Complement modulation to improve donor organ quality

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C1-inhibitor treatment decreases renal injury in an established rat brain death model

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Abstract

Background

Kidneys derived from brain-dead donors have lower graft survival rates than kidneys derived from living donors. Complement activation plays an important role in brain death. The aim of our study was therefore to investigate the effect of C1-inhibitor (C1-INH) on brain death-induced renal injury.

Methods

Brain death was induced in rats by inflating an epidural placed balloon catheter. Thirty minutes after induction, rats were treated with saline, low-dose or high-dose C1-INH. Sham-operated rats served as controls. After 4 hours of brain death, renal function, injury, inflammation and complement activation were analyzed.

Results

High-dose C1-INH treatment of brain-dead donors resulted in significantly lower renal gene expression and serum levels of IL-6. Treatment with C1-INH also improved renal function and reduced renal injury, reflected by the significantly lower KIM-1 gene expression and lower serum levels of LDH and creatinine. Furthermore, C1-INH effectively reduced complement activation by brain death and significantly increased functional levels. However, C1-INH treatment did not prevent renal cellular influx.

Conclusions

Targeting complement activation after the induction of brain death reduced renal inflammation and improved renal function, before transplantation. Therefore, strategies targeting complement activation in human brain-dead donors might clinically improve donor organ viability and renal allograft survival.

Introduction

Today, brain-dead donors still comprise the majority of deceased organ donors. It is well known that fully mismatched living donors have better early graft function and improved long-term outcome compared to kidneys from fully matched deceased donors.[1] This illustrates that donor origin is an important cause of allograft injury. Brain death is characterized by hemodynamic and hormonal instabilities resulting in immune activation and eventually decreased organ viability before and after transplantation.[2,3] Therefore, attenuating brain death-induced renal injury in the donor seems to be a promising therapeutic strategy to improve outcome after renal transplantation.[3,4] The harmful effect of brain death on renal allograft survival is in part explained by immunological changes.[5,6] Brain-dead donors have increased levels of cytokines (IL-1β, IL-6, IL-8), chemokine's (MCP-1) and adhesion molecules (ICAM-1, VCAM-1, E- and P-selectin).[7] Subsequently, increased numbers of infiltrating macrophages (MΦ) and polymorphonuclear (PMN) cells are found in renal tissue of brain-dead donors.[8,9] Ultimately, this leads to tissue damage and repair as reflected by increased expression of KIM-1, a marker of brain death-induced renal injury.[10] Consequently, recipients of renal allografts from brain-dead donors have a higher chance to develop delayed graft function (DGF, defined as the need for dialysis within the first 7 days post-transplant) and a lower long-term renal graft survival.[1,11] There is circumstantial evidence for a prominent role of complement activation in the pathogenesis of renal injury in deceased donors.[12] In previous studies we demonstrated that circulating complement components are activated in deceased donors and are associated with acute rejection after renal transplantation.[13] Furthermore, in kidneys from brain-dead donors, complement component 3 was upregulated and activated before kidney retrieval without additional expression after cold ischemia or reperfusion.[14] Moreover, microarray analysis of 554 kidney biopsies showed enrichment of complement pathways in brain-dead donors prior to organ retrieval.[15] In addition, genetic variation of donor C3 was associated with allograft outcome.[16,17] Altogether, these data demonstrate that complement activation plays a pivotal role in brain death-associated renal injury and may impact long-term allograft survival rates. In accordance with this, inhibition of the complement system in brain-dead donors rats was shown to improve renal allograft function in the recipient after transplantation.[18] C1-inhibitor (C1-INH) is one of the central regulators of the complement system, as it interacts with different complement factors. Additionally, C1-INH plays a key role in the kinin- and coagulation system.[19] Previously, it has been shown that C1-INH is able to inhibit complement activation in vitro of the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP).[20–22] The clinical value of C1-INH has been well established since it has been used extensively for the prophylaxis and treatment of hereditary angioedema (HAE).[23] Preliminary clinical studies in transplantation show the therapeutic potential of C1-INH in the treatment of renal ischemia-reperfusion injury and antibody-mediated rejection.[24] The aim of our study was to investigate the effect of C1-INH on brain death-induced renal injury. Rats were treated after the induction of brain death, since it is analogous to a potential clinical intervention strategy that would involve treating human brain-dead donors prior to procurement for transplantation.

Materials and Methods

Rats

Adult male Fisher F344 rats (260–300 g) were used. Rats received care in compliance with the guidelines of the local animal ethics committee according to Experiments on Animals Act (1996) issued by the Dutch Ministry of Public Health, Welfare and Sports.

Experimental protocol

Brain death was induced as described previously.[25] In short, rats were anaesthetized, intubated and ventilated. A baseline blood sample was drawn. Temperature and blood pressure were continuously monitored and regulated. Through a frontolateral borehole in the skull, a catheter was placed epidural and inflated slowly. The induction was completed after 30 minutes and brain death was confirmed by an apnea test. After another 30 minutes a blood sample was taken (before treatment). During brain death, rats received no anesthesia. If blood pressure fell below 80 mmHg, it was restored by the administration of 10% hydroxyethyl starch solution (HAES) or noradrenaline. After 4 hours of brain death, blood was collected from the aorta and organs were flushed with cold saline and retrieved. Retrieved organs were paraffin-embedded or snap frozen in liquid nitrogen stored at −80°C together with collected serum. In sham-operated rats, a hole was drilled in the skull without insertion of the balloon catheter and rats were ventilated for half an hour under anesthesia before they were sacrificed (Figure 1). Four rats per group were included in the sham-operated groups (groups 1-3). Ten rats per group were included in the brain-dead groups. Rats were randomly divided into six groups**.** In all groups, C1-INH or saline, were administered via the femoral vein.

Group 1: sham-operated rats treated with saline.
Group 2: sham-operated rats treated with a low-dose of C1-INH (100 U/kg).
Group 3: sham-operated rats treated with a high-dose of C1-INH (400 U/kg).
Group 4: brain-dead rats treated with saline.
Group 5: brain-dead rats treated with a low-dose C1-INH (100 U/kg), 0.5hr after brain death.
Group 6: brain-dead rats treated with a high-dose C1-INH (400 U/kg), 0.5hr after brain death.

