Effects of brain death on donor organ viability in transplantation

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Summary and perspective

Organ transplantation has evolved from an experimental procedure in the 1950's and 60's to the therapy of choice for end-stage organ failure. The first solid organ to outgrow the experimental transplantation setting was the kidney. At that time the successful transplant programs were those in which donor organs from living family members were used for transplantation in their ill relatives (living-related transplant combination). Due to the success of these transplantations and an increasing number of patients with endstage renal disease opting for this new treatment regimen a discrepancy between demand and supply of kidney grafts rapidly developed and additional donor sources were sought to enlarge the donor organ pool. These remained limited to living related and a limited number of non-heart beating donors until the late 1960's, when post-mortal organ donation became possible after the institution of brain death. At that time, technical innovations made it possible to maintain an adequate oxygen concentration in peripheral tissues by mechanical ventilation in patients without a ventilatory drive. This implied a major step in anesthesiology, while at the same time in head traumatized heart-beating cadavers a phase of temporary stabilization could be achieved. Thus introducing a new medical entity, now referred to as brain death. After thorough discussion and overcoming moral, legal, and religious issues now considering this phase as a terminal end point (1), cadaveric donors became the main source of donor organs in solid organ transplantation . A number of physiological and pathophysiological changes induced by brain death have since then been studied (2-6). However, potential deleterious effects of brain death on donor organ viability have not been the subject of any previous study. This is surprising since a significant difference in transplant results can be observed when outcome of living related and unrelated (mostly spouses) transplant combinations are compared to those of cadaveric transplant combinations (7-9). Outcome after transplantation is significantly better in the living unrelated transplant combination despite the fact that HLA matching is better in the cadaveric transplant combination. These results cannot entirely be explained by shorter cold ischemia times in the elective living (un) related setting. These differences have been consistently observed and support our hypothesis that the unphysiological state of brain death in the cadaveric donor does contribute to decreased graft survival. This effect could be caused by immune activation of the potential donor organ. The concept of immune activation is based on statements of several authors in the past who repeatedly have pointed at the fact that the driving force behind immune responses is the need to recognize danger, instead of the historical opinion of discrimination between self and non-self (10-12). The phase of brain death could very well be interpreted as a situation of significant danger, thus leading to an adequate immune response. Immune activated donor organs

would then be transplanted in HLA non-identical recipients subsequently triggering host allo-responsiveness and increasing the rate of acute and chronic rejection.
The aim of this thesis was to analyze whether the phase of brain death in the donor had deleterious effects, presumably due to immune activation of the potential donor organ, and thus at least in part- explaining above-mentioned differences in graft survival after transplantation.
To study this hypothesis first a brain death model in the rat was developed. After validation of this model and obtaining consistent results a series of subsequent experiments were carried out.

In the first series of experiments, presented in **Chapter III** the effects of brain death in hypotensive donors are studied. Hypotension will inevitably occur after onset of brain death, unless adequately treated. In donors with persisting hypotension, referred to as marginal donors, potential donor organs will suffer more than in normotensive stable donors as reflected by the increased risk of primary dysfunction once organs retrieved from these donors are transplanted. Short (1h) and longer term (6h) exposure to a period of brain death was studied and immune cell activation in potential donor liver and kidney tissue was evaluated. Standard serum parameters were also determined. The data obtained were compared to those of a normotensive non-brain dead control group. In addition, hypotensive non-brain dead controls were used to study potential effects of hypotension as such. First, acute and prolonged effects on organ function was shown as demonstrated by increased concentrations of the liver function marker aspartate transaminase (113 vs. 253 vs. 272 in controls, 1h BD an 6h rats respectively) and kidney function marker creatinine (43 vs. 87 vs. 178). Furthermore, definite tissue activation was shown by the increased expression of cell adhesion molecules and a marked influx of leukocytes in kidney (9.1 vs. 17 vs. 20 in controls, 1h BD and 6h BD rats respectively) and liver tissue (12.3 vs. 13 vs. 27). These effects were not caused by hypotension as such, since no differences between normotensive non-brain dead controls and hypotensive non-brain dead controls were shown in any of the studied parameters.

