Association of Graft Effluent Parameters with Donor Body Mass Index, Graft Quality, and Post-Transplant Events

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Background: We evaluated whether effluent parameters prior to reperfusion correlate with post-transplant outcomes in liver transplant recipients.

Material/Methods: Concentrations of high mobility group box 1 protein (HMGB1), uncleaved cytokeratin-18 (M65), caspase-cleaved cytokeratin 18 fragment (M30), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP) were measured in effluent samples from 53 adult liver recipients (42 survived for 1 year and 11 did not survive).

Results: Effluent concentrations of ALP (p=0.006), AST (p=0.050), and Ca²⁺ (p=0.003) were higher in patients with bacteremia in the first post-transplant year and ALP (p=0.015) was higher in patients with early graft dysfunction (EAD). Multivariate analysis of effluent parameters showed that Ca²⁺ >0.30 mmol/l (p=0.012, odds ratio [OR]=7.12, confidence interval [CI]=1.56–32.58), and ALP ≥27 IU/l (p=0.033, OR=5.31, CI=1.14–27.74) were significantly associated with 1-year post-transplant bacteremia, whereas ALP ≥27 IU/l (p=0.020, OR=5.56, CI=1.32–23.46) was significantly associated with EAD. HMGB1 >54 pg/ml (p=0.008, OR=6.05, CI=1.59–23.00) was significantly associated with the donor body mass index (p=0.008, OR=6.05, CI=1.59–23.00) and fatty liver (p=0.005, OR=11.68, CI=2.10–64.01).

Conclusions: Effluent parameters are indicators of liver quality and predict the outcome of liver transplantation. High effluent Ca²⁺ and ALP are risk factors of post-transplant bacteremia. In addition, high ALP is a risk factor of EAD, and high HMGB1 is an indicator of liver quality.

MeSH Keywords: Bacterial Infections • Calcium • Flushing • Liver Transplantation

Abbreviations: ALP – alkaline phosphatase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; BMI – body mass index; CMV – cytomegalovirus; EAD – early graft dysfunction; GGT – gamma-glutamyl transpeptidase; HBV – hepatitis B virus; HCV – hepatitis C virus; HMGB1 – high mobility group box 1 protein; INR – international normalized ratio; IU – international unit; LDH – lactate dehydrogenase; M30 – Caspase-cleaved cytokeratin 18 fragment; M65 – uncleaved cytokeratin-18; MELD – model for end-stage liver disease; OR – odds ratio; ROC – receiver operating characteristic

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Background

Predicting postoperative complications may help to promote patient and graft survival after liver transplantation [1,2]. Early post-transplant complications, including hemorrhage, vascular dysfunction, vascular leakage, bacterial infection, and early allograft rejection and dysfunction, are the main postoperative problems impacting patient and graft survival [1,2]. Postoperative bacterial infections, especially bacteremia, are associated with morbidity and mortality after liver transplantation [3,4]. These bacterial infections in liver transplant recipients are influenced by allograft, operation, donor, and recipient factors [4–6].

The association between effluent parameters and liver transplant outcome has been studied. Ischemia time was recently shown to correlate with the release of injury markers in the liver effluent [7–9]. We have reported that damaged epithelial cells and hepatocytes can be detected by purine nucleoside phosphorylase levels in donor plasma and transplant effluent in pigs [10]. In another study, effluent parameters were associated with survival rate in an experimental porcine liver transplant model; low effluent aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) levels were associated with higher survival rates [11]. These findings were confirmed in humans; higher effluent aminotransferase and LDH levels correlated with 1-month survival in liver transplant recipients [12,13]. Likewise, levels of xanthine oxidoreductase, pro-inflammatory cytokines, hyaluronic acid, von Willebrand factor, and other immune responses in caval effluent correlated with early graft dysfunction (EAD) [14–18].

