Donor pre-treatment with the heat shock protein-inducer geranylgeranylacetone reduces brain death-associated inflammation in the kidney at organ retrieval
Abstract in English

Brain dead-derived kidney grafts have inferior transplantation outcomes compared to living donated kidneys. Factors leading to inferior organ quality are diminished organ perfusion and pro-inflammatory changes during the brain death period. The protective heat shock proteins (HSPs) are known to be up-regulated, but at the end of brain death. To reduce the brain death-related kidney injury, we want to increase the Hsp72 expression at the start of brain death. In this study we investigated whether geranylgeranylacetone (GGA), a Hsp72 inducer, can reduce the pro-inflammatory changes and improve kidney donor quality in an in vivo brain death rat model.

Male F344 rats (275-300g, n=15) underwent slow induction of brain death and were kept brain dead for 4 hours. We administered GGA (400 mg/kg orally) or a saline vehicle 20h and 1h prior to brain death induction. Sham-operated animals (n=14) received the same treatment.

At the moment of organ retrieval, the expressions of Hsp72 or other HSPs are not increased by GGA. However, kidney interleukin-6 (IL-6) mRNA levels in GGA pre-treated brain dead rats were lower compared to saline-treated controls. Systemic ASAT levels were also reduced by GGA, indicating decreased inflammation.

These results suggest that GGA reduces pro-inflammation during the brain death period, despite the unchanged expression of Hsp72. To further explore the benefits of GGA on organ quality, we would need to transplant the GGA pre-treated brain death kidneys.

Samenvatting in het Nederlands

Nieren verkregen van hersendode donoren hebben een verminderde overleving vergeleken met nieren verkregen van levende donoren. Factoren die gedurende de hersendode periode leiden tot deze verminderde orgaan kwaliteit zijn verminderde orgaan perfusie en verhoogde inflammatie. Van de beschermende heat shock eiwitten (HSPs) is bekend dat deze zijn opgereguleerd aan het einde van de hersendode periode. Om de nier schade verbonden met hersendood te verminderen willen we de Hsp72 expressie al aan het begin van de hersendode periode verhogen. In deze studie is onderzocht of geranylgeranylaceteton (GGA), een Hsp72 inducer, de inflammatoire veranderingen kan verminderen en de nier donor kwaliteit kan verhogen in een in vivo hersendood rat model.

Mannelijke F344 ratten (275-300g, n=15) ondergingen langzaam inductie van hersendood en werden stabiel gehouden gedurende 4 uur. We hebben GGA (400 mg/kg oraal) of een zoutoplossing toegediend, 20 uur en 1 uur voorafgaand aan de hersendood inductie. Controlegeopereerde ratten (n=14) kregen dezelfde behandeling.

Opvallend is dat de expressie van Hsp72 of andere HSPs niet zijn verhoogd met GGA voorbehandeling. Echter, nier interleukine-6 (IL-6) mRNA niveaus in GGA voorbehandelde hersendode ratten waren lager dan in zoutoplossing behandelde controles. Systemische ASAT niveaus werden ook verminderd door de GGA voorbehandeling, wijzend op een verminderde ontsteking.

Deze resultaten suggereren dat GGA de ontsteking vermindert tijdens de hersendode periode, ondanks de ongewijzigde expressie van Hsp72. Om de positieve effecten van GGA op orgaan kwaliteit verder te onderzoeken, zouden we GGA voorbehandelde hersendode nieren moeten transplanteren.
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Introduction

Kidney transplantation is the treatment of choice for most people with end-stage renal failure. In the Netherlands, the majority of donor kidneys are obtained from deceased brain-dead (BD) donors with a cerebrovascular haemorrhage on trauma (1), although due to persistent organ shortage the number of living kidney donations are increasing, as well as deceased cardiac donors.

Pathophysiologic changes during BD affect organ quality. Deceased BD donor kidneys have lower survival rates and higher rates of delayed graft function compared to living donated kidneys (2). These differences in graft survival cannot be fully explained by differences in warm and cold ischemia times, or human leukocyte antigen (HLA) mismatches between donor and recipient (2), suggesting a role for brain death. Brain ischemia results in non-function of the central nervous system associated with hemodynamic instability, systemic hormonal changes, and diminished perfusion of peripheral organs (3). This stress reaction results in a progressive up-regulation of pro-inflammatory mediators, increased cellular infiltration, and other inflammatory changes in the donor organs (4,5). These changes may enhance the immunogenicity of the grafts and increase the risk of acute graft rejection (6).

Pharmacological treatment of the BD donor to improve organ quality by reducing the exposure to stress and inflammation is a novel approach for improving transplantation outcome. Up-regulation of heat shock proteins (HSPs) is such a pharmacological treatment option which might benefit donor organ quality.