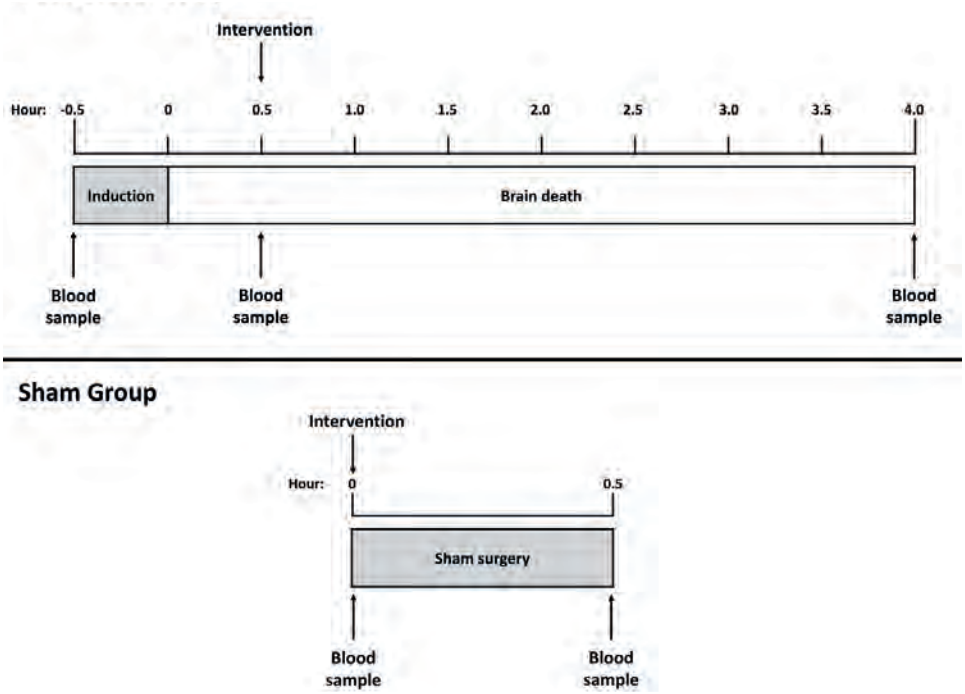


Figure 1. Schematic presentation of the protocol in brain-dead rats and sham-operated rats.
To induce brain death, a catheter was placed epidural through a frontolateral borehole in the skull and inflated slowly. After 30 minutes, brain death was confirmed with an apnea test. Rats were treated with saline, a low-dose of C1-INH (100 U/kg) or a high-dose of C1-INH (400 U/kg) 30 minutes after the confirmation of brain death. After 4 hours of brain death, rats were sacrificed and organs were collected. Blood samples were collected at baseline, 30 minutes after brain death (before intervention) and after 4 hours of brain death. In the sham-operated rats, a hole was drilled in the skull without insertion of the balloon catheter and rats were ventilated for half an hour under anesthesia before sacrifice. Directly after sham-surgery rats were treated with saline, low-dose or high-dose of C1-INH. Blood samples were collected at baseline (before intervention) and 30 minutes after sham surgery. Abbreviations: C1-INH, C1-inhibitor.

Biochemical determinations

Creatinine and lactate dehydrogenase (LDH) were determined in serum samples in a routine fashion (Mega, Merck, Amsterdam, The Netherlands).

Complement activation and functional levels

Rat complement pathway activity was assessed by functional complement ELISAs for the CP, LP and AP. The sC5b-9 ELISA was used for the detection of complement activation.[26] Per rat the baseline values were set at a 100% or 1 AU/mL and consecutive samples were calculated accordingly.

RNA isolation and qPCR

Total RNA was extracted from rat kidneys using the TRIzol reagent method (Invitrogen, Breda, the Netherlands). The isolated RNA was then treated with DNAse I to remove any contamination with genomic DNA (Invitrogen). The integrity of isolated RNA was analyzed by using gel electrophoresis. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the M-MLV Reverse Transcriptase enzyme kit (Invitrogen), oligo dT primers and RNase inhibitor (Invitrogen) according to the manufacturer's protocol. Primer sequences (Table 1) were designed based on Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using emission from SYBR green master mix (Applied Biosystems). All samples for RT-PCR were done in triplicate. The expression of genes was normalized to β-actin. The data are presented as fold increase compared to the housekeeping gene. Fold increase of the sham-operated rats treated with saline was set at 1, and the other groups were calculated accordingly.

Table 1.

Gene-specific qPCR primers and their respective PCR fragment lengths

Primers	Primer sequences	Amplicon length (bp)
β-actin	5′-GGAAATCGTGCGTGACATTAAA-3′ 5′-GCGGCAGTGGCCATCTC-3′	74
BAX	5′-GCGTGGTTGCCCTCTTCTAC-3′ 5′-TGATCAGCTCGGGCACTTTAGT-3′	74
Bcl-2	5′-CTGGGATGCCTTTGTGGAA-3′ 5′-TCAGAGACAGCCAGGAGAAATCA-3′	70
IL-6	5′-CCAACTTCCAATGCTCTCCTAATG-3′ 5′-TTCAAGTGCTTTCAAGAGTTGGAT-3′	89
P-selectin	5′-TCTCTGGGTCTTCGTGTTTCTTATCT-3′, 5′-GTGTCCCCCTAGTACCATCTGAA-3′	80
VCAM-1	5′-TGTGGAAGTGTGCCCGAAA-3′ 5′-ACGAGCCATTAACAGACTTTAGCA-3′	84
MCP-1	5′-CTTTGAATGTGAACTTGACCCATAA-3′ 5′-ACAGAAGTGCTTGAGGTGGTTGT-3′	78
KIM-1	5′-AGAGAGAGCAGGACACAGGCTT-3′ 5′-ACCCGTGGTAGTCCCAAACA-3′	75

Abbreviations: β-actin, βeta-actin; BAX, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma protein 2; IL, Interleukin; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

Immunohistochemistry

Frozen sections were fixed with acetone, followed by endogenous peroxidase blocking. Paraffin-embedded tissue sections were deparaffinized and antigen retrieval was performed, followed by endogenous peroxidase blocking. Sections were incubated with a primary antibody for 1 hour at room temperature (Table 2). After washing, sections were incubated with appropriate secondary and tertiary antibodies (Dako, Glostrup, Denmark). The reaction was developed by the addition of 3-amino-9-ethylcarbazole (AEC) and counterstained with haematoxylin solution (Merck, Darmstadt, Germany). For HIS48 and ED-1 (for granulocytes and macrophages respectively), in each section the number of glomerular and interstitial positive cells was assessed as the average count from 20 different microscopic fields, randomly divided throughout the renal cortex, using a light microscope (objective x50 – x200).

Table 2.

Primary antibodies used for immunohistochemistry

Specifity	Sections	Antibody	Supplier	2nd/3rd antibodies
HIS48	Frozen	Mouse monoclonal anti-rat granulocytes	IQ Products, Groningen, The Netherlands	RbαM^{PO}/GαRb^{PO}
ED-1	Paraffin	Mouse monoclonal anti-rat macrophages/ monocytes (ab31630)	Abcam, Oxford, UK	RbαM^{PO}/GαRb^{PO}

Abbreviations: M, mouse; PO, polyclonal; Rb, rabbit.