High mobility group box 1 protein (HMGB1) is a member of the HMGB family and protects cells from injury in normal organs [19]. However, in the liver, HMGB1 plays a critical role in hepatic ischemia/reperfusion and acetaminophen-induced liver necrotic injury and some cancers [19]. Cytokeratin-18 (M65) is a major intermediate filament protein in the liver and is released into the extracellular space during cell death [20]. CK18 can be cleaved into fragments of approximately 30 and 45 kDa by caspases. The 30 kDa fragment can be detected by a specific antibody (M30). The M30: M65 ratio effectively differentiates between apoptotic and necrotic cell death [20].

The aim of this study was to evaluate whether effluent parameters prior to reperfusion correlate with post-transplant outcomes such as EAD, acute rejection, viral and bacterial infections, and mortality in liver transplant recipients.

Material and Methods

Patients

Ninety-seven liver transplantations were performed at the Department of General, Visceral, and Transplant Surgery, University of Heidelberg between January 2009 and December 2009. Eleven patients died during the first post-transplant year (11% mortality rate). Pre-transplant concentrations of HMGB1, M65, M30, alanine aminotransferase (ALT), AST, gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP) were measured in effluent samples from 11 recipients that did not survive the first post-transplant year (age 57.1±9.5 years; 2 females) and 42 recipients who survived the first post-transplant year (age 51.2±11.0 years; 10 females). The transplantation was necessary because of liver failure caused by chronic hepatitis (H) C virus (V) (HCV) and/or HBV infection in 19 patients; alcohol abuse in 17 patients; and congenital, autoimmune disease, and/or diseases with unknown etiology (including cryptogenic cirrhosis, biliary disease, metabolic liver disease, autoimmune hepatitis, and amyloidosis) in 15 patients. Two patients had acute toxic hepatitis.

To diagnose EAD, total bilirubin, international normalized ratio (INR), ALT, AST, ALP, GGT, and LDH plasma levels were measured daily before transplantation until the tenth post-transplant day. Disease severity was based on MELD staging and varied between patients. Demographic data of surviving and non-surviving patients are shown in Table 1. Post-transplant anti-infection prophylaxis included 3 days of cefuroxime and metronidazole treatment, 3 months of cotrimoxazole treatment, and 10 days of itraconazole, voriconazole, or caspofungin treatment. Recipients of cytomegalovirus (CMV)-positive donors were treated with an oral prophylaxis of valganciclovir for 3 months.

Demographics and patient characteristics

Eleven patients died during the first post-transplant year due to graft failure and sepsis. Fourteen patients experienced EAD and acute rejection occurred in 11 patients. Demographic, pre-transplant characteristics, post-transplant characteristic, and laboratory findings were similar between rejectors and non-rejectors. Demographic data, including age, sex, original liver diseases, pre-transplant CMV, HBV, and HCV IgG status, as well as kidney and liver function (bilirubin and INR), were similar in survivors and non-survivors (Table 1). Three patients with EAD and 8 patients without EAD (p=0.94) died during the first post-transplant year. Demographic data including age, sex, original liver diseases, pre-transplant CMV, HBV, and HCV IgG status as well as kidney function were similar in patients with and without EAD and in bacteremic and non-bacteremic patients.
**Effluent sampling**

The graft was flushed with 500 mL of chilled (4°C) histidine-tryptophan-ketoglutarate (HTK) solution through a catheter placed in the portal vein both on the back table and during cold ischemia. The first 20 mL of rinsing effluent was collected shortly before transplantation from the inferior caval vein and aliquoted. All samples were stored at below –40°C until the day of analysis. All effluent parameters in all samples were investigated in duplicate.

**Determination of effluent AST, ALT, ALP, GGT, and LDH levels**

Effluent AST, ALT, ALP, GGT, and Ca++, Na+ and Mg++ ions were assessed in a certified laboratory of the Limbach group in Heidelberg.

**Determination of serum immune parameters**

Cell apoptosis markers (M30 and M65) and HMGB1 were measured by ELISA using Quantikine Kits (R&D Systems, Wiesbaden, Germany).