Stressed cells induce the expression of protective genes, known as the heat shock protein genes. These highly conserved HSPs were first discovered in Drosophila. Pre-treating Drosophila for several minutes with an increased temperature (7), also known as a sub-lethal heat shock, markedly improved tolerance to ischemia. Other stressors to induce HSPs are e.g. ischemia, anoxia, surgical stress, heavy metal ions, ethanol, and viral agents.

HSPs are classified into six families according to their molecular size in kDa: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small HSPs (e.g. HO-1). Within a family, there are different HSPs with different functions. There is also a distinction between constitutively or inductively expressed HSPs. Hsp72 is the inducible HSP in the Hsp70 family of which expression is rapidly increased in response to stress (8).

Intracellular HSPs function as molecular chaperones, assisting in the folding of native proteins in their correct formation. HSPs also assist in refolding, or degeneration of damaged and denatured proteins. In this way, HSPs prevent accumulation of protein aggregates and thereby inhibit the induction of apoptosis (9), and prevent the release of pro-inflammatory mediators through inhibition of the NF-kB-gene (10). However, when a cell becomes necrotic, its cytoplasm with the HSPs are released. These extracellular HSPs can then activate the innate immune system (11,12) by antigen presentation of chaperoned molecules (13).

Despite this immunomodulating effect, studies have demonstrated that up-regulation of HSPs in mice and rats provides cytoprotection against ischemia reperfusion injury (IRI) in vivo in kidney (10), heart (14), lung (15), small intestine (16), and liver (17).

The role of HSPs has also been suggested to be protective in human heart- and liver transplantations, since lower expression of Hsp72 levels in grafts correlates with early rejection (18,19).

Thermal stress was the first discovered method to provide HSP-induced cytoprotection, however, this is not a suitable procedure for use in donors. A drug known to rapidly increase HSP
expression is geranylgeranylace-tone (GGA) which has been developed and used in Japan to treat gastritis and gastric ulcers for more than 25 years without serious adverse effects.

HSP up-regulation by GGA was first reported in gastric mucosa, where its working mechanism has been partly elucidated in rodents. GGA directly stimulates transcriptional activation of the Hsp72 gene (20). Binding of GGA to the hydrophobic c-terminal of Hsp72 results in the dissociation of Heat Shock Factor 1 (HSF1) (21). Free HSF1 becomes phosphorylated and binds to the Heat Shock Element (HSE) in the promoter region of the Hsp72 gene (22). HSF1 is considered to be most important for the stress induced expression of Hsp72 (23). In order for GGA to induce HSP expression, its hydrophobic region has to be maintained. This makes GGA impossible to solubilise, but it can be emulsified. Therefore, GGA can only be administered orally or peritoneally.

The optimal dosage for up-regulating HSPs in rat kidney was found to be orally 2 times 400mg/kg, 24h and 1h before harvesting the organs (24). Administration of GGA to healthy rats 1 day before harvesting the kidney increased Hsp72 expression with 200% (24,25,26), whereas no difference was found in the expression of other HSPs (24,26). However, the combination of GGA with a known stress stimulus for HSP expression increases the heat shock response even more (27). The combination of BD stress with GGA has never been investigated before and might also induce other HSPs that were not up-regulated in the kidney by GGA treatment alone. GGA administration appears to have systemic effects since increased Hsp72 expression was also found in vivo in rat liver(17,28), heart(28,29), lung(30), intestine(31), and gastric mucosa(20).

Therefore, GGA seems to be a suitable drug for up-regulating HSPs, protecting multiple organs against the stress reaction during BD.

Protective effects of GGA pre-treatment have been shown in several in vivo experiments. GGA pre-treatment greatly increased the survival rate in a syngeneic rat liver transplantation model (17). In this experiment the survival of vehicle-treated rats was 0% after two days, whereas the survival in GGA pre-treated rats was 100% after 7 days. These protective effects of GGA were attributed to the increased expression of Hsp72 and Hsp90 (17). GGA also exerts protective effects in vivo against IRI in heart (29),(32), and drug induced damage in rat lung (30). In a kidney IRI model, pre-treatment of rats with GGA improved the survival rate, and protected the kidney against apoptosis and infiltration of monocytes (24). This effect was abolished after administration of Quercetin, which is an Hsp72 inhibitor (24). Similarly, the protective effects of GGA were abrogated in Hsp72 knockout mice which were subjected to kidney IRI (25).

Although brain death-related stress increases Hsp72 expression after 30 min BD, and HO-1 expression after 4 hours BD (33), we think that this up-regulation of HSPs is not high enough and develops not fast enough to protect the donor kidney against the pro-inflammatory changes during brain death. To protect the donor kidney, we want to up-regulate HSP expression before the induction of brain death by administration of GGA in a brain death rat model.
Aim
The aim of this study is to investigate whether administration of geranylgeranylacetone (GGA) to brain dead rats can improve the quality of donor kidneys by increasing the expression of heat shock proteins.

