 is a strong anti-apoptotic protein, and is also known for its anti-inflammatory functions (57,58). Its activation is dependent on heat shock factor (HSF)-1. In unstressed cells, it is bound to HSF-1 in the cytoplasm of every cell. In stressed cells, HSP-72 binds to damaged proteins, and is therefore dissociated from HSF-1. Free activated HSF-1 is then oligomerized, as seen in tripling of their molecular mass, before entering the nucleus. In the nucleus, HSF-1 binds to the heat shock responsive element (HSE) in the promoter of HSPs genes inducing transcription and translation HSPs including HSP-72 (59,60).

Besides HSP-72, another protective protein has been often studied: heme oxygenase (HO)-1 (also known as HSP-32). HO-1 is the inducible member of the HO family but its protective
effects are different. HO-1 has antioxidant capacities and acts as potent anti-inflammatory and anti-apoptotic protein whenever oxidative injury takes place (61-63). HO-1 is an enzyme that catalyze the conversion of heme into biliverdin, carbon monoxide and iron. The degraded heme products act as an antioxidant, a vasodilator, and inhibitor of platelet aggregation. This prevents tissues from oxidative injury, or reactive oxygen species (ROS). The activation mechanism of HO-1 has not been unraveled yet but could be related to the HSF-1 associated HSE-binding in the HSPs genes.

In brain dead human and animal donors however, the up-regulation of both HSP-72 and HO-1 is insufficient to protect renal cells (34,36,51). To increase the protecting abilities of HSP-72 and HO-1 in brain dead donors, their expression should be increased. Therefore, several attempts have been made to enhance the up-regulation with drugs administration for a better functioning graft after transplantation. Up-regulation of HSPs occurs, besides of the effects of brain dead, trough hyperthermia and drug administration. Hyperthermia is inducing a thermal stress response, and both HSP-72 and HO-1 expression has been successfully increased by administrating a heat shock to rats and in ischemia/reperfusion experiments (64,65). However, this has not been studied in humans because of the clinical problems of dispensing a heat shock to a donor. The idea is simple and effective, but in order to attempt this with a human donor, with a body hundred times as a rat, more research has to been done with bigger animals first. Administrating of a locally heat shock to the human body will probably result in an intolerable dermal pain and is therefore not a clinical applicable alternative. Therefore, a therapeutic approach prevails to enhance HSP up-regulation.

Up-regulation of HSP-72 has been successfully attempted in several studies by administrating Taurine (66), Herbimycin-A (67), mercury (68), and dexamethasone (69). HO-1 has, among others, been up-regulated due to cobalt-potoporphyrin (70) and the gene transfer of the recombinant adenovirus encoding rat HO-1 cDNA (71). Furthermore, HO-1 is up-regulated in ischemic experiments (72-74).

However, many of the HSP-72 and HO-1 inducers are toxic for humans. Therefore, studies started to experiment with geranyl-geranylacetone (GGA). GGA is an anti-ulcer drug developed in Japan which has been used clinically to treat gastritis or gastric ulcers since 1984 without serious adverse reactions. GGA boosts HSP-72 expression in gastric mucosa, small intestine, hearts, livers, and kidneys, via HSF-1 dissociation (Figure 7 in attachments) (75-79). However, GGA administration in animal experiments is orally and is therefore dependent on the metabolic activity of the animal. Intravenous administration therefore prevails, but GGA is difficult to dissolve. Recently, Nyk has been synthesized as a soluble derivate of GGA.

In this research, we will study the effects of Nyk pre-treatment on kidney graft quality in a brain dead rat model. Several inflammatory parameters will be measured in renal tissue after a four hour brain dead experiment.

**Aim**
The aim of this experiment is to study whether Nyk pre-treatment to brain dead rats can reduce the inflammatory active state and of the kidney after donation.

**Main objectives**
First we want to study whether Nyk pre-treatment reduces the adhesion molecules,
interleukin-6 expression, and infiltration of neutrophils and monocytes/macrophages in the kidney graft. Secondly, we want to study if Nyk pre-treatment increases the expression of HSP-72 and HO-1 in renal tissue. Thirdly, we want to study if the inflammatory expression with Nyk pre-treatment is lower compared to GGA pre-treatment.
Materials and Methods

Animals
Adult male Fischer F344 rats (N=36) (Harlan, Horst, the Netherlands), weighing 278-310 gram (293±1.75 gram) were used in this experiment. The animals were housed in the Central Animal Facility of the University Medical Centre Groningen (UMCG), and animal care was according to Experiments on Animals Act (1996) issued by the Dutch Ministry of Public health, Welfare and Sports. Freedom of access to food and water was accomplished. The animals were caged in groups in a light-dark cycle of 12h-12h and a temperature-controlled (22ºC) environment.

Nyk, GGA, and Saline preparation
Nyk was kindly provided by Nyken BV (Groningen, the Netherlands) and solubilized in 0.9% Sodiumchloride (NaCl) for infusion (Baxter BV, Utrecht, the Netherlands) and administered in a concentration of 0.56mg Nyk/kg rat weight.
GGA was purchased at Eisai Co., Ltd (Tokyo, Japan), and solubilized in 100% dimethyl sulfoxide(DMSO) (Merck KGaA, Darmstadt, Germany) and diluted in 10% Kleptose HPB parenteral grade (Roquette Co., Lestrem, France) with a final DMSO concentration of 0.5%. GGA pre-treatment was given in the same concentration as Nyk pre-treatment. The concentration are based on previous experiments with GGA administration in mice. Standard sterile bags with NaCl were used for Saline administration.