**Table 1.** Demographic and characteristic data of patients with and without one year survival.

| Parameters                            | Non-survivor (n=11) | Survivor (n=42) | p   |
|---------------------------------------|---------------------|----------------|-----|
| Age (mean ±SD; years)                 | 57.1±9.5            | 51.2±11.0      | 0.07|
| Female/male (n)                       | 2/9                 | 10/32          | 0.69|
| Original liver disease hepatitis/alcoholic/others (n) | 5/3/3               | 14/16/12       | 0.72|
| Re-transplantation (n)                | 4                   | 6              | 0.10|
| Encephalopathy (n)                    | 4                   | 11             | 0.50|
| MELD score                            | 18.9±9.0            | 18.8±9.0       | 0.91|
| preTx bil (mg/dl) (component of meld score) | 9.8±11.1            | 7.2±7.4        | 0.88|
| preTx INR (component of meld score)   | 1.4±0.5             | 1.4±0.4        | 0.91|
| preTx serum albumin (g/L)             | 28.6±6.6            | 31.7±5.9       | 0.11|
| HBV-ab+ (n)                           | 0                   | 0              | 0.18|
| HCV-ab+ (n)                           | 5                   | 9              | 0.11|
| CMV-ab+ (n)                           | 5                   | 25             | 0.40|
| Donor BMI                             | 27.3±3.8            | 25.8±4.2       | 0.22|
| Donor age (mean±sd years)             | 59.1±23.3           | 55.7±17.1      | 0.28|
| Donor gender female (n)               | 5                   | 20             | 0.90|
| Donor CMV+ (n)                        | 7                   | 25             | 0.80|
| Cold ischemia time (H)                | 8.6±2.1             | 9.9±2.5        | 0.06|
| Operation time (H)                    | 6.6±1.4             | 5.3±1.2        | 0.009|
| Blood loss (L)                        | 4.2±1.5             | 4.5±4.4        | 0.33|
| Intra-OP packed red cells transfusion (unit) | 11±11               | 9±9            | 0.35|
| EAD (n)                               | 3                   | 11             | 0.94|
| One year bacteremia (n)               | 10                  | 6              | <0.001|
| AR (n)                                | 3                   | 8              | 0.55|

Mann-Whitney-U test, chi square Kruskal- Wallis and Fisher exact tests were used. Tx – transplantation; BMI – body mass index; HBV – hepatitis B virus; HCV – hepatitis C virus; CMV – cytomegalovirus; OP –operation; EAD – early allograft dysfunction; AR – acute rejection; L – liter; H – hour.
Definition of EAD

EAD was diagnosed postoperatively by laboratory tests that measure liver injury and dysfunction, such as bilirubin ≥10 mg/dL on day 7, INR ≥1.6 on day 7, or ALT and/or AST >2000 IU/L within the first 7 post-transplant days.

Statistical analyses

Categorical and continuous variables were analyzed using chi-square, Fisher exact, and Mann-Whitney U tests. Continuous variables were modeled and stratified by median. The most sensitive cut-off values were calculated by receiver operating characteristic (ROC) curve analysis. Univariate and multivariate logistic regression analyses identified the greatest predictive risk factors for EAD, bacteremia, graft loss, and mortality. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) 22.0. After Bonferroni correction, p values <0.05 were defined as statistically significant.