Main objectives
First we want to investigate whether GGA induces expression of HSPs in kidney. Secondly, we want to investigate whether the administration of GGA in brain dead rats can modulate the pro-inflammatory response in the kidney by decreasing interleukine-6 levels, influx of neutrophils and macrophages, and prevent cellular injury. Third, we want to investigate whether GGA has systemic effects in this brain death model by analysing the liver for Hsp72 expression and inflammation.
Materials and Methods

Animals
Adult male Fischer F344 rats (N=32), weighing 278-310 gram, were used (Harlan, Horst, the Netherlands). Animals were housed in cages at 22˚C with a light-dark cycle of 12/12h and were allowed free access to food and water. Acclimatization period was 1 week before starting experiments. The experiments were approved by the local animal care committee and performed according to the guidelines of the Institutional Animal Care and Use Committee following National Institutes of Health guidelines.

Experimental groups
To study the effects of oral GGA administration in the brain dead (BD) donor, 4 groups (n=8) were studied. Group 1: sham-operated treated with saline, group 2: sham-operated treated with GGA, group 3: 4h BD treated with saline, group 4: 4h BD treated with GGA.
GGA (400 mg/kg) was administered 20h and 1h before the BD induction or the sham operation (group 2 and 4). GGA was purchased at Eisai CO., Ltd (Tokyo, Japan), and emulsified in 5% arabic gum (51198-250G, Sigma Aldrich). Control rats received saline with 5% arabic gum 20h and 1h before the BD induction or the sham operation (group 1 and 3).

Brain death model
Brain death was induced as described previously (34). The procedure was as follows. Rats were anesthetised using isoflorane (Pharmachemie BV, Haarlem, the Netherlands) with 100% O2. One cannula was inserted in the femoral artery to monitor blood pressure (BP), a second cannula was inserted in the femoral vein to administer drugs. Animals were intubated via a tracheostomy and ventilated throughout the experiment. a no. 4 Fogarty catheter (Edwards Lifesciences Co., Irvine, CA) was placed subdurally via a frontolateral drillhole in the skull. The catheter was slowly inflated (16µl/min) with saline using a syringe pump (Terufusion, Termo Co., Tokyo, Japan).
This slow inflation model simulates an epidural hematoma leading to brain death. During balloon inflation, a hypotensive period occurred. When BP returned to 80 mmHg, inflation of the balloon and anaesthesia were stopped. The balloon was kept inflated throughout the experiment. BD was confirmed by the absence of corneal and pupillary reflexes, and an apnoea test. After 30 min of BD the ventilated air was switched from 100% O2 to 50% O2 in air. Temperature was monitored rectally and kept constant at 37˚C. Animals were kept BD for 4 hours (Supplementary figure 1). If BP 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 lung pressure, decreasing ventilation rate, administering HAES 10% (Fresenius Kabi AG, Bad Homburg, Germany), administrating noradrenalin 0.1mg/ml (Centrafarm services BV, Nieuwe Donk, the Netherlands).
Exclusion criteria were a BP below 80 mmHg for more than 10 min, or a maximal administration of 10 ml of liquids.
After 4 hours of brain death, 500 IU heparin (Leo Pharma BV, Breda, the Netherlands) was used 5 min before the end of the BD period. Recuronium bromide 0.6mg/kg (D50162.A, Sandoz BV, Almere, the Netherlands) was used 10 min before the end of the BD period to achieve full muscle relaxation and allow abdominal surgery. Blood and urine were collected from the aorta and bladder. Organs were flushed with ice-cold saline via the aorta. Kidney and liver were removed and snap frozen in liquid nitrogen or fixated in 4% paraformaldehyde. Blood was centrifuged for 10 min at 960g and 4˚C. Blood plasma was collected and stored at -80˚C. Sham
operated rats (same operation except insertion of the balloon catheter) served as controls. Anaesthesia continued for 30 minutes after the sham operation to mimic the BD induction period. Blood, urine, and organs were collection directly hereafter.

**Histology and immunohistochemistry**

Staining for polymorphonuclear cells (PMNs) was performed on kidney and liver cryosections (5µm). Sections were fixated using acetone. Endogenous peroxidise was blocked using 0.01% H2O2 in phosphate-buffered saline (PBS) for 30 min. Sections were stained with primary antibody His-48 mAB (supernatant, two times diluted).