Experimental groups
To study the effects of intravenously (iv.) Nyk pre-treatment in the brain dead donor, 4 groups (n=8-9) were studied. All the groups received their primarily treatment for including 20 hours before brain death induction. The second treatment occurred 0 hours before induction. Brain death induction was the same in all animals, as described below.
Group 1: iv. Saline at 20h and 0h, group 2: iv. Nyk at 20h and 0h, group 3: iv. Saline at 20h and iv. Nyk at 0h, group 4: iv. GGA at 20h and 0h.

Brain death model
Brain death was induced as described previously (42). The procedure was as follows. Rats were anesthetised using isoflurane (Pharmachemie BV, Haarlem, the Netherlands) with 100% O2. Brain death induction started with placing a balloon-tipped cannula (Edwards Lifesciences Co., Irvine, CA) through a 1x1 mm frontolateral hole in the epidural space with the tip pointed towards caudal. Slow inflation of the balloon (16µl/min) simulated an epidural hematoma, leading to brain death. Blood pressure monitoring occurred with cannula insertion in the femoral artery. A second cannula in the femoral vein was used for hemodynamic drugs administering. Animals were intubated via a tracheostomy and mechanically ventilated throughout the experiment. Ventilation occurred with a small rodent ventilator (683 small animal ventilator, Harvard Apparatus, UK).
As a result of inflation, hemodynamic changes occurred starting with a hypotensive period followed by a graduate blood pressure increase. When the blood pressure reached 80 mmHg, balloon infiltration was stopped. The balloon was kept infiltrated throughout the experiment. Brain death was confirmed approximately 30 minutes after balloon inflation stop by dilated and fixed pupils, absence of corneal reflexes and a negative apnea test. After 30 min of brain death, the ventilated air was switched from 100% O2 to 50% O2 in air. Throughout the experiment, the temperature was maintained at 37ºC using a heating pad.
Animals were kept brain dead for 4 hours. If the blood pressure fell below 80 mmHg, it was restored with the following order of actions: compressing the rats body, lifting the backside of the rats body, decreasing post end expiratory pressure, decreasing ventilation rate, administering hydroxyethyl starch (Haes) 10% (Fresenius Kabi AG, Bad Homburg, Germany), administering noradrenalin 0.01mg/ml (Centrafarm services BV, Nieuwe Donk, the Netherlands). Exclusion criteria were a blood pressure below 80 mmHg for more than 10 min, or a maximal administration of 10 ml of liquids.

15 min before the end of the 4 hours of brain death, Suxamethoniumchloride (Centrafarm BV, Etten-Leur, the Netherlands) was given iv. to achieve full muscle relaxation to allow abdominal surgery. This was followed 10 min later with the administration of 500IU heparin (Leo Pharma BV, Breda, the Netherlands) iv. After 4 hours of brain death, blood and urine were collected from the aorta and bladder prior to aorta-flush with ice-cold NaCl. Right kidneys were removed and sliced in parts. One part was snap frozen in liquid nitrogen and one part was fixated in 4% paraformaldehyde. Blood was centrifuged for 10 min at 960g at 4°C. The resulting plasma were collected and stored at -80°C.

**Histology and immunohistochemistry**

Staining for neutrophils was performed on kidney cryosections (4µm) according to protocol. Sections were fixed using acetone. Endogenous peroxidise was blocked using 0.01% Hydrogen peroxide (H2O2) in phosphate-buffered saline (PBS) for 30 min. Sections were stained with primary granulocytes antibody His48 (Stressgen). Staining for monocytes/macrophages, cleaved caspase-3, and p38 were performed on kidney paraffin sections (4µm) according to protocol. Sections were de-waxed, rehydrated and subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl buffer at 80°C (pH=9.0, ED-1). Endogenous peroxidase was blocked with 0.03% H2O2 in PBS for 30 min. Primary antibody for p38 was anti-p38 antibody (Stressgen). Monocyte/macrophage primary antibody was ED-1 (Stressgen). Cleaved caspase-3 primary antibody was Asp175 (Stressgen).

Primary antibody was incubated on paraffin- and cryosections for 60 min at room temperature. After the initial primary antibody binding, the sections were washed with PBS for 5 min. Secondary and tertiary antibody (Dakopatts, Glostrup, Denmark) incubation lasted for 30 min at room temperature to detect binding of the primary antibody. Secondary and tertiary antibodies were added in PBS, which was supplemented with 1% bovine serum albumin (BSA) and 1% normal rat serum. Peroxidase activity was visualised using 9-aminoethylcarbazole (AEC) for cryosections, and 3,3′-diaminobenzidine tetrahydrochloride (DAB+, K3468; DAKO) for paraffin sections. Sections were counterstained with haematoxylin. Negative antibody controls were performed in all staining analysis.