Results

Demographics and patient characteristics

Patients who died during the first post-transplant year were not significantly older (p=0.07) and had longer operation times (6.6±1.4 h vs. 5.3±1.2 h, p=0.009) than patients who survived the first year after transplantation (Table 1). Six patients in the survivor group and 4 patients in the non-survivor group had re-transplantations (p=0.10). Thirty-four patients experienced bacterial infections, including urinary tract infection, blood stream infection, pneumonia, wound infection, and cholangitis, during the first 42±52 post-transplant days (11/11 non-survivors vs. 23/42 survivors, p=0.001). Sixteen of 34 patients with bacterial infection had bacteremia (10/11 non-survivors vs. 6/42 survivors, p=0.001). Compared with patients with early allograft function, EAD patients had significantly higher pre-transplant serum bilirubin (component of MELD score; p=0.003), slightly higher MELD score (p=0.02), higher INR (component of MELD score, p=0.020), and longer ICU stay (p=0.05). The HCV and HBV statuses were negative in all donors. Bacteremia was slightly more frequent in patients with EAD than in patients with early allograft function (7/14 vs. 9/39, p=0.06). Pre-transplant serum bilirubin was similar in bacteremic and non-bacteremic and in 1-year survivor and non-survivor patients (p=n.s.). The immunosuppression regimen included cyclosporine plus prednisolone in 26 patients and tacrolimus plus prednisolone in 22 patients. Nine patients in the cyclosporine group and 5 patients in the tacrolimus group also received mycophenolate mofetil.

Effect of effluent parameters on postoperative outcome

Effluent parameters were not significantly different between 1-year survivors and non-survivors, rejectors and non-rejectors, patients with and without post-transplant CMV infection, and recipients with 1-year graft survival and without graft survival. There were no significant differences in effluent AST, ALT, AST, M65, and M30 levels between bacteremic and non-bacteremic patients. Effluent concentrations of Ca²⁺ (p=0.001), ALP (p=0.002), and HMGB1 (p=0.040) were significantly higher in patients with bacteremia in the first post-transplant year than in patients without bacteremia (Figure 1). Effluent ALP concentrations were lower in patients with early allograft function than in EAD patients (p=0.016) (Figure 1).

Sensitivity and specificity of parameters in patients with post-transplant events

We performed ROC curve analysis to calculate cut-off values for significant effluent parameters. For bacteremia, the sensitivity and specificity values were 78% and 71% for effluent Ca²⁺ >0.30 mmol/l (area under curve=80%), 79% and 71% for effluent ALP >27 IU/l (area under curve=75%), and 64% and 75% for effluent HMGB1 >50 mg/ml (area under curve=69%), respectively. For EAD, effluent ALP >27 IU/l had a sensitivity of 79% and a specificity of 65% (area under curve=71%) (Figure 2A, 2B).

Regression analysis of significant parameters

Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate associations between significant parameters and bacteriemia/EAD using univariate and multivariate logistic regression models. Multivariate regression analysis showed that effluent Ca²⁺ >0.30 mmol/l (p=0.022, OR=5.96, CI=1.30–27.45) and ALP >27 IU/l (p=0.008, OR=7.74, CI=1.71–35.00) are significantly associated with 1-year post-transplant bacteremia and that an effluent ALP level of ≥27 IU/l (p=0.010, OR=6.55, CI=1.56–27.48) is significantly associated with EAD in liver transplant recipients.

Association between effluent parameters and donor BMI

Effluent concentrations of HMGB1 (90±75 vs. 33±32 pg/ml, p=0.002) and M65 (4122±2662 vs. 2151±1652 pg/ml, p=0.031) were higher in donors with a BMI >25 than in donors with a BMI ≤25. The sensitivity and specificity of significant parameters were calculated and are depicted in Figure 2C. Univariate and multivariate regression analyses of effluent parameters showed that only effluent HMGB1 >54 pg/ml (OR=6.05, CI 1.59–23.00, p=0.008) was significantly associated with the donor BMI.
Association between effluent parameters and donor fatty liver

Donors with ≥20% fatty liver (n=10) had significantly higher effluent HMGB1 (140±87 vs. 42±38 pg/ml, p<0.001), M65 (5452±2173 vs. 2514±2155 pg/ml, p=0.003), Ca²⁺ (0.49±0.28 vs. 0.26±0.15 mmol/l, p=0.012), and M30 (917±996 vs. 330±526 pg/ml, p=0.014) concentrations and a higher BMI (28±3.8 vs. 25±2.9, p=0.039) than did patients with <20% fatty liver (n=43). The sensitivity and specificity of significant parameters were calculated and are shown in Figure 2D. Univariate and multivariate regression analyses of effluent parameters showed that effluent HMGB1 >54 pg/ml (OR=11.68, CI=2.10–64.01, p=0.005) was significantly associated with fatty liver. Eight out of 10 patients with ≥20% fatty liver and 13 out of 43 patients with <20% fatty liver experienced bacterial infection (p=0.004).