IL-6 ELISA

IL-6 production in serum was determined using Rat IL-6 ELISA Kit (R&D Systems, Abingdon, UK) according to the supplied protocol.

Statistics

Statistical analysis was performed with StatsDirect (v 3.0.133, Cheshire, UK). The Kruskal–Wallis test and Mann–Whitney U test were used to assess differences between groups of non-parametric data and one-way analysis of variance and t test for normally distributed data. All statistical tests were 2-tailed with $P<0.05$ regarded as significant. Results are presented as a median and interquartile range for non-parametric data and mean and standard error of the mean (SEM) for parametric data.

Results

Treatment with C1-INH protects kidneys from brain death-induced injury

In brain-dead rats creatinine levels were significantly higher after 4 hours than in sham-operated rats, indicating reduced renal function (Figure 2A). In accordance with these observations, renal gene expression of kidney injury molecule-1 (KIM-1) was also significantly upregulated in kidneys from brain-dead rats compared to controls (Figure 2B). Since complement is known to play a role in brain death-induced renal injury, we investigated the effect of C1-INH treatment on kidneys of brain-dead donors. High-dose C1-INH treatment significantly reduced creatinine levels (Figure 2A). Median creatinine levels in brain-dead rats treated with high-dose C1-INH were 64 µM, compared to 96 µM in saline-treated brain-dead rats. Sham-operated rats had a median creatinine of 34 µM. In accordance, high-dose C1-INH treatment reduced renal injury in brain-dead rats as seen by the lower renal gene expression of KIM-1 (Figure 2B). Renal KIM-1 expression was 47% lower in the C1-INH-treated rats than saline-treated rats. Low-dose C1-INH treatment was unable to improve renal function or injury.

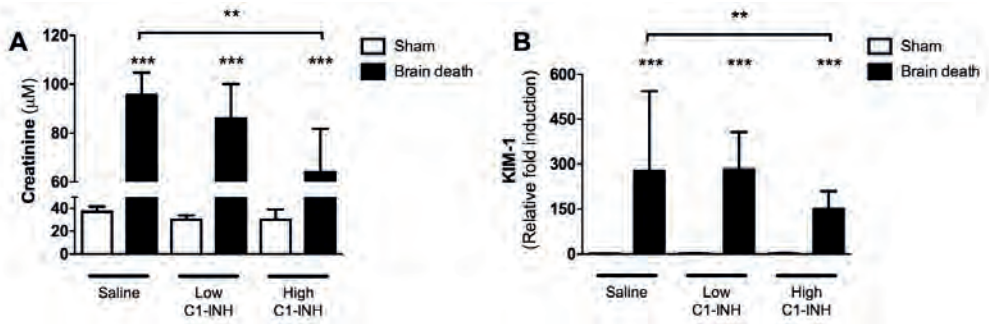


Figure 2. **C1-inhibitor treatment in brain-dead rats reduced renal injury.**
(A) Plasma creatinine levels of brain-dead or sham-operated rats treated with saline, 100 U/kg (low) C1-INH or 400 U/kg (high) C1-INH. (B) KIM-1 gene expression in kidneys of rats after brain death or sham operation. Quantitative real-time RT-PCR was performed. The gene expression of KIM-1 relative to β-actin was set at 1 in sham-operated rats treated with saline and the other values are calculated accordingly. Data are shown as median and interquartile range and were analyzed by Kruskal-Wallis test with an option for multiple comparisons (**p<0.01, ***p<0.001). Asterisks above the bars denote significant differences between brain-dead and sham-operated rats of the same treatment, while asterisks above the capped line indicate significant differences between brain-dead groups from the different treatments.

Furthermore, brain-dead rats had significantly higher levels of LDH than sham-operated rats. High-dose C1-INH treatment led to significantly lower LDH levels than treatment with saline upon brain death (Figure 3A). On the other hand, brain death-induced renal apoptosis was not altered by C1-INH treatment since the BAX/Bcl-2 ratio was not significantly different between the groups (Figure 3B).

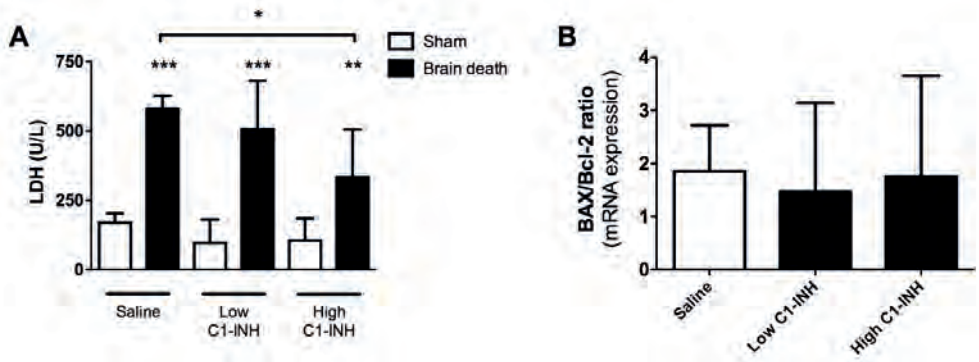


Figure 3. C1-inhibitor treatment reduces tissue damage in brain-dead rats but not renal apoptosis. (A) Plasma LDH levels of brain-dead or sham-operated rats treated with saline, 100 U/kg (low) C1-INH or 400 U/kg (high) C1-INH. (B) BAX/Bcl-2 gene expression ratio in kidneys of rats after brain death. Renal apoptosis reflected by the BAX/Bcl-2 ratio. Data are shown as median and interquartile range and were analyzed by Kruskal-Wallis test with an option for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001). Asterisks above the bars denote significant differences between brain-dead and sham-operated rats of the same treatment, while asterisks above the capped line indicate significant differences between brain-dead groups from the different treatments.