**Morphometric analysis of histology and immunohistochemistry**

Stained paraffin- and cryosections were scanned with NanoZoomer 2.0-HT (Hamamatsu Photonics). Digital slides were assessed with the software program Aperio Imagescope (version 11.1.2.760, Aperio Thechnologies). Scoring occurred by two observers independently from each other. For each kidney section, positive cells were counted in 10 microscopic fields of the cortex and medulla at 200x magnification.
Western blotting
Per sample, six 20 μm cryosections were lysed in 200 μL RIPA buffer (1% NP40, 0.1% SDS, 10 mM β-mercaptoethanol) containing protease inhibitors (Complete, Roche). Samples were lysed on ice, centrifuged for 15 min at 16000g (4°C), and supernatant was collected. Protein concentrations were measured using the Lowry Protein assay (BioRad).

Equal amounts of protein were loaded on to SDS/PAGE (10% polyacrylamide gels). Proteins were transferred on to nitrocellulose membranes and incubated with HSP-72 antibody SPA-810 (StressGen). The house keeping-gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as loading control and was detected with mouse antibody (RDI Research Diagnostics). Blots were subsequently incubated with HRP (horseradish peroxidase)-conjugated antimouse secondary antibody (Amersham), and visualization was performed with ECL and hyperfilm. Detected signal was quantified and normalized for the GAPDH signal on the same blot.

RNA isolation and semi-quantitative RT-PCR
Total RNA was isolated from kidney cryosections using TRIzol™ (15504-020, Invitrogen) method and using a DNase treatment step with deoxyribonuclease I (AMP-D1, sigma Aldrich). The RNA quantity was measured using a spectrophotometer. RNA quality control was secured using electrophoresis. Positive samples showed no DNA contamination. Using Primer express 2.0 (Applied Biosystems, Foster city, CA), gene-specific primers were designed and gene-sequences were published (Table 1). Amplification and detection of the PCR products were performed with 7900 HT real-time polymerase chain reaction systems (Applied Biosystems) using SYBR Green (SYBR Green master mix; Applied Biosystems). All assays were performed in triplet. The samples were amplified as follows, first an activation step at 50°C for 2 min and a hot start at 95°C for 10 min. The PCR step consisted 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Specificity of the PCR products was routinely assessed by performing a dissociation curve at the end of the amplification program. Gene expression was related to the mean ß-actin gene expression from the same cDNA.

Table 1  qRT-PCR primer sequences for the genes (forward, reverse)  

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Bp</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>5’-GGAAATCGTGCGTGACATTAA-3’, 5’-GCCGCACGTGCGCATCTCC-3’</td>
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<tr>
<td>IL-6</td>
<td>5’-CCAACCTCCAATGCTCTCTCTAATG-3’, 5’-TTCAAAGTGCTTTCAAGAGTGGAT-3’</td>
<td>89</td>
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<tr>
<td>E-selectin</td>
<td>5’-GTCTGGATGCTGCTCTTTGTTAC-3’, 5’-CTGCCACAGAAAGTGCCACTAC-3’</td>
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<tr>
<td>Icam-1</td>
<td>5’-CCAGACCCCTGGAGATGAGAA-3’, 5’-AAGCGCTTTGTGATGATCC-3’</td>
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<tr>
<td>Bax</td>
<td>5’-CTGGGATGCGCTTGGGAGAA-3’, 5’-TCAGAGACAGCCAGAGAAAT-3’</td>
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<tr>
<td>Bcl-2</td>
<td>5’-CGCGCGCTGGTGATAA-3’, 5’-CTGTAAGGCCACCCAGAGTAT-3’</td>
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<td>HO-1</td>
<td>5’-ACTTTCAGAAGGGTCAAGTGGTCC-3’, 5’-TTGAGCAGAGGCGGTCATTG-3’</td>
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<tr>
<td>Hsp72</td>
<td>5’-CTGACAAAGAAGGGTCTGG-3’, 5’-AGCAGCCTCAAGAGTCTGCTC-3’</td>
<td>302</td>
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IL-6= interleuking-6, Icam-1= Intracelllar adhesion molecule-1, HO-1= heme oxygenase-1, HSP-72= heat shock protein-72, BAX and Bcl-2 are respectively pro- and anti-apoptotic genes; BP=base-paired.

Statistical analyses
All data were tested for normality using Q-Q plots and the Kolmogorov-Smirnov test. Normally distributed data were expressed as mean ± standard deviation of the mean. Non-normally distributed data are expressed as median± standard error and differences were tested with Mann-Whitney U tests. Correlations between variables were assessed with bivariate correlation and Pearson correlation coefficients. A P value < 0.05 was considered significant. Statistical analyses were performed using SPSS version 21.0 (SPSS Inc, Chicago, US).
Results

Surgery and brain death induction

The 4 hour brain death experiment succeeded with thirty-four of the thirty-six rats used for this experiment, resulting in an success rate of 94%. One rat of the Nyk group (group 2) died during the experiment as the result of a human error, one GGA pre-treated rat experiment was stopped due to an excessive hypotensive period. All rats received the same surgery and brain death induction method, performed by one of the two surgeons specialized in rodent-surgery working in the Surgical Research Laboratory. After anesthesia, the rats were prepared for brain death induction according to protocol. Group inclusion occurred 20 hours before, with the administration of either Nyk, GGA, or Saline iv. The inflammatory response of group 3 (iv. Saline at 20h and iv. Nyk at 0h), is not included in this report as this group was not included in the objectives.