Staining for Hsp72 and ED-1 (monocytes) were performed on kidney paraffin sections (4µm). Sections were de-waxed, rehydrated and subjected to heat-induced antigen retrieval by microwave heating in 1 mM EDTA (pH=8.0, Hsp72), or 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 antibodies included Hsp72: SPA-810 (Stressgen), ED-1: MCA341R (Serotech). Incubation of the primary antibodies on paraffin- and cryosections lasted for 60 min at room temperature (RT), binding of the antibody was detected by incubation with appropriate peroxidase-labelled secondary and tertiary antibodies (Dakopatts, Glostrup, Denmark) for 30 min at RT. Antibody dilutions were made in PBS 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 order to assess morphologic changes, kidney paraffin sections (4µm) were de-waxed, rehydrated and subjected to periodic acid-Schiff (PAS) reagent.

**Morphometric analysis of histology and immunohistochemistry**

PAS-stained kidney sections were assessed by an experienced pathologist in a blind fashion. 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).

Infiltration of PMNs in renal and liver tissue was assessed in His-48-stained cryosections. Kidney sections were scored by two observers in a blind fashion. For each tissue section, His-48 positive cells were counted in 10 microscopic fields of the cortex at 200x magnification. Positive staining in renal and liver sections were also calculated with Aperio Imagescope software including a cytoplasmic IHC algorithm. This algorithm is based on the spectral differentiation between red (positive) and blue (counter) staining (supplementary figure 2). Values are expressed as percentage strong positive surface area.

Infiltration of monocytes in renal tissue was assessed in ED-1-stained paraffin sections. Renal cortex tissue was assessed with Aperio Imagescope software using the same cytoplasmic IHC algorithm. Values are expressed as percentage strong positive surface area.

Hsp72 expression in renal cortex tissue was assessed in Hsp72-stained paraffin sections. Positive staining was assessed with Aperio Imagescope software using the same cytoplasmic IHC algorithm. Values are expressed as percentage strong positive surface area.

Hsp72 was also stained in liver paraffin sections. Hsp72 expression could not be assessed with Aperio Imagescope software.
Biochemical determinations
At the Laboratory Centre of the University Medical Centre Groningen, the following measurements were determined in a routine fashion: Creatinine in plasma and urine, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) enzyme activity in plasma and urine, lactate dehydrogenase (LDH) activity in plasma (based on the conversion rate of lactate to pyruvate), and urea concentration in plasma. Sodium and potassium in plasma and urine was determined with flow photometry.

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-Nitrophenol and N-acetyl-β-D-glucosaminidase. Enzymatic activity was expressed as the amount of enzyme required to release 1µmol of product per minute. NAG levels were normalized for urine creatinine levels and expressed as U/mmol UCr.

Interleukin-6 (IL-6) levels in plasma were measured using a rat IL-6 duoset ELISA (DY506, R&D systems, Abingdon, UK). IL-6 levels were expressed as ng/mL.

Western blotting
Per sample, six 20 μm cryosections were lysed in 200 µL RIPA buffer (1% NP_40, 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 Hsp72 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 qRT-PCR
Total RNA was isolated from kidney and liver cryosections using the TRIzol (15504-020, Invitrogen) method and using a DNase treatment step with deoxyribonuclease I (AMP-D1, sigma Aldrich). RNA quality was verified for absence of DNA contamination by performing real-time polymerase chain reactions (RT-PCR) in which reverse transcriptase was omitted using glyceraldehyde-3-phosphate dehydrogenase primers. Gene-specific primers were designed using Primer express 2.0 (Applied Biosystems, Foster city, CA) and published gene-sequences. Primers are shown in (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 β-actin gene expression from the same cDNA.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Bp</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>5′-GGAAATCGTGCGTGACATTAA-3′, 5′-GGGCCAGTGCCATCTC-3′</td>
<td>74</td>
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<tr>
<td>IL-6</td>
<td>5′-CCAATTCGCTGCCTCCTTCTAATG-3′, 5′-TTCAAGTGGCTTTCAAGAGTTGGAT-3′</td>
<td>89</td>
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<td>E-selectin</td>
<td>5′-GTCTCGATGCTGCCTACTTG-3′, 5′-CTGCCAGAAAGTGCCACTAC-3′</td>
<td>73</td>
</tr>
<tr>
<td>Bax</td>
<td>5′-CCGCGCGTCGTTGTTAAT-3′, 5′-CTGTAAAGGCCACCCAGTAGTAT-3′</td>
<td>70</td>
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<tr>
<td>Bcl-2</td>
<td>5′-AAGGCTTCTACCTGGCTCCAG-3′, 5′-ACATGGCTACATCTCTCGGTG-3′</td>
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<tr>
<td>Hsp27</td>
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<td>HO-1</td>
<td>5′-CTGACAAGAAAGGTTGCTGG-3′, 5′-AGCAGCCATCAAGGCTCTGTC-3′</td>
<td>302</td>
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<td>Hsp72</td>
<td>5′-CATGCCACACAGATTCTTTAATGGTT-3′, 5′-GATGCTTACCTTTACCTTGATAAT-3′</td>
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**Statistical analyses**