Brain death leads to local and systemic complement activation, resulting in complement consumption

Next, we investigated complement activation and functional levels during brain death. The sC5b-9 levels increased significantly during brain death from 1.00 AU/mL at baseline to 1.95 AU/mL after 4 hours (Figure 4A). Between 0.5 and 4 hours after the induction of brain death the sC5b-9 levels rose as well although not significant (0.5h: 1.42 AU/mL, 4h: 1,95 AU/mL). In sham-operated rats, there was no significant increase in sC5b-9 levels (supplementary data). Subsequently, we assessed whether treatment with C1-INH was able to prevent systemic complement activation in rats when administered 0.5 hours after the induction of brain death. High-dose C1-INH treatment prevented further increase between 0.5 and 4 hours in brain-dead rats (0.5h: 1.47 AU/mL, 4h; 1.53 AU/mL). However, after 4 hours sC5b-9 levels were still significantly higher than baseline samples in the C1-INH-treated brain-dead rats. We next determined whether the systemic complement activation was accompanied by complement depletion by measuring functional complement levels. In brain-dead rats, functional levels were significantly lower after 4 hours compared to baseline levels for all three pathways. After 4 hours, functional levels were 72%, 74% and 74% for the CP, LP and AP, respectively (Figure 4B-D). There was a drop in functional levels for all pathways between 0.5 and 4 hours as well, however this was not significant. In the sham-operated groups, functional levels of the LP and AP were not significantly altered (supplementary data). However, there was a significant decrease in the functional levels of the CP in the sham-operated rats (15% reduction compared to baseline). Next, we determined whether treatment with C1-INH prevented complement consumption in rats when administered 0.5 hour after the induction of brain death. For the CP, functional levels rose between 0.5 and 4 hours after both low-dose and high-dose C1-INH

treatment (Figure 4B). After 4 hours, functional levels were no longer significantly lower than at baseline. In the sham-operated rats, high-dose C1-INH significantly increased CP functional complement levels as well (supplementary data). For the AP, a similar protection was seen after both low-dose and high-dose C1-INH treatment (Figure 4D). Strikingly, high-dose C1-INH treatment even significantly increased functional AP levels compared to baseline, indicating prevention of complement consumption of the AP. For the LP, the significant decrease in functional levels by brain death could not be prevented by C1-INH (Figure 4C).

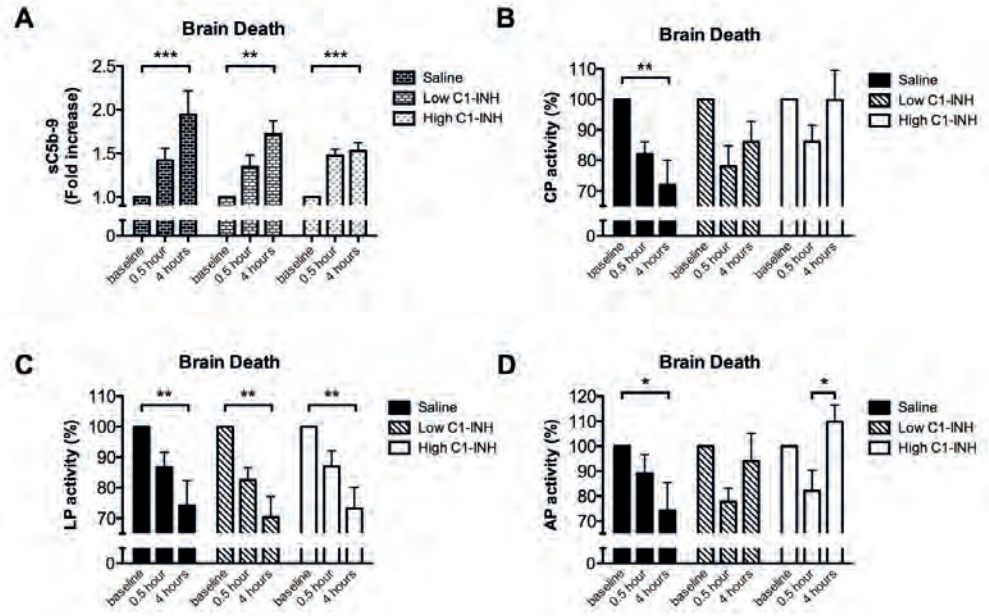


Figure 4. C1-inhibitor treatment prevented further complement activation and consumption of the classical and alternative pathway, but not the lectin pathway.
(A) Plasma soluble C5b-9 (sC5b-9) levels and functional complement levels for (B) the classical pathway, (C) lectin pathway and (D) alternative pathway of brain-dead rats. Levels were determined at baseline (t = 0 hour), prior to treatment (t = 0.5 hours) and prior to sacrifice (t = 4 hours). Values at baseline are set at 1.0 or 100% per rat, and other consecutive levels are calculated accordingly. Data is expressed as mean percentage ± SEM and analyzed by ANOVA with a Bonferroni post-hoc test. Significant differences are indicated (*p<0.05; **p<0.01 and ***p<0.001). Abbreviations: sC5b-9, soluble C5b-9; SEM, standard error of the mean.

C1-INH treatment reduces both renal IL-6 expression and systemic IL-6 levels

Subsequently, we assessed the gene expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), chemokine's (MCP-1), and adhesion molecules (VCAM-1, ICAM-1, E-selectin and P-selectin). In brain-dead rats, renal expression levels of IL-6, MCP-1, VCAM-1 and P-selectin were significantly increased compared to sham-operated rats (Figure 5). No significant increase was found for renal expression of IL-1β, TNF-α, E-selectin and ICAM-1 in brain-dead rats (data not shown). Next, we evaluated the effect of C1-INH treatment on the expression

of IL-6, MCP-1, VCAM-1 and P-selectin. A clear downward trend was visible for IL-6 expression in the brain-dead rats treated with C1-INH compared to saline-treated rats (Figure 5A). Treatment with both high-dose and low-dose C1-INH significantly reduced VCAM-1 expression (Figure 5B).

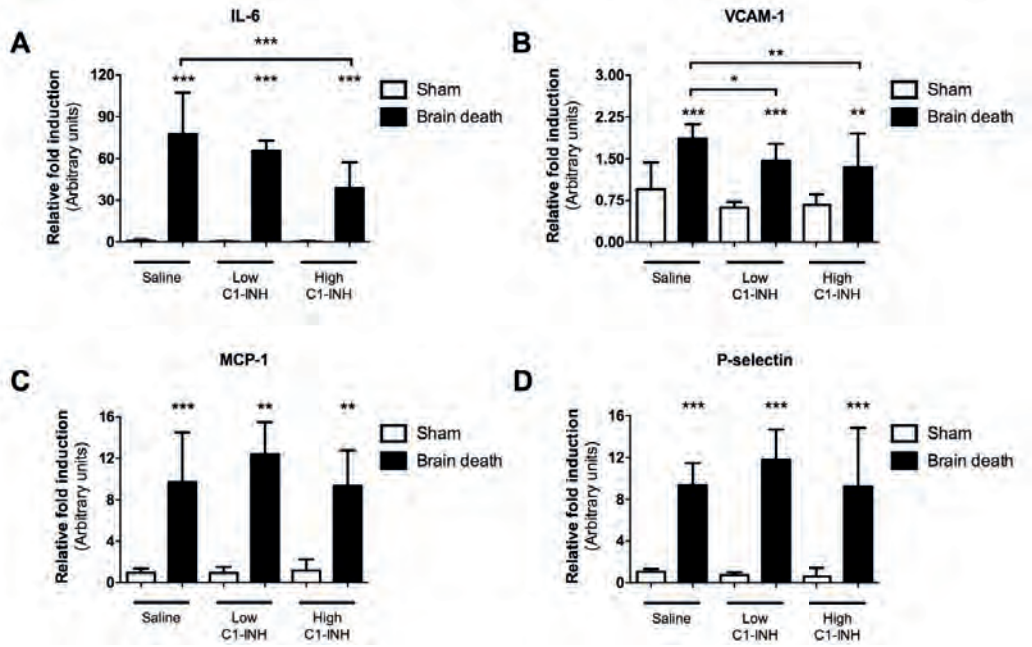


Figure 5. Reduction in renal gene expression of inflammatory mediators in brain-dead rats after C1-inhibitor treatment.