Figure 2 shows the mean arterial pressure (MAP) of the groups during the brain death experiment, grouped as the induction period (-30 till 0 min) and the 4 hour brain death period. After the initial hypotensive period, an increase is seen in blood pressure. However, we did not see a sharp peak as seen in humans and animal experiments (42). We see normotensive levels for the first 1.5 hours after confirmed brain death, followed by hyper-/normotensive levels for the Saline pre-treated group, and normotensive levels for the Nyk and GGA pre-treated group. The MAP difference between the groups were not significant (Table 2). The prominent drop in MAP at the end of the brain death period is due to surgical procedures, which always started 3.75 hours after confirmed brain death for ureter cannulation, followed by aorta perfusion at exactly 4 hours of confirmed brain death.

In our experiments, after reaching the threshold MAP level we stopped balloon inflation and tried to maintain normotensive levels. Hypertensive levels were not corrected. Hypotensive levels which were not correctable with body changing or ventilator adjustments were corrected with Haes and/or noradrenalin. The amount of Haes and noradrenalin administration in the groups were not significant different from each other (Table 2).
We furthermore evaluated if administration of either one or both could influence the inflammatory response outcome, therefore we tested for correlation (Table 3 and 4).

### Table 2

<table>
<thead>
<tr>
<th>Mann-Whitney U results of independent parameters (median±standard error)</th>
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<td>Nyk</td>
</tr>
<tr>
<td>Brain death induction time (min)</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
</tr>
<tr>
<td>Weight (gram)</td>
</tr>
<tr>
<td>Haes administration (ml)</td>
</tr>
<tr>
<td>Noradrenalin administration (ml)</td>
</tr>
</tbody>
</table>

*Haes = Hydroxyethyl starch; GGA=geranyl-geranylace tone*

### Table 3

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Nyk</th>
<th>GGA</th>
</tr>
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<tr>
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<td>Noradrenalin</td>
<td>Haes</td>
</tr>
<tr>
<td>E-selectin</td>
<td>0.33 (P=0.42)</td>
<td>0.54 (P=0.17)</td>
</tr>
<tr>
<td>Icam-1</td>
<td>0.56 (P=0.15)</td>
<td>0.46 (P=0.25)</td>
</tr>
<tr>
<td>WB HSP-72</td>
<td>0.69 (P=0.06)</td>
<td>0.39 (P=0.34)</td>
</tr>
<tr>
<td>mRNA HSP-72</td>
<td>0.56 (P=0.15)</td>
<td>0.27 (P=0.52)</td>
</tr>
<tr>
<td>mRNA HO-1</td>
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<td>0.25 (P=0.56)</td>
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<tr>
<td>mRNA IL-6</td>
<td>0.71 (P=0.05)*</td>
<td>0.46 (P=0.25)</td>
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<tr>
<td>Neutrophil infiltration</td>
<td>0.36 (P=0.38)</td>
<td>0.31 (P=0.46)</td>
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<tr>
<td>M/M infiltration</td>
<td>-0.41 (P=0.31)</td>
<td>-0.11 (P=0.79)</td>
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<tr>
<td>mRNA BAX</td>
<td>0.28 (P=0.50)</td>
<td>-0.03 (P=0.95)</td>
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<tr>
<td>mRNA Bcl-2</td>
<td>0.24 (P=0.56)</td>
<td>0.31 (P=0.46)</td>
</tr>
<tr>
<td>mRNA BAX/Bcl-2 ratio</td>
<td>0.13 (P=0.76)</td>
<td>-0.20 (P=0.64)</td>
</tr>
</tbody>
</table>

*P-value is significant (< 0.05) WB= Western blot; mRNA= messenger ribonucleic acid; M/M= monocyte/macrophage; GGA= geranyl-geranylace tone; Haes= hydroxyethyl starch*

### Table 4

<table>
<thead>
<tr>
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<td>Noradrenalin</td>
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<tr>
<td>E-selectin</td>
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</tr>
<tr>
<td>Icam-1</td>
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</tr>
<tr>
<td>WB HSP-72</td>
<td>0.13 (P=0.76)</td>
</tr>
<tr>
<td>mRNA HSP-72</td>
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</tr>
<tr>
<td>mRNA HO-1</td>
<td>0.07 (P=0.85)</td>
</tr>
<tr>
<td>mRNA IL-6</td>
<td>0.71 (P=0.03)*</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0.38 (P=0.31)</td>
</tr>
<tr>
<td>M/M infiltration</td>
<td>-0.37 (P=0.32)</td>
</tr>
<tr>
<td>mRNA BAX</td>
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<tr>
<td>mRNA Bcl-2</td>
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</tr>
<tr>
<td>mRNA BAX/Bcl-2 ratio</td>
<td>-0.77 (P=0.02)</td>
</tr>
</tbody>
</table>

*P-value is significant (< 0.05) WB= Western blot; mRNA= messenger ribonucleic acid; M/M= monocyte/macrophage; GGA= geranyl-geranylace tone; Haes= hydroxyethyl starch*
Real-Time Polymerase Chain Reaction (RT-PCR)

Multiple genes were tested to determine the renal response to brain death in both groups. Levels of mRNA expression were measured for interleukin (IL)-6, E-selectin, and Icam-1 for the inflammatory response. HSP-72 and HO-1 were tested for the protective response. Pro-apoptotic BAX and anti-apoptotic Bcl-2 were measured for the intrinsic apoptotic cascade. (Figure 4 and Table 5).