The distribution of data was assessed with Q-Q plots and the Kolmogorov-Smirnov test for normality. Normally distributed data are expressed as mean ± standard error of the mean. Non-normally distributed data are expressed as median [IQR] and differences were tested with Mann-Whitney U tests. Correlations between variables were assessed with one-way ANOVA, after logarithmic transformation if required. A p value < 0.05 was considered significant. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc, Chicago, US)
Results

Brain death experiments

Induction of brain death showed a consistent blood pressure pattern as described before (34). The induction period took approximately 30 minutes. Blood pressure was kept at a mean arterial pressure of at least 80 mmHg during the 4h BD period. Brain dead groups had an average infusion of 3.2 mL HAES 10% and 1.6 mL noradrenalin (NA) to maintain stable blood pressure. There was no difference in administration of HAES (P=0.34) or NA (P=0.54) between brain dead groups.

Protein expression of Hsp72 in kidney and liver

Hsp72 quantities in kidney and liver were measured with western blotting and normalized for GAPDH on the same blot. No difference was found in renal and liver Hsp72 protein expression between BD or sham-operated rats. GGA pre-treatment in BD and sham-operated rats did not increase Hsp72 expression measured in renal and liver tissue measured with western blotting (figure 1). Hsp72 expression was also assessed with IHC for kidney and liver tissue. Hsp72 expression in liver could not be quantified with Aperio Imagescope software. In cortical renal tissue no difference was found in Hsp72 protein expression between BD or sham operated rats. GGA pre-treatment did not affect liver (data not shown) or cortical renal Hsp72 expression measured with IHC (figure 2).

Figure 1 (A) Kidney and (B) Liver westernblots at 72 kDa (C) Western blotting showed no difference in Hsp72 expression between BD and sham-operated rats in kidney and liver tissue. GGA pre-treatment did not increase the Hsp72 expression in both kidney and liver tissue. GGA: geranylgeranylacetone; BD: brain dead
Hsp72-staining in the renal cortex. (A) Sham-operated rat, (B) brain dead rat, (C) sham-operated rat pre-treated with geranygeranylacetone (GGA), (D) brain dead rat pre-treated with GGA. (E) No difference was found in Hsp72 expression between BD and sham-operated rats in kidney tissue. GGA pre-treatment did not increase the amount of Hsp72 expression in kidney tissue.

**Gene expression of heat shock proteins in kidney and liver**
qRT-PCR showed an increase in renal Hsp27 (P=0.013), HO-1 (P<0.01), and Hsp90a (P<0.01) mRNA levels of brain dead rats compared to sham-operated rats. qPCR showed no difference in renal Hsp72 mRNA levels of brain dead rats compared to sham-operated rats (figure 3). GGA pre-treatment did not increase renal HSP mRNA expression. In liver tissue, Hsp72 mRNA levels are increased in brain dead rats compared to sham-operated controls (P<0.01). GGA pre-treatment had no effect on liver Hsp72 mRNA levels in brain dead rats (Figure 4).
Pro-inflammatory gene expression in the kidney and liver

qRT-PCR showed an increase in renal mRNA levels of IL-6 (P<0.01) and E-selectin (P<0.01) of brain dead rats compared to sham-operated rats. The ratio of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) expression was calculated, reflecting the amount of apoptosis.

The renal Bax/Bcl-2 mRNA ratio was increased (P<0.01) in brain dead rats compared to sham-operated rats. GGA pre-treatment decreased IL-6 mRNA levels in brain dead rats (P=0.011). The effect of GGA in brain dead rats was not significant (P=0.148).

GGA: geranylgeranylacetone; BD: brain dead

Figure 4  qRT-PCR showed an increase in liver (A) Hsp72 (P<0.01), (B) IL-6 (P<0.01) mRNA levels of brain dead rats compared to sham-operated rats. GGA pre-treatment decreased IL-6 mRNA levels in brain dead rats (P=0.011). The effect of GGA in brain dead rats was not significant (P=0.148).

GGA: geranylgeranylacetone; BD: brain dead

Figure 5  qRT-PCR showed an increase in renal mRNA levels of (A) IL-6 (P<0.01) and (B) E-selectin (P<0.01) of brain dead rats compared to sham-operated rats. (C) Bax/Bcl-2 mRNA ratio was increased (P<0.01) in brain dead rats compared to sham-operated rats. GGA pre-treatment decreased IL-6 mRNA levels in brain dead rats (P=0.015). GGA pre-treatment did not affect E-selectin mRNA levels or the Bax/Bcl-2 ratio in brain dead rats.