Gene expression of inflammatory markers in kidneys of brain-dead or sham-operated rats. Gene expression levels of (A) IL-6, (B) VCAM-1, (C) MCP-1, (D) P-selectin. Data are shown as expression relative to β-actin, were sham-operated rats treated with saline are set at 1 and the rest is calculated accordingly. Data are shown as median and interquartile range and were analyzed by Kruskal-Wallis test with an option for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001). Asterisks above the bars denote significant differences between brain-dead and sham-operated rats of the same treatment, while asterisks above the capped line indicate significant differences between brain-dead groups from the different treatments.

In brain-dead rats with high-dose C1-INH renal expression of IL-6 was reduced by 55%. Serum levels of IL-6 were also determined to assess whether systemic levels of IL-6 were also lower in C1-INH treated rats (Figure 6). Levels of IL-6 were not determined in the sham-operated rats. The median IL-6 serum concentration was 107 pg/mL in high-dose C1-INH treated rats compared to 206 pg/mL in saline-treated brain-dead rats.

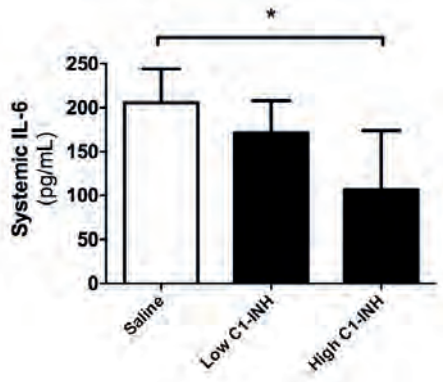


Figure 6. C1-inhibitor treatment led to reduced systemic IL-6 in brain-dead rats.
Serum levels of interleukine-6 (IL-6) in brain-dead rats, treated with saline or C1-INH. Data are shown as median and interquartile range and were analyzed by Mann–Whitney U test (*p<0.05). N is 10 per group.

To further characterize local renal inflammation upon brain death, infiltrating neutrophils (PMN) and macrophages (MΦ) were quantified by immunohistochemistry. There was a significant increase in the number of infiltrating PMN, but not in MΦ in the renal cortex of brain-dead rats compared to sham-operated rats. Treatment with C1-INH, low or high-dose, did not attenuate the increase in infiltrating PMN in the renal cortex (Figure 7A). However, treatment with C1-INH in the brain-dead rats appears to lead to increased number of MΦ compared to saline-treated brain-dead rats and C1-INH sham-operated rats (Figure 7B).

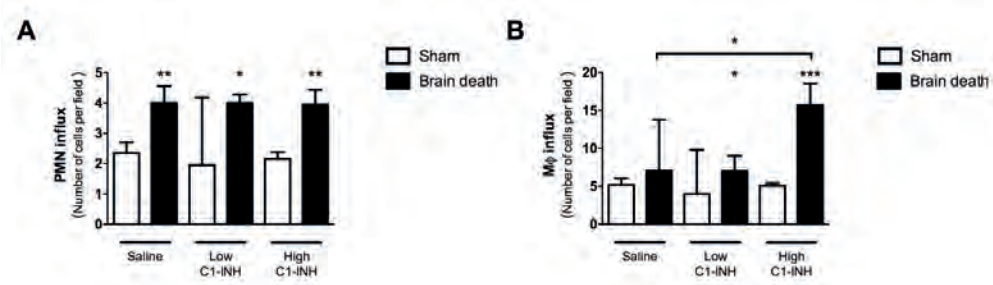


Figure 7. C1-inhibitor did not decrease the number of infiltrating granulocytes or macrophages in the renal cortex of brain-dead rats.
Renal influx of (A) polymorphonuclear (PMN) leukocytes and (B) macrophages (Mϕ) was evaluated by immunohistochemistry. The HIS48 monoclonal antibody reacts with a molecule expressed on the surface of all rat granulocytes. The monoclonal antibody ED-1 is a marker for rat macrophages. In each section the number of interstitial positive cells were assessed by the average count from 10 different microscopic fields, randomly divided throughout the renal cortex, using a light microscope (objective 200x). Data are shown as median and interquartile range and were analyzed by Kruskal-Wallis test with an option for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001). Asterisks above the bars denote significant differences between brain-dead and sham-operated rats with the same treatment, while asterisks above the capped line indicate significant differences between brain-dead groups with the different treatments.

Discussion

In this study, we demonstrated that complement inhibition in brain-dead donors attenuates renal injury before organ retrieval. In our model, rats were treated after the induction of brain death, since this closely resembles the potential clinical situation that would involve treating donors after the diagnosis of brain death. We found that high-dose C1-INH treatment after the induction of brain death decreased local renal and systemic inflammation and renal injury and improved renal function. Therefore, strategies targeting complement activation in human brain-dead donors might improve donor organ viability and improves renal allograft survival.

Donor organ injury induced by brain death is considered to be an important risk factor for DGF, renal allograft graft rejection and allograft survival after transplantation.[1,11,27–29] The complement system has been shown to play an important role in brain death-induced renal injury.[13,14,30] In addition, treatment with complement inhibiting drugs has proven to be beneficial in preclinical models of DGF, emphasizing that strategies targeting complement activation are potentially beneficial in the transplantation setting.[18]

The present study investigated the effect of human plasma-derived C1-INH treatment in brain-dead rats. C1-INH is a complement inhibitor of all three pathways, and this already-available therapeutic agent is currently used for the treatment of HAE.[20–22] C1-INH treatment has already been shown to improve outcome in a number of disease models, including ischemia-reperfusion injury, antibody-mediated transplant rejection and xenotransplantation models.[43] The use of C1-INH may have greater benefits in brain-dead donors than other complement inhibitors. Plasma-derived C1-INH appears to be extremely safe, since it has been used for over three decades.[31] C1-INH also regulates the coagulation system and can inhibit leukocyte adhesion to the endothelium, which also contributes to brain death-induced renal injury.[14,15,32] It has been suggested that C1-INH has additional anti-inflammatory functions and anti-apoptotic effects independent of protease inhibition.[40-42] C1-INH also regulates the coagulation and the kallikrein-kinin systems, which also contributes to brain death-induced organ injury.[14,15,33]