**Table 5**

| mRNA expression levels of the inflammatory and protective response (median±standard error) |
|-----------------------------------------------|-----------------------------------|
| **Nyk** | **GGA** | **Saline** |
| E-selectin | 0.48±0.08 | 0.76±0.21 | 0.87±0.14 |
| Icam-1 | 0.48±0.07 | 0.67±0.06 | 0.76±0.08 |
| IL-6 | 0.54±0.46 | 1.44±0.39 | 1.43±0.70 |
| HSP-72 | 0.92±0.20 | 0.91±0.27 | 0.64±0.12 |
| HO-1 | 0.60±0.09 | 0.63±0.13 | 0.61±0.07 |
| BAX | 1.89±0.61 | 1.99±0.17 | 1.81±0.17 |
| Bcl-2 | 0.59±0.04 | 0.69±0.04 | 0.78±0.08 |
| BAX/Bcl-2 ratio | 3.28±0.39 | 2.77±0.34 | 2.32±0.21 |

RT-PCR results showed a significant difference in the renal up-regulation of the genes coded for E-selectin, Icam-1 and Bcl-2 between Nyk pre-treatment and Saline pre-treatment. E-selectin was decreased 1.81 fold (P=0.00), Icam-1 1.58 fold (P=0.04) and Bcl-2 1.32 fold (P=0.02). IL-6 mRNA expression was decreased 2.65 fold but no significant value was reached. No difference in BAX expression was seen.

Significance difference between Nyk pre-treatment and GGA pre-treatment were seen in Icam-1 and IL-6 expression. Icam-1 mRNA expression was decreased 1.40 fold (P=0.04), and IL-6 mRNA 2.67 fold (P=0.05) with Nyk pre-treatment. The expression of E-selectin mRNA was reduced 1.58 fold, but the result was insignificant. No difference was seen in BAX and Bcl-2 expression.

The protective response showed no significant differences between Nyk, GGA, and Saline pre-treatment. mRNA HSP-72 expression was the highest with Nyk pre-treatment and lowest with Saline pre-treatment. HO-1 mRNA expression was highest with GGA treatment, and lowest with Nyk pre-treatment. However, mRNA HO-1 expression were low in all animals.
Western Blot
HSP-72 protein expression was measured with Western blotting. One sample of the Saline pre-treated group was not included in this test due to technical problems. Western blotting showed an increase of 1.88 fold in HSP-72 expression in the Nyk pre-treated compared to Saline pre-treatment, and an increase of 1.36 fold compared to GGA pre-treatment, but both results were not significant (Figure 3). A broad range is seen in the HSP-72 expression in the Nyk pre-treated group compared to the GGA and Saline pre-treated group.

Figure 3 HSP-72 protein expression between Nyk, GGA, and Saline pre-treatment.
Differences were not significant.
Broad expression of HSP-72 was seen in the Nyk pre-treated group.

The protein expression of HSF-1 showed no differences between Nyk, GGA, or Saline pre-treatment (results not shown).

Immunohistochemistry
The inflammatory response was besides RT-PCR and Western blot measured with immunohistochemistry. Cellular infiltration of the cortical and medullar segments of the right kidney was measured with staining with granulocytes antibody (HIS48) for neutrophil infiltration and anti-CD68 antibody (ED-1) for monocytes/macrophages infiltration.

Infiltration of neutrophils was significantly reduced with Nyk pre-treatment compared to GGA pre-treatment or Saline pre-treatment. The average infiltration per microscopic field for Nyk pre-treated rats was 3.14±0.23 versus 4.26±0.27 for GGA pre-treated rats (P=0.01) and 5.89±0.35 for Saline pre-treated rats (P=0.00) (Figure 5).

Monocytes/macrophages infiltration showed no significant difference between the pre-treated groups. Nyk pre-treatment showed an average infiltration per microscopic field of 7.73±1.71. Infiltration with GGA pre-treatment was 8.05±1.56 and Saline pre-treatment levels were 8.52±2.37 (results not shown).

Staining for cleaved caspase-3 and p38 showed no difference in expression, as concluded by an immunohistochemistry expert (results not shown)
**Figure 4** mRNA expression of E-selectin, Icam-1, IL-6, HSP-72, HO-1, and BAX/Bcl-2 ratio with Nyk, GGA, and Saline pre-treatment. Significant differences between Nyk and Saline pre-treatment were seen in E-selectin and Icam-1. Between Nyk and GGA pre-treatment, significant differences were seen in Icam-1 and IL-6. HSP-72 and HO-1 were both insignificant different between all pre-treated groups.
Figure 5 Neutrophil infiltration stained with HIS48 antibody in Nyk (E&F), GGA (C&D), and Saline (A&B) pre-treatment. Infiltration was significantly reduced with Nyk pre-treatment compared to Saline or GGA pre-treatment. Median±standard error values infiltration per field for Nyk pre-treatment was 3.14±0.23, for GGA pre-treatment 4.26±0.27 and for Saline pre-treatment 5.89±0.35. Magnification=200x.
Discussion

Patients with end-stage renal failure (ESRF) are in need of a kidney graft to maintain life. There is a significant difference in short- and long-term outcomes between living kidney donation and heart-beating (HB) kidney donation. The latter provides inflammatory active kidney grafts with histological damage that results in lower short- and long-term outcomes. To improve kidney graft outcomes from HB donors, influencing the inflammatory state of the organ donor has been the focus of experiments the last years. One option to improve HB kidney grafts is reducing the inflammatory state of the graft. However, the HB donor cannot be treated before brain death is confirmed and a lot of damage has already occurred before confirmation. Therefore, increasing the response of the kidney to the damaging process associated with brain death is a more clinically relevant option. This study is based on earlier results with geranyl-geranylacetone (GGA), but elaboration takes place with the recently developed drug Nyk. As a derivate of GGA, it is better soluble and should theoretically accomplish improvement in outcomes. Described here is a first attempt with Nyk pre-treatment, to test if it can reduce the inflammatory active state of the kidney in HB donors via heat shock protein (HSP)-72 and heme oxygenase (HO)-1 expression.