GGA: geranylgeranylacetone; BD: brain dead; IL-6: interleukin-6
**Biochemistry after brain death in blood and urine**

Blood and urine were collected after the end of the animal experiments. After brain death, creatinine levels in plasma were 88.7 µL ± 6.9, compared to 40.6 µL ± 2.9 in sham operated rats (P<0.01). In urine, no differences were found in creatinine levels between brain dead and sham groups (5.8 mmol/L ± 0.52, P=0.71). After correcting urine samples for urine creatinine levels, no difference was found between sodium (10 ± 1.56, p=0.32) and potassium (21.6±2.1, P=0.09) concentrations between BD and sham-operated rats.

To evaluate cellular damage, plasma lactate dehydrogenase (LDH), plasma and urine aspartate aminotransferase (ASAT), plasma aminotransferase (ALAT), plasma urea concentration, plasma interleukine-6 (IL-6), and urine N-acetyl-β-D-glucosaminidase (NAG) were measured (table 2). ASAT plasma levels were increased after BD, and GGA pre-treatment significantly reduces ASAT plasma levels (P=0.04). The differences found in plasma IL-6 (P=0.07) (figure 6), LDH (P=0.19), and ALAT (P=0.23) levels were not significant between BD control and GGA pre-treated rats. No effect of GGA administration was seen on the other injury parameters.

**Table 2**  Cellular injury parameters in plasma and urine (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=7)</th>
<th>Sham + GGA (n=7)</th>
<th>BD (n=7)</th>
<th>BD + GGA (n=8)</th>
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<tbody>
<tr>
<td>Plasma Urea (mmol/L)</td>
<td>12.1 ± 0.4</td>
<td>11.4 ± 0.3</td>
<td>20.6 ± 0.8 *</td>
<td>20.0 ± 0.6</td>
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<tr>
<td>Plasma LDH (U/L)</td>
<td>120.4 ± 8.4</td>
<td>148.1 ± 15.2</td>
<td>342.1 ± 50.1 *</td>
<td>251.1 ± 38.2</td>
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<tr>
<td>Plasma ASAT (U/L)</td>
<td>79.6 ± 5.2</td>
<td>74.7 ± 3.4</td>
<td>166.3 ± 15.0 *</td>
<td>120.1 ± 9.4 †</td>
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<tr>
<td>Plasma ALAT (U/L)</td>
<td>66.3 ± 10.1</td>
<td>58.0 ± 3.2</td>
<td>114.3 ± 14.6 *</td>
<td>84.6 ± 5.6</td>
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<tr>
<td>Plasma IL-6 (ng/mL)</td>
<td>1.5 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>260 ± 55.8 *</td>
<td>134 ± 16.6</td>
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<tr>
<td>Urine ASAT (U/mmol UCr) ‡</td>
<td>3.3 ± 0.5</td>
<td>6.1 ± 1.7</td>
<td>9.4 ± 1.8 *</td>
<td>9.6 ± 1.3</td>
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<tr>
<td>Urine NAG (U/mmol UCr) ‡</td>
<td>0.11 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.13 ± 0.03</td>
</tr>
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</table>

BD: brain dead; GGA: geranylgeranylacetone; LDH: lactate dehydrogenase; ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase; IL-6: Interleukine-6; NAG: N-acetyl-β-D-glucosaminidase

* significant at P<0.05 as compared to sham-operated controls
† significant at P<0.05 as compared to BD
‡ Urine samples are corrected for urine creatinine levels

**Figure 6**   Concentration of systemic interleukin-6 (IL-6) levels in plasma, measured in brain dead (BD) rats. Pre-treatment of brain dead rats with geranylgeranyllactone did not significantly decrease systemic IL-6 levels (P=0.075). Systemic IL-6 levels in sham-operated rats were too low measure.
Histological findings in renal and liver tissue
The renal cortex showed no morphologic changes between sham-operated and brain dead rats. GGA pre-treatment did not affect the renal morphology (figure 7). The number of infiltrating monocytes in the renal cortex were not increased by brain death, GGA pre-treatment did not affect the number of infiltrating monocytes (figure 8). Brain death did increase the amount of infiltrating polymorphonuclear cells (PMNs) in kidney (P<0.01) (figure 9) and liver tissue (P<0.01) (figure 10). No effect of GGA was found on the number of infiltrating PMNs.

To validate the quantification method with Aperio Imagescope software, we have quantified His-48-stained kidney sections by hand in a blind fashion and with Aperio Imagescope software. The results of both quantification methods were comparable (Supplementary figure 3).

Figure 7  PAS-staining in the renal cortex. (A) Sham-operated rat, (B) brain dead rat, (C) sham-operated rat pre-treated with geranygeranylacetone (GGA), (D) brain dead rat pre-treated with GGA. No morphological changes of the renal cortex was found between sham-operated and brain dead rats. Images were taken at 200x magnification.