A vital consideration for any intervention in brain-dead donors is that it should not impact the successful utilization of other organs. This is a potential limitation of complement inhibition in the donor, since it is not known if all donor organs would benefit from such therapy. Nevertheless, C1-INH treatment has already shown promising results in lung transplantation.[34] In addition to the trials for the treatment of renal ischemia-reperfusion injury and renal antibody-mediated rejection, C1-INH treatment is also being tested in liver transplantation (NCT01886443 and NCT02251041).[24, 36-40] In accordance, in our study C1-INH did not negatively impact the liver since specific injury markers, AST and ALT, were unaltered (supplementary data). Lastly, C1-INH inhibits at an early point and could thereby decrease organ injury, whereas later blockage can only eliminate effector functions of complement activation.

The cause of the immune activation in brain-dead donors is not well understood but it's thought to be initiated by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) in the systemic circulation as well as in the local

environment. Studies on the contribution of complement in brain death have mostly focused on local C3 expression and synthesis.[14,35,36] However, we show that brain death induces systemic complement activation already after 0.5 hours. Between 0.5 and 4 hours of brain death, there was a further stepwise increase of sC5b-9. Furthermore, induction of brain death led to significant lower functional levels of all three pathways, which reflects significant systemic complement activation. This is in line with findings in human brain-dead donors that showed increased plasma levels of sC5b-9, C4d and Bb.[13]

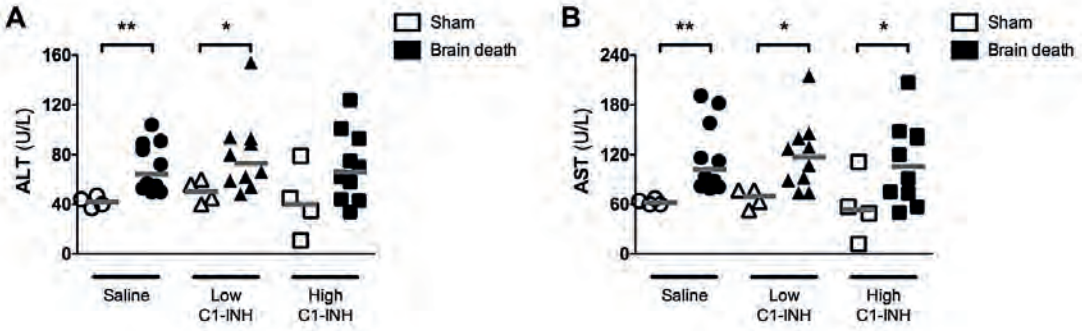
In our rat model, significant complement consumption of the CP and AP in brain-dead rats was prevented by treatment with high-dose C1-INH, thereby increasing functional levels of these pathways. No effect of C1-INH treatment could be seen on the LP. However, human C1-INH was used to inhibit complement in rats and the biggest discrepancies among the complement pathways between rodents and humans are found in the LP.[37] Altogether, these data show that high-dose C1-INH prevented the further increase of sC5b-9 by brain death and thereby increased functional levels of the CP and AP. This excludes the possibility that the *in vivo* administered C1-INH is interfering with the *in vitro* activity assay. Furthermore, the concentration of C1-INH used here is comparable to previous studies in pigs and human.[38,39]

Our study shows that renal inflammation is significantly reduced after treatment of brain-dead rats with C1-INH. High-dose C1-INH treatment partly prevented the brain death-induced gene expression of IL-6 and VCAM-1. Furthermore, also systemic IL-6 was significantly reduced by C1-INH treatment. These findings suggest that complement inhibition by C1-INH protects allografts from development of inflammation. Previous studies have shown that IL-6 is important in the pro-inflammatory reaction of brain death.[40–43] Recently it was demonstrated that higher levels of IL-6 in the donor are associated with a worse outcome in the recipient.[44] Thus, C1-INH treatment leads to a better renal function in brain-dead donors, which could be ascribed to a consequence of improved organ allograft quality by reducing inflammatory cytokines IL-6.

C1-INH significantly reduced creatinine levels in our brain death model before organ retrieval. Yet, creatinine levels remained significantly higher than sham-operated rats. Hemodynamic and hormonal instabilities should also be considered in brain death since these changes additionally affect renal function. KIM-1 expression was also significantly lower by C1-INH treatment. KIM-1 has been shown to be a specific marker of brain death-induced kidney damage. In human brain-dead donors, urinary KIM-1 has been shown to be associated with renal function after transplantation.[10] In addition, levels of LDH significantly increased during brain death compared to the sham-operated rats, indicating progressive cell damage. C1-INH reduced LDH levels, although this could not be explained by changes in renal apoptotic in the mitochondria-mediated (intrinsic) pathway. However, the extrinsic pathway of apoptosis and necrosis was not assessed. Despite the significant protection demonstrated by C1-INH in preserving the kidney in our model, further clinical investigations are necessary to evaluate the effect of C1-INH on brain death-induced renal injury.

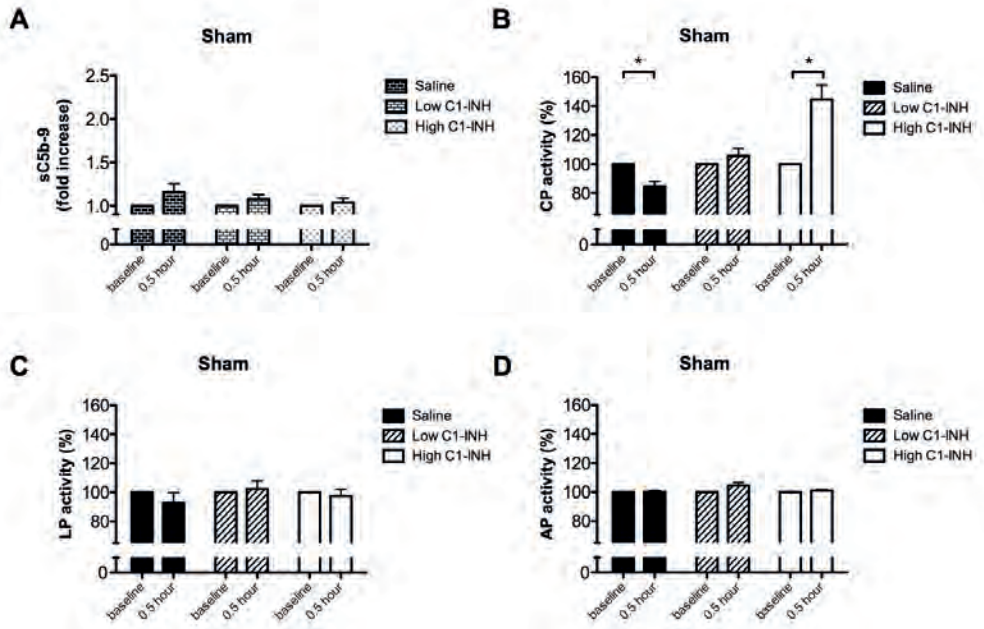
In conclusion, an important finding of this study is that treatment of the donor with a single dose of C1-INH after the induction of brain death protects against brain death-induced renal injury. These findings open a new window of opportunity for complement inhibition in brain-dead donors to potentially improve kidney grafts for transplantation.