To determine the effects of brain death without interference of hypotension, however, a normotensive brain death model was used in following studies. In **Chapter IV** and **V** effects of brain death on liver and kidney tissue were separately investigated. In both series of experiments short and longer periods of brain death in the presence and absence of hemodynamic instability were applied. In these studies, rats were randomly allocated to one of six groups: two sham operated non-brain dead control groups killed 1h or 6h after onset of the experiment or four brain dead groups. These rats were killed 1h or 6h

after onset of brain death. During the phase of brain death two of these groups, the 1h and 6h optimal donors received hemodynamic support to maintain normotension. The two other brain dead groups remained hypotensive. Again, standard serum parameters were monitored. Immune activation of organs was investigated in more detail. In **Chapter IV** the effects of brain death on liver tissue are described. This study showed progressive liver dysfunction by increased α-GST levels, most pronounced in hemodynamic unstable brain dead donors (1h: 25 vs. 428 vs. 168 and 6h 18 vs. 919 vs. 642 in controls, marginal and optimal donors, respectively). Irrespective of hemodynamic status, a progressive inflammatory activation was observed in brain dead rats compared to controls. Increased expression of cell adhesion molecules facilitated influx of leukocytes into the potential donor liver. The immune activation resembled a non-specific immune response with the influx of leukocytes consisting primarily of polymorfo mononuclear cells.
In **Chapter V** similar effects on kidneys are described showing kidney dysfunction expressed as increased serum creatinine levels (1h: 59 vs. 87 vs. 60; 6h: 43 vs. 178 vs. 98 in controls, marginal and optimal brain dead donors, respectively). Both, activation of cell adhesion molecules and influx of leukocytes in the donor kidney are similar to the observations in liver tissue. From these studies it was concluded that the phase of brain death in the donor causes progressive liver and kidney dysfunction, which is enhanced by hemodynamic instability in the donor. Also, a non-specific immune activation is induced by the onset of brain death, irrespective of hemodynamic stabilization with influx of primarily polymorphomononuclear cells. A similar response is shown in both studied organs indicating a generalized response instead of a specific organ oriented reaction.

As part of the inflammatory response, as shown in these liver and kidney biopsies, inflammatory mediators and acute phase proteins are upregulated. In addition of being involved in inflammation, these substances can influence vital processes such as apoptosis and drug metabolism capacity of the liver. Especially TNF-α, the key inflammatory mediator, can activate a potentially detrimental process of apoptosis. On the other hand, binding of TNF-α on its ligand can also counteract the onset of apoptosis by activation of the NF-κB regulated survival pathway. In **Chapter VI** we investigated whether the inflammatory response in the liver of brain dead donors is accompanied by changes in apoptosis and expression of apoptosis related proteins, in particular those regulated by NF-κB. Next, we analyzed which of the two major pathways leading to apoptosis, the cell surface mediated pathway or the mitochondrial pathway, was activated when induction of apoptosis occurred. In this experiment caspase 3 enzyme activity, a downstream effector caspase, was significantly increased in liver tissue of brain dead rats compared to non-brain dead control rats (38.1 vs. 17.1 AFU/μg DNA in 6h brain dead rats vs. non-brain dead controls

respectively). TUNEL staining revealed that the apoptotic cells were primarily hepatocytes. mRNA levels of all cytokine induced activators (Fas, Bid) and inhibitors (A1, BCL-xl, cIAP2) of both apoptotic pathways were significantly increased in liver tissue of brain dead donors versus non-brain dead controls. Based on these results we concluded that the phase of brain death induces increased apoptosis of hepatocytes despite enhanced expression of NF-κB dependent anti-apoptotic genes. Apoptosis in hepatocytes is mediated by both pathways, as has been shown previously under different circumstances. The increased rate of apoptosis will contribute to a decreased graft viability. Thus, prevention of induction of apoptosis in the future might indeed improve donor organ viability.