Figure 8  Infiltrating monocytes positive for ED-1 in the renal cortex. (A) Sham-operated rat, (B) brain dead rat, (C) sham-operated rat pre-treated with geranygeranylacetone (GGA), (D) brain dead rat pre-treated with GGA. (E) No difference in the infiltration of monocytes was found between sham-operated and brain dead rats. No effect of GGA was found on the number of infiltrating monocytes. Images were taken at 160x magnification.
Figure 9  Infiltrating polymorphonuclear cells (PMNs) positive for His48 in the renal cortex. (A) Sham-operated rat, (B) brain dead rat, (C) sham-operated rat pre-treated with geranygeranylacetone (GGA), (D) brain dead rat pre-treated with GGA. (E) Brain dead rats show an increased infiltration of PMNs compared to sham-operated rats (P<0.01), no effect of GGA was found on the number of infiltrating PMNs. Images were taken at 110x magnification.

Figure 10  Infiltrating polymorphonuclear cells (PMNs) positive for His48 in the liver. (A) Sham-operated rat, (B) brain dead rat, (C) sham-operated rat pre-treated with geranygeranylacetone (GGA), (D) brain dead rat pre-treated with GGA. (E) Brain dead rats show an increased infiltration of PMNs compared to sham-operated rats (P<0.01), no effect of GGA was found on the number of infiltrating PMNs. Images were taken at 110x magnification.
**Linear regression analyses**

Renal Hsp72 protein expression was found to correlate significantly with systemic IL-6 expression and renal IL-6 mRNA expression. Hsp72 protein expression also correlates with the influx of PMNs and monocytes in renal cortex tissue. Hsp72 expression did not correlate with the Bax/Bcl-2 ratio or E-selectin mRNA levels (table 3).

Renal Hsp72 mRNA expression correlates with renal Bax/Bcl-2 ratio, however, Hsp72 mRNA levels did not correlate with renal IL-6 or E-selectin mRNA levels (table 4).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Univariate linear regression outcomes of Hsp72 protein expression in the renal cortex on different inflammatory parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Adjusted R²</td>
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<td>Systemic IL-6 plasma levels</td>
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<td>Renal IL-6 mRNA levels</td>
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<td>Renal PMNs infiltration</td>
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<td>Renal monocyte infiltration</td>
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<td>Renal Bax/Bcl-2 mRNA ratio</td>
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<tr>
<td>Renal E-selectin mRNA levels</td>
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PMNs, polymorphonuclear cells; IL-6, interleukin-6; Bax/Bcl-2 mRNA ratio, reflecting the degree of apoptosis

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Univariate linear regression outcomes of Hsp72 mRNA expression in the renal cortex on different inflammatory parameters</th>
</tr>
</thead>
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<td>Variable</td>
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<td>Renal Bax/Bcl-2 mRNA ratio</td>
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<tr>
<td>Renal IL-6 mRNA levels</td>
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<td>Renal E-selectin mRNA levels</td>
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PMNs, polymorphonuclear cells; IL-6, interleukin-6; Bax/Bcl-2 mRNA ratio, reflecting the degree of apoptosis
Discussion

GGA has been shown to have HSP-inducing capacity in kidneys of orally pre-treated rats (24). The enhanced expression of HSPs in rat kidney reduces the harmful effects of ischemia reperfusion injury (IRI) (24) inevitably related to transplantation. This effect is also documented in other organs (17,30,32), suggesting a systemic effect of GGA.

In this present study we show that GGA reduces the pro-inflammatory in rat kidney related to brain death. IL-6 mRNA levels in GGA pre-treated brain dead rat kidneys and livers are lower compared to untreated brain dead rat kidneys. However, systemic IL-6 plasma levels from GGA pre-treated brain dead rats are not significantly different from untreated brain dead rats.

After brain death induction by balloon inflation, the severely damaged brain could leak IL-6 in the systemic circulation via the blood brain barrier, increasing the systemic IL-6 concentrations. We suspect this could mark the decreased release of IL-6 from other transplantable organs, which are less inflamed by the pre-treatment of GGA. GGA also reduces systemic ASAT levels, a different inflammatory marker, released by the mitochondria of several organs like the liver, kidney, heart, muscles, and the brain. Combining these results, we show that GGA reduces inflammation in the brain dead rat kidney at the moment of organ retrieval.

Remarkably, GGA does not increase the expression of HSPs in renal or liver tissue in sham-operated rats. We hypothesized that GGA needs a stress stimulus to increase or boost HSP expression. However, the HSP expression is also not increased in our brain dead rat kidneys or livers pre-treated with GGA. We can not explain this result, since our GGA pre-treated brain dead rat kidneys show decreased IL-6 mRNA levels, proving that GGA does exert a protective function. Hsp72 is one of the inducible HSPs, which should be up-regulated in the presence of a stress stimulus (8). The brain dead rats did receive stress since Hsp27, HO-1, and Hsp90a are all up-regulated in brain dead rat kidneys compared to sham-operated controls. In a previous study Hsp72 expression was increased after 30 min. of brain death, however the Hsp72 expression was already reduced at the time point of 4h (33). An explanation for the unchanged expression of Hsp72 at 4h in GGA pre-treated brain dead rats in this present study is that the combination of GGA with the brain death stress stimulus is not stressful enough to maintain up regulation of Hsp72 in this window of 4h.