Using Nyk pre-treatment in a graduate onset brain death model with rats, we observe a significant reduction in inflammatory parameters compared to Saline pre-treatment or GGA pre-treatment.

The significant differences with Saline pre-treatment were seen in the inflammatory parameters E-selectin, Icam-1, and neutrophil infiltration. The expression of mRNA IL-6 was more decreased after Nyk pre-treatment, but the result did not reach a significance value.

Significant differences with GGA pre-treatment were seen in the mRNA expression of IL-6 and Icam-1, and neutrophil infiltration. The results of mRNA E-selectin expression were lower in the Nyk pre-treated group, but the result was insignificant.

Discrepancy was seen in the infiltration of monocytes/macrophages compared with other studies (15,44,80). This could be the result of a different antigen staining. We have used ED-1, which is specific for circulating monocytes who have infiltrated into the tissue. The studies which showed increased monocytes/macrophages infiltration have stained with ED-2, specific for infiltrating monocytes and resistant macrophages. Both the monocytes and macrophages are inducing histological damage, but ED-2 was not available in our experiment period. Therefore, staining for ED-2 has to be performed in the future as we do believe that the low results are contradictory with the other anti-inflammatory results. Furthermore, one study found high levels of monocyte chemoattractant protein (MCP)-1. It might help to measure MCP-1 as well to know whether MCP-1 expression is decreased as well.

Neutrophil granulocytes are known to be increased in several inflammatory diseases, and induce histological damage by releasing of cytokines and phagocytosis (81-83). As described in the introduction, inflammation is the main insult in HB kidney donation and provides the opportunity for neutrophil granulocytes to enter the tissue and induce histological damage. The result of histological damage is seen as an increased amount of histocompatibility antigens. These increase the ischemia/reperfusion injury and initiate a more severe immune response in the recipient after transplantation. Neutrophil granulocytes infiltration is therefore associated with accelerated graft rejection in several studies (13-15,84). In theory, the reduced infiltration due to Nyk pre-treatment should therefore reduce the immunogenicity of the kidney graft, improve short-term and eventually long-term outcomes. We have tried to measure histological damage with apoptosis and necrosis.
analyzing. However, necrosis analysis could not be done during the analyzing period. Apoptotic analyzing showed no differences in BAX/BCL-2 ratio, initiators of the intrinsic apoptosis cascade (Table 5 and Figure 4). To test if apoptosis was executed we have stained for cleaved caspase-3. The staining provided no differences between the groups, as concluded with inspection from an immunohistochemistry expert (results not shown). However, we believe that only limited histological damage have occurred at the time of donation. As described, the damage done by brain death leads to a pronounced expression of histocompatibility antigens. Histological damage itself, by apoptosis and necrosis, is more the result of ischemia/reperfusion injury than brain dead injury (85). However, this conclusion cannot be made without necrotic analyzing.

How Nyk induces a reduction in the inflammatory parameters is still unknown. In contrast to the described hypothesis, we did not find a significant up-regulation of HSP-72 and HO-1 in the Nyk pre-treated rats. The mRNA expression of HSP-72 was increased 1.33 fold, whereas HO-1 mRNA expression was even reduced compared to the other groups. However, because of the possibility of only a short-term up-regulation of mRNA expression, we have measured HSP-72 protein expression with Western blot. Western blot material for HO-1 was not available during the analyzing period. HSP-72 protein expression was increased 1.95 fold and 2.16 fold compared to respectively GGA and Saline pre-treatment, but the results were still insignificant. This could be due to the small size group, but as seen in Figure 3, HSP-72 protein expression has a broad expression in the Nyk pre-treated group. We tried to find an answer to the broad protein expression in the Nyk pre-treated group. A correlation with one of the other measured parameters like hemodynamic drug administration, brain death induction time, mean arterial pressure during the brain death experiment, or the weight of the rats was not found. None had a correlation level sufficient enough to explain the differences (Table 9 in attachments). Furthermore, we looked at the possibility if gaining experience with the experiment was a factor, that is, if the HSP-72 expression was highest or lowest at the last rats compared to the first ones. But the expression was various high and low among the rats so no explanation could be found there as well. Therefore, it is possible that the insignificant results are dose-dependent combined with a still unknown other factor. As described, our experiment was done with one more group, group 3 (Nyk and Saline). Group 3 received half of the dosage for group 2 (Nyk). In group 3, mRNA expression and Western blot showed a higher up-regulation of both mRNA and protein expression compared to the Saline group, but the increase was less compared to group 2. Still, no correlations could be made which explain the differences in HSP-72 up-regulation but the comparison revealed a dose-dependent up-regulation of HSP-72. To test if our animals induce a different HSP-72 expression compared to other studies, a heat shock induction could be valuable. Heat shock is known to increase HSP-72 up-regulation, and it should be interesting to see if there will still be various HSP-72 up-regulations after heat shock induction (64,65). If so, an independent factor is influencing the HSP-72 expression and should be ruled out for further experiments. Lastly, the dosage for each rat has been measured according to their metabolic weight and the metabolic weight of the GGA mouse treatment. It is possible that the comparison with mice was not sufficient and therefore lower dosages were administered for the rats who showed low expressions of HSP-72.