Supplementary Figures



Supplementary Figure 1. C1-inhibitor treatment in brain-dead rats did not interfere with liver function.

Plasma (A) ALT and (B) AST levels in brain-dead or sham-operated rats treated with saline, 100 U/kg (low) C1-INH or 400 U/kg (high) C1-INH. Data are shown as median and interquartile range and were analyzed by Kruskal-Wallis test with an option for multiple comparisons (*p<0.05, **p<0.01). Asterisks above the capped line indicate significant differences between brain-dead and sham-operated rats of the same treatment. There was no significant difference between brain-dead groups from the different treatments.



Supplementary Figure 2. Plasma sC5b-9 and functional levels of the classical, lectin and alternative pathway in sham-operated rats.

(A) Plasma soluble C5b-9 (sC5b-9) levels and functional complement levels for (B) the classical pathway, (C) lectin pathway and (D) alternative pathway of sham-operated rats. Levels were determined at baseline and prior to sacrifice. Per rat baseline values are set at 100%, and other consecutive levels are calculated accordingly. Data is expressed as mean percentage ± SEM and analyzed by ANOVA with a Bonferroni post-hoc test. Significant differences are indicated (*p<0.05; **p<0.01 and ***p<0.001).

References

1. Terasaki P, Cecka J, Gjertson D, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. *N Engl J Med*. 1995;10(6):333-336. doi:10.1056/NEJM199508103330601
2. Citerio G, Cypel M, Dobb GGJ, et al. Organ donation in adults: a critical care perspective. *Intensive Care Med*. 2016;42(305):305-315. doi:10.1007/s00134-015-4191-5
3. Fung A, Zhao H, Yang B, Lian Q, Ma D. ischaemic and inflammatory injury in renal grafts from brain death donation: an update review. *J Anesth*. 2016;30(307):307-316. doi:10.1007/s00540-015-2120-y
4. Bos EM, Leuvenink HGD, Van Goor H, Ploeg RJ. Kidney grafts from brain dead donors: Inferior quality or opportunity for improvement? *Kidney Int*. 2007;72(7):797-805. doi:10.1038/sj.ki.5002400
5. Westendorp WH, Leuvenink HG, Ploeg RJ. Brain death induced renal injury. *Curr Opin Organ Transplant*. 2011;16(2):151-156. doi:10.1097/MOT.0b013e328344a5dc
6. Watts RP, Thom O, Fraser JF. Inflammatory signalling associated with brain dead organ donation: from brain injury to brain stem death and posttransplant ischaemia reperfusion injury. *J Transplant*. 2013;2013:521369. doi:10.1155/2013/521369
7. Bouma HR, Ploeg RJR, Schuurs TA. Signal transduction pathways involved in brain death-induced renal injury. *Am J Transplant*. 2009;9(5):989-997. doi:10.1111/j.1600-6143.2009.02587.x
8. Nijboer WN, Schuurs TA, van der Hoeven JAB, et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. *Transplantation*. 2004;78(7):978-986. doi:10.1097/01.tp.0000135565.49535.60
9. Schuurs TA, Morariu AM, Ottens PJ, et al. Time-Dependent Changes in Donor Brain Death Related Processes. *Am J Transplant*. 2006;6(12):2903-2911. doi:10.1111/j.1600-6143.2006.01547.x
10. Nijboer WN, Schuurs TA, Damman J, et al. Kidney Injury Molecule-1 is an Early Noninvasive Indicator for Donor Brain Death-Induced Injury Prior to Kidney Transplantation. *Am J Transplant*. 2009;9(8):1752-1759. doi:10.1111/j.1600-6143.2009.02713.x
11. Ounissi M, Cherif M, Abdallah T Ben, et al. Risk factors and consequences of delayed graft function. *Saudi J Kidney Dis Transpl*. 2013;24(2):243-246. doi:10.4103/1319-2442.109564.
12. Damman J, Schuurs TA, Ploeg RJ, Seelen MA. Complement and renal transplantation: from donor to recipient. *Transplantation*. 2008;85(7):923-927. doi:10.1097/TP.0b013e3181683cf5
13. Damman J, Seelen MA, Moers C, et al. Systemic complement activation in deceased donors is associated with acute rejection after renal transplantation in the recipient. *Transplantation*. 2011;92(2):163-169. doi:10.1097/TP.0b013e318222c9a0
14. Damman J, Nijboer WN, Schuurs TA, et al. Local renal complement C3 induction by donor brain death is associated with reduced renal allograft function after transplantation. *Nephrol Dial Transplant*. 2011;26(7):2345-2354. doi:10.1093/ndt/gfq717
15. Damman J, Bloks VW, Daha MR, et al. Hypoxia and Complement-and-Coagulation Pathways in the Deceased Organ Donor as the Major Target for Intervention to Improve Renal Allograft Outcome. *Transplantation*. 2015;99(6):1293-1300. doi:10.1097/TP.0000000000000500
16. Damman J, Daha MR, Leuvenink HGH, et al. Association of complement C3 gene variants with renal transplant outcome of deceased cardiac dead donor kidneys. *Am J Transplant*. 2012;12(3):660-668. doi:10.1111/j.1600-6143.2011.03880.x
17. Brown KM, Kondeatis E, Vaughan RW, et al. Influence of donor C3 allotype on late renal-transplantation outcome. *N Engl J Med*. 2006;354:2014-2023. doi:10.1056/NEJMoa052825
18. Damman J, Hoeger S, Boneschansker L, et al. Targeting complement activation in brain-dead donors improves renal function after transplantation. *Transpl Immunol*. 2011;24(4):233-237. doi:10.1016/j.trim.2011.03.001
19. Zeerleder S. C1-inhibitor: more than a serine protease inhibitor. *Semin Thromb Hemost*. 2011;37(4):362-374. doi:10.1055/s-0031-1276585
20. Poppelaars F, Damman J, de Vrij EL, et al. New insight into the effects of heparinoids on complement inhibition by C1-inhibitor. *Clin Exp Immunol*. 2016;184(3):378-388. doi:10.1111/cei.12777
21. Jiang H, Wagner E, Zhang H, Frank MM. Complement 1 Inhibitor Is a Regulator of the Alternative Complement Pathway. *J Exp Med*. 2001;194(11):1609-1616. doi:10.1084/jem.194.11.1609
22. Nielsen EW, Waage C, Fure H, et al. Effect of supraphysiologic levels of C1-inhibitor on the classical, lectin and alternative pathways of complement. *Mol Immunol*. 2007;44(8):1819-1826. doi:10.1016/j.molimm.2006.10.003