Discussion

Early identification of transplant recipients who are at risk of mortality, rejection, and infection, improves transplant outcome. The rate of blood stream infection is high after liver transplantation and is associated with high mortality rates [4]. Known risk factors of bacteremia include repeat surgery, ABO incompatibility, preoperative massive pleural effusion or ascites, Child-Pugh class C, postoperative cytomegalovirus infection, massive operative blood loss, and older age [4]. Donor-related risk factors of bacteremia after liver transplantation have not been well defined. Donor age, prolonged ICU stay, quality of the donor liver, donor infection, and donor viral status have been suggested as donor-related risk factors of bacteremia [5,21]. The use of hepatocellular parameters to preoperatively evaluate transplant quality had been studied by Lange et al. [22]. The authors concluded that effluent components are sensible markers for preexisting damage or acquired damage during cold ischemia [22]. Our data (Mann-Whitney test) show that effluent Ca²⁺ and ALP concentrations are increased in patients with post-transplant bacteremia (Figure 1). Interestingly, we also found an association of effluent Ca²⁺ concentration with biliary stasis (ALP and GGT), inflammatory markers of macrophage/monocyte activation (HMGB1) and epithelial cell death (M65). To the best of our knowledge, this is the first report showing that effluent Ca²⁺ concentrations may be a marker.
of allograft damage. Previous experimental and clinical studies showed that Ca$^{++}$ concentration plays a critical role in toxic cell death and programmed cell death [23–25]. Reduced apoptosis and cellular changes reduce mitochondrial Ca$^{++}$ concentrations, and excessive Ca$^{++}$ concentrations have been shown during ischemia/reperfusion [26,27]. Taylor et al. suggested that increased ionized Ca$^{++}$ concentrations in ischemic-damaged kidney effluents are caused by intracellular calcium release after organelle damage and lysis [28]. The present study shows there is a strong association between effluent Ca$^{++}$ and post-transplant bacteremia. Effluent Ca$^{++}$ levels also correlated with inflammatory and cell death markers, in agreement with previous findings from Taylor et al. [28]. The effect of intracellular Ca$^{++}$ on cell death is known [29], but it has both beneficial and detrimental effects on hepatocellular apoptosis and injury.

An influx and accumulation of extracellular Ca$^{++}$ions often contributes to lethal cell injury [30]. Extracellular and cytosolic Ca$^{++}$ concentrations differ considerably [31]. The continuous inflow of Ca$^{++}$ through the plasma membrane is balanced by specific Ca$^{++}$-ATPases, which extrude Ca$^{++}$ from cells [31]. A high extracellular Ca$^{++}$ concentration is toxic for hepatocytes [32]. We speculate that a higher effluent Ca$^{++}$ concentration is a marker of organ injury and might be caused by apoptosis and necrosis of hepatic cells [26–28]. In this study, all allografts were conserved and perfused using a HTK solution containing 0.015 mmol/l Ca$^{++}$. Almost all cases of bacteremia during the first 3 post-transplant months occurred in patients with an effluent Ca$^{++}$ concentration >0.3 mmol/l. Therefore, we believe that the Ca$^{++}$ contained in the perfusion solution did not affect the incidence of bacteremia in our patients.