Suzuki et al. (24) have shown up regulation of Hsp72 after GGA pre-treatment in rats subjected to 15 min of IRI. IRI produces massive stress and injury to the kidney, reflected in a great amount of morphological changes in the renal cortex. In our experiment, brain death stress does not produce any morphological changes like necrosis or swollen cytoplasm. Another explanation for the unchanged Hsp72 expression could be the used antibody, although this is very unlikely since we have used the same antibody as Suzuki et al. (24). Also, our PCR data show the same results as our westernblots and Hsp72-stainings.

In this study we have also correlated the renal protein and mRNA expression of Hsp72 to several pro-inflammatory parameters. We show that higher Hsp72 protein expression in the kidney is correlated with lower systemic IL-6 and lower renal mRNA IL-6 levels. This indicates that Hsp72 has anti-inflammatory effects, probably by preventing injury in stressed cells. Unexpectedly, we found that Hsp72 also correlates with the infiltration of polymorphonuclear cells and monocytes. This is in contrast with other experiments (24), although it has been suggested that HSPs have immunomodulating functions by presenting antigens to immune active cells in the extracellular environment (37).
**Limitations**

Oral administration of GGA to a brain dead donor would not be practical, intraperitoneal injection of a GGA solution would be more suitable for administration. We also expect that the uptake of GGA is faster via intraperitoneal injection. In our experiment we have treated 3 rats with an intraperitoneal injection of GGA, unfortunately all rats presented with signs of severe inflammation within 20 hours after injection. In our setting, we were not able to administer GGA in a sterile manner. Therefore, we could only test the effects of oral administration of GGA. Administration of GGA 20h before brain death induction is not possible in the human clinical setting. Since GGA has anti-inflammatory effects in this brain death model, the next step would be administration of GGA after the induction of brain death. Another limitation of this study is the brain death rat model. The results in this model are extremely variable. As is shown in the results, there are large variations in the outcomes within the same treatment groups. Furthermore, in this research design it is not possible to predict the outcome of transplantation since there is no IRI or the possibility of rejection.

**Recommendations for further research**

A new development is modified GGA which can be solubilized. This would make it possible to administrate the drug intravenously. Intravenous administration of drugs is the preferred method of administration in BD donors. The next step to evaluate the beneficial effects of HSP up-regulation on kidney donor quality would be to transplant the pre-treated brain dead donor kidneys in a syngeneic transplantation model.

**Conclusion**

Despite the unchanged Hsp72 levels and the unchanged leukocyte infiltration in this experiment, we believe that GGA might be a suitable drug for transplantation. At the moment of organ retrieval GGA does reduce IL-6 mRNA and ASAT levels. Furthermore, GGA has no negative effects on the organ quality during the brain death period. These findings combined with the protective effects of GGA during IRI (24) might make GGA a beneficial adjuvant to improve the outcome of kidney transplantation with brain dead-derived donor kidneys. We would suggest a kidney transplantation rat model with brain dead-derived donor kidneys to evaluate the efficacy of this drug for improving transplantation outcome.

**Conflict of interest**

The author declares that he has no conflict of interest.

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Supplementary figures

**Supplementary figure 1**  Brain dead induced rat via a balloon catheter (BC). The rat was kept viable by mechanical ventilation (MV). Blood pressure is monitored by an arterial cannula (AC) inserted in the femoral artery and connected to a pressure monitor (PM). Blood pressure was kept stable by administration of starch (HAES) or noradrenalin (NA) to a venous cannula (VC). Temperature kept stable at 37˚C and monitored with a rectal probe (RP).

**Supplementary figure 2**  (A) Infiltrating polymorphonuclear cells (PMNs) positive for His48 in the renal cortex. (B) Positive staining was calculated with Aperio Imagescope software using an algorithm based on the spectral differentiation between red (positive) and blue (counter) staining. In the algorithm, yellow represents negative staining, orange represents positive staining, and red represents strong positive staining. Images were taken at 200x magnification.
Supplementary figure 3  

(A) Infiltrating polymorphonuclear cells (PMNs) in the renal cortex, stained positive for His48. The amount of infiltration was expressed as percentage positive per area (%) calculated with Aperio Imagescope software. (B) Average number of His48-positive cells in the renal cortex scored in random 10 fields at 200x magnification. The results of the quantitative analysis with the Aperio Imagescope software are comparable to the results scored in a blind fashion.