The low expression of mRNA HO-1 is indicating that Nyk pre-treatment does not increase the up-regulation of mRNA HO-1. In other studies, up-regulation of HO-1 was successfully and showed to be protective, as described in the introduction. It is possible that lower expressions are seen due to the ischemic state of our rat kidney grafts. All of the described studies
included ischemic periods. It is possible that our low HO-1 expression is associated with limited ischemia due to maintaining the blood pressure >80 mmHg. In Figure 2 is seen that after brain death induction, no hypotensive period has occurred in both groups. Also, no hypertensive period right after induction is seen which is different from the described animal model (42). We have not applied different animals or material in our method, but the discrepancy could be explained by a different end induction points and differences in blood pressure stabilization. One hypothesis is that we were more anticipating for the high peak pressure and therefore we stopped the induction period sooner. Another (additional) hypothesis is that we maintained normotensive values with less constricting renal vessels and subsequently higher renal perfusion by altering the respiratory frequency and –amplitude prior to hemodynamic drugs administration.

Besides the measured expression of HSP-72 and HO-1, they showed furthermore no significant correlation with the inflammatory parameters to explain the reduction in E-selectin, Icam-1, and neutrophil infiltration (Tables 6, 7 and 8 in attachments). Therefore, a possibility for the low HSP-72 and HO-1 outcomes is that other HSPs are functioning instead with Nyk pre-treatment. Therefore, HSF-1 expression has been measured, as HSF-1 is not only inducing HSP-72 expression. The HSF-1 expression was indifferent between the groups, but the 4 hours of brain death could be too long for HSF-1 up-regulation due to Nyk pre-treatment. Therefore, HSF-1 should be tested with Western blot and other HSPs should be measured with RT-PCR and Western blot. The functioning of Nyk pre-treatment on inflammatory reduction could be related to other HSPs than HSP-72 or HO-1. Plain brain death induction induced only the expression of HSP-72 and HO-1, and not of HSP-27 and HSP-40 (52). It could be possible that the latter two are up-regulated due to Nyk pre-treatment and therefore they should be analyzed. Other studies showed an increased expression of HSP-90, HSP-25, and HSP-27 after (pre-)treatment (86-89). Therefore, other HSPs should be analyzed prior to other experiments to see how Nyk pre-treatment reduces the inflammatory response.

The reduction of inflammatory parameters with Nyk pre-treatment could be, regardless the HSPs expression, due to inhibition of mitogen-activated protein (MAP) kinases. Other studies have described that several cellular pathways are involved in the brain dead damaged kidneys, like up-regulation of MAP kinases. In these studies, the produced inflammatory cytokines in brain death activate the cell’s MAP kinases by binding to the gp130 receptors, which induce phosphorylation of JNK, p38, and ERK pathways through the JAK/STAT pathways. As a result of p38 activation, nuclear-factor-kappa-bêta (NFkB) is activated, and is translocated to the nucleus where it influence the gene-expression of among others IL-6, E-selectin, and Icam-1 (27, 30-38). Because we observe a reduced expression of E-selectin and Icam-1, the possibility that the MAP-kinases pathways are inhibited by Nyk pre-treatment arise. We have not measured all the MAP-kinases, because that would be too expensive and time-taken. Because NFkB staining was not available in the analyzing period, we stained for p38, as this is the main inducer of NFkB and therefore the pro-inflammatory state in the cell. No differences were seen between Nyk, GGA, or Saline pre-treatment (results not shown). However, the indifferent result does not have to be contradictory because p38 is involved in many cellular processes (37, 90). Therefore, more analysis have to be done to analyze the effect of Nyk pre-treatment on MAP-kinases pathways, because the inflammatory influence of JNK, p38 and NFkB inhibition has been observed in other studies (91-95). Because NFkB is involved in many cellular processes, an ischemia/reperfusion experiment should be performed to analyze the anti-inflammatory response to see if NFkB is directly inhibited by Nyk pre-treatment.
Secondly, if NFκB effects on the inflammatory response is inhibited, the complete pathway from pro-inflammatory cytokines binding till NFκB expression should be analyzed to understand how Nyk pre-treatment reduces the up-regulation of the adhesion molecules and subsequently neutrophil infiltration. At last, a transplantation model should be done with Nyk treatment after brain death induction to analyze the inflammatory reduction when the donor is not pre-treated, as is the clinical manner.