**Figure 2.** ROC curve analysis of the effect of significant effluent parameters on: (A) bacteriemia; (B) early graft dysfunction (EAD); (C) body mass index (BMI), and (D) fatty liver. (A) Alkaline phosphatase (ALP) and Ca$^{++}$ levels in bacteremic (n=15) vs. non-bacteremic (n=38) patients. (B) ALP levels in patients with early graft dysfunction (EAD) (n=14) vs. patients without EAD (n=39). (C) M65 and HMGB1 levels in patients with a BMI ≥25 vs. a BMI <25. (D) ROC curve analysis of effluent M65, M30, HMGB1, and Ca$^{++}$ concentrations, and ≥20% fatty liver (n=10) vs. <20% fatty liver (n=43).
ALPs are present in many human tissues, including bone, intestine, kidney, liver, placenta, and white blood cells [33]. Liver ALP originates from the hepatobiliary tree and high ALP is a marker of intra- or extra-hepatic cholestasis, liver infiltrative diseases, hepatotoxicity, and primary sclerosing cholangitis [34]. Interestingly, high Ca++ and ALP concentrations in the effluent significantly increased the risk of bacteriemia during the first post-transplant year.

Remarkably, of all known liver enzymes, only ALP was associated with EAD. EAD is a multifactorial condition and is related to ischemia/reperfusion injury. We have shown that serum IFN-γ levels measured immediately before a transplant may predict EAD [35]. The rate of EAD after deceased donor transplantation is about 20%. Therefore, predicting and estimating EAD is very important [35]. The clinical impact of effluent parameters on EAD has not been studied in detail [14,36–38]. In a review article, Bolondi et al. suggested that the donor-risk index and extended criteria donor score cannot determine short-term graft and patient survival [39]. In contrast, we have shown that effluent ALP is a predictive marker of EAD and shows good sensitivity and specificity for predicting EAD. High serum ALP is a marker of progressive disease and poor outcome in patients with primary biliary cirrhosis and liver failure [40]. The recurrence of HCV infection and the progression of liver fibrosis are accelerated after liver transplantation in patients with biochemical cholestasis, which is defined by an increase in ALP and GGT [41].

BMI and M6S are independent predictors of non-alcoholic steatohepatitis and HMGB1 plays a critical role in pathogenesis of this disease [42]. HMGB1 and M6S are apoptosis markers and regulate the balance of autophagy and apoptosis. They are also released from cells with damaged membranes [43,44]. These 2 markers are associated with high BMI, suggesting that more cells are damaged in obese people. Our present findings are in agreement with those of previous studies [45].

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Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide [46]. Macrophase steatosis in >25% of hepatocytes defines a marginal donor liver [47]. Fatty liver is associated with changes in immune responses, including inflammation, oxidative stress, lipid peroxidation, mitochondrial dysfunction, and cell proliferation, such as CD4(+) and CD8(+) CD45RO subsets in the liver [46,48,49]. Hepatic steatosis is associated with an increase in mitochondrial complex I (C-I) activity-mediated leaks and decreased respiratory control ratios after cold ischemia [50]. Chu et al. suggested that severe steatosis (>60%) increases the risk of poor graft function, while moderate-to-severe steatosis (>30%) reduces graft survival [51]. The role of Toll-like receptors in NAFLD has been investigated in human and experimental studies [52–54]. HMGB1 mediates TLR4-induced activation of TLR4/MyD88 signaling in liver cells during the early progression of NAFLD. Furthermore, inhibiting HMGB1 prevents steatosis and inflammation through SIRT1-mediated HMGB1 deacetylation [55,56]. One clinical report has shown high plasma HMGB1 levels in pediatric patients with NAFLD [57]. Our study shows for the first time that effluent HMGB1 can be used as a marker of liver quality and fatty liver.

Conclusions

Effluent parameters are indicators of liver quality and can predict the outcome of liver transplantation. High effluent Ca++ and ALP are risk factors of post-transplant bacteriemia. In addition, high effluent ALP increases the risk of EAD, and effluent HMGB1 levels can indicate liver quality.

Conflict of interests

The authors declare that they have no competing interests.
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