Similar cytokines involved in the process of apoptosis can affect the metabolic liver capacity contributing to the observed decreased organ viability. Therefore, metabolic capacity was evaluated using livers of 6h normotensive brain dead rats in **Chapter VII**. The livers from these animals were flushed with cold University of Wisconsin (UW) solution during explantation. Once cold stored, a liver slice model was used to prepare the liver tissue. This model enabled us to perform multiple tests on each liver and study the maintenance of the liver architecture and important cell-cell interactions. Tests were performed after flush-out and reoxygenation of the liver in the incubation medium. Liver cell integrity was studied by measuring ATP content and ATP driven urea synthesis. To investigate whether immune activation induces cytokine production in non-parenchymal cells of the liver (i.e. Kupffer and endothelial cells), cytokine production (IL-10 and IL-1B) and inducible nitric oxide synthesis (iNOS) upregulation was measured. Effects on metabolic capacity were studied by determination of phase 1 and 2 metabolism of model compounds. Results show that the phase of brain death, subsequent preservation and re-oxygenation had no effect on the general cell integrity. Activation of non-parenchymal cells occurred with a temporary onset of Kupffer cells and a lasting activation of endothelial cells. Upregulation of iNOS and NOx production was shown indicating cytokine release, although no significant differences in metabolic liver capacity could be observed.

To this point, our studies indicated that the phase of brain death produces less inert and activated donor organs that will likely provoke an increased immuneresponse after transplantation resulting eventually in a decreased graft survival. To analyze this a transplant study was performed that is described in **Chapter VIII**. Donor livers used were derived either from 6h normotensive brain dead rats or non-brain dead control rats. Livers were flushed with UW solution and immediately transplanted or flushed and cold stored for 20h in UW solution prior to transplantation in syngeneic recipients. Graft function in the recipient an animal survival was monitored for 14 days. Serum levels of LDH and AST

LDH and AST were monitored after transplantation. Graft survival of livers derived from non-brain dead donors both immediately transplanted and/or stored for 20h was 100%. Survival decreased significantly when livers were procured from brain dead donors even without any preservation to 75% (6/8). Only 20% (2/10) of the animals survived when livers retrieved from brain dead donors were cold stored for 20h prior to transplantation. Biopsies of these livers in the latter group showed pericentral necrosis and vacuolization of hepatocytes. In human transplants these findings are associated with primary dysfunction. This study showed that brain death induced alterations in the donor liver render it more sensitive to preservation and ischemia/reperfusion injury than livers used from living donors which will result in an increased immune-response and decreased graft survival.

Simultaneous to our efforts to better evaluate the effects of brain death on organ viability the group of Tilney at Harvard University (13) performed kidney allograft experiments. In their study, they transplanted kidneys retrieved from brain dead donor rats using a slow rejecting donor-recipient combination . They were able to show that kidney grafts harvested from 6h normotensive brain dead donors experienced an accelerated rejection and had a significantly decreased graft survival compared to those transplanted from non-brain dead controls. These results confirmed the hypothesis of brain death related injury in the allogeneic kidney transplant model and are in accordance with our study in the liver transplant model.

Following our experimental results demonstrating consistent and mounting evidence of brain death induced alterations to potential donor organs effects of brain death in our human donors were studied. We initiated the routine collection of kidney biopsies prior to organ preservation in the donor, after preservation and after reperfusion in the kidney graft recipient. Immunohistochemistry and semiquantitative reverse transcriptase-polymerase chain reaction were performed on the biopsy specimens of 27 cadaveric and 34 living (un)related donor derived biopsies (14). Data from these biopsies confirmed brain death induced endothelial cell activation by a significant increase in E-selectin expression and interstitial leukocyte invasion in the cadaveric biopsies. No significant differences in intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) between both groups, however, were detected. Furthermore, protective proteins heme oxygenase (HO)- 1 and heat shock protein (Hsp)70 were significantly increased after brain death. Evaluation of clinical outcome confirmed a significantly higher delayed graft function rate and inferior survival rates and creatinine levels in the brain dead group compared with the living group. The amount of ICAM, VCAM and leukocytes in the biopsy specimens was shown to be associated with elevated serum creatinine values one and three years after transplantation and poorer creatinine clearance, which was most