**Conclusion**

We believe that Nyk pre-treatment is a potential suitable drug for the heart-beating donor to reduce the inflammatory state of the kidney graft. The inflammatory expression is decreased compared to GGA pre-treatment. At the moment of organ retrieval, Nyk pre-treatment reduces the expression of adhesion molecules and subsequently infiltration of neutrophil granulocytes and possible monocyte/macrophages. If the reduction is due to HSP-72 expression or HO-1 expression is unlikely, but more analysis has to be performed to measure the expression of other HSPs first. The result of Nyk pre-treatment should be further studied with ischemia/reperfusion experiments to analyze short-term outcomes.

**Acknowledgements**

Above all, I want to thank Leon van Dullemen and Henri Leuvenink for their supervising, their approach to introduce me to translational research, and for the discussions and advisements. Secondly, I am truly thankful for the introduction to the many analyzing techniques and advice of Petra Ottens, Susanne Veldhuis, Janneke Wiersma, Rik Mencke and Jacco Zwaagstra.

Lastly, I could not perform the research without the help of Dane Hoeksema and Jelle Adelmeijer and I want to thank them for their positive influence.
References


**Attachments**

**Table 6**  Correlation levels of Nyk pre-treated HSPs expression with inflammatory parameters

<table>
<thead>
<tr>
<th>HSP</th>
<th>HO-1</th>
<th>WB HSP-72</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-selectin</td>
<td>0.04 (P=0.92)</td>
<td>0.43 (P=0.40)</td>
</tr>
<tr>
<td>Icam-1</td>
<td>-0.05 (P=0.91)</td>
<td>0.60 (P=0.12)</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.20 (P=0.63)</td>
<td>0.55 (P=0.16)</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>-0.69 (P=0.06)</td>
<td>0.03 (P=0.94)</td>
</tr>
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<td>M/M infiltration</td>
<td>0.65 (P=0.08)</td>
<td>0.79 (P=0.02) *</td>
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</table>

HSPs= heat shock proteins, HO-1 = heme oxygenase-1, WB = Western blot, M/M= monocyte/macrophage, IL-6 = interleukin-6. * P-value is significant (<0.05)

**Table 7**  Correlation levels of GGA pre-treated HSPs expression with inflammatory parameters

<table>
<thead>
<tr>
<th>HSP</th>
<th>HO-1</th>
<th>WB HSP-72</th>
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<tbody>
<tr>
<td>E-selectin</td>
<td>-0.21 (P=0.62)</td>
<td>-0.61 (P=0.11)</td>
</tr>
<tr>
<td>Icam-1</td>
<td>-0.49 (P=0.22)</td>
<td>0.31 (P=0.45)</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.04 (P=0.94)</td>
<td>0.46 (P=0.31)</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0.36 (P=0.38)</td>
<td>0.07 (P=0.88)</td>
</tr>
<tr>
<td>M/M infiltration</td>
<td>0.36 (P=0.38)</td>
<td>0.07 (P=0.88)</td>
</tr>
</tbody>
</table>

HSP= heat shock proteins, HO-1 = heme oxygenase-1, WB = Western blot, M/M= monocytes/macrophage, IL-6 = interleukin-6. * P-value is significant (<0.05)

**Table 8**  Correlation levels of Saline pre-treated HSPs expression with inflammatory parameters

<table>
<thead>
<tr>
<th>HSP</th>
<th>HO-1</th>
<th>WB HSP-72</th>
</tr>
</thead>
<tbody>
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<td>E-selectin</td>
<td>0.10 (P=0.80)</td>
<td>0.16 (P=0.65)</td>
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<td>Icam-1</td>
<td>-0.09 (P=0.82)</td>
<td>0.12 (P=0.76)</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.40 (P=0.29)</td>
<td>-0.36 (P=0.35)</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>-0.60 (P=0.09)</td>
<td>-0.65 (P=0.06)</td>
</tr>
<tr>
<td>M/M infiltration</td>
<td>-0.21 (P=0.60)</td>
<td>-0.07 (P=0.87)</td>
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</tbody>
</table>

HSP= heat shock proteins, HO-1 = heme oxygenase-1, WB = Western blot, M/M= monocytes/macrophage, IL-6 = interleukin-6. * P-value is significant (<0.05)
Table 9  Correlation level between HSP-72 protein expression and parameters associated with brain death induction and brain death experiment.

<table>
<thead>
<tr>
<th>WB HSP-72</th>
<th>Noradrenalin</th>
<th>0.69 (P=0.06)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haes</td>
<td>0.39 (P=0.34)</td>
</tr>
<tr>
<td></td>
<td>Weight rats</td>
<td>-0.07(P=0.88)</td>
</tr>
<tr>
<td></td>
<td>Brain death induction time</td>
<td>0.20 (P=0.64)</td>
</tr>
<tr>
<td></td>
<td>Mean arterial pressure</td>
<td>-0.40(P=0.33)</td>
</tr>
</tbody>
</table>

* P-value is significant (<0.05). Haes= Hydroxyethyl starch; WB= Western blot

Figure 6. Brain dead associated peripheral inflammation, as described in article reference number 26. Pro-inflammatory cytokine IL-6 produced in the brain bind to IL-6 receptors in the kidney. It activates MAP kinases pathway, in which NFκB induce transcription of adhesion molecules genes and further pro-inflammatory cytokines. IL-6= interleukin-6, MAP= mitogen active protein, NFκB = nuclear factor kappa B
Figure 7. Induction with GGA of HSP-72 (here described as HSP70) via HSF-1 activation.
GGA= geranyl-geranylacetone, HSF-1= heat shock factor-1