23. Zuraw, Bruce L. MD, Busse, Paula J MD, White, Martha MD, Jacobs, Joshua MD, Lumry, William MD, Bake, Jamesr MD. Nanofiltered C1 Inhibitor Concentrate for Treatment of Hereditary Angioedema. *N Engl J Med*. 2010;363:513-522. doi:10.1056/NEJMoa1404595
24. Berger M, Baldwin WM, Jordan SC. Potential Roles for C1 Inhibitor in Transplantation. *Transplantation*. 2016;100(7):1415-1424. doi:10.1097/TP.0000000000000995
25. Kolkert JLPJ, 't Hart NAN, Van Dijk A, Ottens PPJ, Ploeg RJR, Leuvenink HHGD. The gradual onset brain deat model: a relevant model to study organ donation and its consequences on the outcome after transplantation. *Lab Anim*. 2007;41(363). doi:10.1258/002367707781282848
26. Kotimaa J, van der Pol P, Leijtens S, et al. Functional assessment of rat complement pathway activities and quantification of soluble C5b-9 in an experimental model of renal ischemia/reperfusion injury. *J Immunol Methods*. 2014;412:14-23. doi:10.1016/J.JIM.2014.06.011
27. Pratschke J, Wilhelm MJ, Kusaka M, et al. Accelerated rejection of renal allografts from brain-dead donors. *Ann Surg*. 2000;232(2):263-271. doi:10.1097/00000658-200008000-00017
28. Pratschke J, Wilhelm MJ, Laskowski I, et al. Influence of donor brain death on chronic rejection of renal transplants in rats. *J Am Soc Nephrol*. 2001;12(11):2474-2481. doi:10.1097/01.tp.0000134769.65550.70
29. Sánchez-Fructuoso AI, Prats D, Marques M, et al. Does donor brain death influence acute vascular rejection in the kidney transplant? *Transplantation*. 2004;78(1):142-146.
30. Sheen JH, Heeger PS. Effects of complement activation on allograft injury. *Curr Opin Organ Transplant*. 2015;20(4):468-475. doi:10.1097/MOT.0000000000000216
31. De Serres J, Gröner A, Lindner J. Safety and efficacy of pasteurized C1 inhibitor concentrate (Berinert P) in hereditary angioedema: a review. *Transfus Apher Sci*. 2003;29(3):247-254. doi:10.1016/j.transci.2003.08.006
32. Gasser M, Waaga-Gasser AM, Grimm MW, et al. Selectin Blockade Plus Therapy with Low-Dose Sirolimus and Cyclosporin A Prevent Brain Death-Induced Renal Allograft Dysfunction. *Am J Transplant*. 2005;5(4):662-670. doi:10.1111/j.1600-6143.2005.00763.x
33. Davis AE, Cai S, Liu D. C1 inhibitor: biologic activities that are independent of protease inhibition. *Immunobiology*. 2007;212(4-5):313-323. doi:10.1016/j.imbio.2006.10.003
34. Sommer W, Tudorache I, Kühn C, et al. C1-Esterase-Inhibitor for Primary Graft Dysfunction in Lung Transplantation. *Transplantation*. 2014;97(11):1185-1191. doi:10.1097/TP.0000000000000034
35. Rebolledo R, Liu B, Akhtar MZ, et al. Prednisolone has a positive effect on the kidney but not on the liver of brain dead rats: a potencial role in complement activation. *J Transl Med*. 2014;12(1):111. doi:10.1186/1479-5876-12-111
36. Atkinson C, Varela JC, Tomlinson S. Complement-dependent inflammation and injury in a murine model of brain dead donor hearts. *Circ Res*. 2009;105(11):1094-1101. doi:10.1161/CIRCRESAHA.109.194977
37. Fujita T, Matsushita M, Endo Y. The lectin-complement pathway-its role in innate immunity and evolution. *Immunol Rev*. 2004;198(1):185-202. doi:10.1111/j.0105-2896.2004.0123.x
38. Curci C, Castellano G, Stasi A, et al. Endothelial-to-mesenchymal transition and renal fibrosis in ischaemia/reperfusion injury are mediated by complement anaphylatoxins and Akt pathway. *Nephrol Dial Transplant*. 2014;29(4):799-808. doi:10.1093/ndt/gft516
39. Montgomery RA, Orandi BJ, Racusen L, et al. Plasma-Derived C1 Esterase Inhibitor for Acute Antibody-Mediated Rejection Following Kidney Transplantation: Results of a Randomized Double-Blind Placebo-Controlled Pilot Study. *Am J Transplant*. Published online June 2016. doi:10.1111/ajt.13871
40. Murugan R, Venkataraman R, Wahed AS, et al. Preload responsiveness is associated with increased interleukin-6 and lower organ yield from brain-dead donors. *Crit Care Med*. 2009;37(8):2387-2393. doi:10.1097/CCM.0b013e3181a960d6
41. Pullerits R, Oltean S, Flodén A, Oltean M. Circulating resistin levels are early and significantly increased in deceased brain dead organ donors, correlate with inflammatory cytokine response and remain unaffected by steroid treatment. *J Transl Med*. 2015;13(1):201. doi:10.1186/s12967-015-0574-1
42. Saat TC, Susa D, Roest HP, et al. A comparison of inflammatory, cytoprotective and injury gene expression profiles in kidneys from brain death and cardiac death donors. *Transplantation*. 2014;98(1):15-21. doi:10.1097/TP.0000000000000136
43. de Vries DK, Lindeman JHN, Ringers J, Reinders MEJ, Rabelink TJ, Schaapherder AFM. Donor Brain Death Predisposes Human Kidney Grafts to a Proinflammatory Reaction after Transplantation. *Am J Transplant*. 2011;11(5):1064-1070. doi:10.1111/j.1600-6143.2011.03466.x
44. Murugan R, Venkataraman R, Wahed AS, et al. Increased plasma interleukin-6 in donors is associated with lower recipient hospital-free survival after cadaveric organ transplantation. *Crit Care Med*. 2008;36(6):1810-1816. doi:10.1097/CCM.0b013e318174d89f