profound in brain dead donors. A positive effect of HO-1 on patient and graft survival could only be observed in kidneys from living donors, most likely due to the superfluous amount of damage occurring during brain death.
Koo from Fuggle's group in Oxford (15) also showed endothelial cell activation in kidney biopsies of cadaveric donors. In their study an immunohistochemical comparison of postpreservation pretransplant biopsies from cadaveric (N=65) versus living related donor (N=29) kidneys was performed. High levels of endothelial E-selectin and proximal tubular expression of HLA-DR antigens were detected. In contrast to our clinical study, they showed increased levels of ICAM-1, and VCAM-1 in biopsies from cadaveric kidneys, whereas expression of these markers was markedly reduced in living related kidney biopsies. The expression of pretransplant tubular antigens in cadaver donor kidneys was found to be significantly associated with early acute rejection following transplantation.

Both, the work by the Oxford group and our own in human donor organs, indicate that similar processes are induced by brain death in humans and in animals that will result after transplantation in increased rates of injury and early acute rejection. Although episodes of early acute rejection can be effectively treated, grafts once involved in delayed graft function or an episode of acute rejection show an inferior graft survival (16,17).

Based on the previously results mentioned, improvement of donor organ viability can be achieved by prevention of upregulation of inflammatory mediators during brain death. Recently, Pratschke et al. performed the first intervention study with promising results (18). In a kidney transplant study four groups of F344 rats were used as organ donors and Lewis rats as recipients. In this weak strain combination the acute rejection process occurs at an attenuated tempo, emphasizing any donor-associated changes. In three of the four donor groups brain death was induced. One group served as non-brain dead control. After brain death induction rats were either not treated, injected with glucocorticoids or injected with soluble P-selectin glycoprotein ligands (sPSGL). Steroids were administered to reduce the release of inflammatory cytokines, sPSGL was given to prevent leukocyte adhesion to the inflamed vascular endothelium of the graft. Infiltration of cells occurred in relative small numbers before transplantation in the kidneys of living donor controls and treated brain dead donor groups. Recipients of kidney allografts from untreated brain dead donors experienced an accelerated rate of acute rejection and died of renal failure significantly earlier than controls. Recipients of treated kidneys showed survival times comparable to living donor controls and significantly better than the untreated allografts. This study suggests that counteracting the effects of brain death is feasible in the rat model. The best treatment options and time to start treatment will be subject to future experimentation.

To improve treatment modalities a better understanding of the cause leading to immune activation is crucial. Therefore, we performed studies that analyse effects of brain death in more detail using the DNA microarray technique (19). Both human and rat tissue biopsies are tested on expression of multiple genes. In the first study involving kidney tissue of 6h brain dead rats most genes were categorized in potential interesting different functional groups: Metabolism/Transport, Inflammation/Coagulation, Cell Division/Fibrosis and Defence/Repair. Also, genes encoding transcription factors and proteins involved in signal transduction were identified. In this study, we could show that brain death leads via activation of transcription factors and signal transduction cascades to differential expression of different 'effector' genes. Not only deleterious processes as inflammation and fibrosis occurred in rat brain dead donor kidneys but genes involved in protection and early-repair processes are activated as well. A similar study is currently being performed using human kidney biopsies taken before and after transplantation.

In conclusion, we have shown that brain death should no longer be considered as a static and given condition, but as a dynamic process that significantly influences the viability of peripheral potential donor organs. Duration of brain death should be kept as short as possible and hemodynamic instability should be counteracted. The first crude treatment regimen in the brain dead donor decreases the alloimmune response after transplantation and improves graft survival in animal models.

The sum of the collected data, on the effects of brain death and unphysiological stress on the donor organ viability, up to now shows great potential for future research. A first impression has been formed concerning mechanisms playing a crucial role in the decreased donor organ quality. In the years to come, the possibilities of targeted cytoprotective interventions in brain dead donors must be explored to improve donor organ viability or maintain it at the current level while expanding the donor criteria. In this process the contribution of this thesis is only the tip of the iceberg.