Original Article
A prospective study of ischemia-type biliary lesions after liver transplantation

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Abstract: The aim of this study was to verify the risk factors of ischemia-type biliary lesions (ITBL) through prospective studies and to observe the relationship between cold preservation injuries of the bile duct (CPI-BD) and ITBL. From August 2012 to July 2013, 50 patients who had orthotopic liver transplantation (OLT) in the Organ Transplantation Center, First Central Hospital of Tianjin, were enrolled and put into the ITBL group (n = 8) and the NITBL group (32 cases) according to their postoperative bile duct ischemic conditions, and confirmed by HE staining and TUNEL apoptotic staining, together with electron microscopy. The risk factors were defined by multivariate analysis, including cold ischemic time, intraoperative fresh frozen plasma use, anhepatic time and intraoperative erythrocyte usage. There were no significant differences in the survival rate (P = 0.599) and graft survival rate (P = 0.073) between the two groups, but there was a significant difference in the cold ischemic time (P = 0.033). From the multivariate analysis, the cold ischemic time, usage of intraoperative fresh frozen plasma and anhepatic time were not independent risk factors for ITBL after liver transplantation. Light microscopy and electron microscopy showed that the epithelial cells in the bile duct of Group ITBL gradually appeared to have more mitochondrial vacuolar degeneration, the cell gap widened, and the biliary fibroblasts and collagen cells also appeared to have different degrees of apoptosis. The cold ischemic time is the risk factor toward the epithelial injury in the biliary tract, and the prolongation of cold ischemic time has an obvious correlation with the injury of donated bile duct tissue.

Keywords: Ischemia-type biliary lesions, liver transplantation, cold ischemia, risk factors, prospective study

Introduction

Liver transplantation is the only effective way to treat end-stage liver diseases [1], such as virus hepatitis [2], cholestatic liver diseases [3], primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis [4]. Studies report that the main indications for transplantation are hepatocellular carcinoma (38%), the hepatitis C virus (33.3%), and alcohol liver cirrhosis (19.6%) [5]. However, there are always some complications which will threaten the survival of grafts as well as affect the patients' quality of life, which are now encountered more commonly as a result of the increased number of liver transplantsations and the prolonged survival of transplant patients [6]. Biliary complications are a major source of morbidity, graft loss, and even mortality after liver transplantation [7]. The most troublesome are the so-called ischemic-type biliary lesions (ITBL), with an incidence varying between 5% and 15% [8, 9], which play an extremely important role in influencing the long-term survival and quality of life of recipients after orthotopic liver transplantation (OLT) [10]. Eventually, up to 50% of the patients with ITBL will require a retransplantation or may die. ITBL is a radiological diagnosis, characterized by intrahepatic strictures and dilatations on a cholangiogram, in the absence of hepatic artery thrombosis [11] and was established only when all other causes of destruction of the biliary tree were ruled out [12]. Hepatic resection and cryopreservation caused ischemic injuries to donors' livers, including any damages occurring during the entire process, namely from harvesting the donor liver to the reconstruction of liver-blood circulation in the recipient [13]. There have been many risk factors been found for ITBL,
including cold ischemia time, warm ischemia time [14], and so on. In this paper, we conducted a prospective study of the risk factors of ITBL in 50 OLT patients from June 2013 to December 2013, verified the risk factors resulting in the occurrence of ITBL, and observed the relationship between cold preservation injury of the bile duct and ITBL.

Materials and methods

Subjects

The 50 patients who underwent OLT in the Organ Transplantation Center from August 2012 to July 2013 were enrolled. Patients with primary sclerosing cholangitis or ABO blood group incompatibility were excluded from this study. All the patients underwent a classic non-bypass liver transplantation. Their ages ranged from 27 to 66 years, with the mean age 51.12 ±8.09 years old, and there were 38 males and 12 females. Primary diseases: 27 cases of hepatitis B related diseases, 8 cases of hepatitis C related diseases, 4 cases of re-transplantation, 3 cases of AIH, 2 cases of alcoholic cirrhosis, 1 case of PBC, 1 case of drug-induced liver disease, and 4 cases of cryptogenic cirrhosis. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Central Hospital of Tianjin. Written informed consent was obtained from all participants.

Specimen collection and grouping

The biliary tract tissue of all 50 patients was sampled individually after they completed their liver transplantations (completed cold ischemia), stored in 10% formalin, electron microscopic preservation solution, and -80°C freezer; the bile duct tissue was sampled after achieving the portal vein reflow (starting reperfusion) and stored at -80°C for future use. All the patients were followed up after surgery and we confirmed their diagnoses of ITBL according to their cholangiographies and imaging results. The patients were then divided into the ITBL group (n = 8) and the NITBL group (n = 42).

Observation indexes

The cold ischemic time, anhepatic time, intra-operative fresh frozen plasma, and intraoperative fresh tissue erythrocyte usage were recorded and compared between the two groups; the bile ducts were sampled and underwent HE staining and TUNEL apoptotic staining, together with electron microscopy (Philips, Eindhoven, Netherlands), to find the differences.

Statistical analysis

All 50 patients were analyzed using SPSS19 (SPSS 19.0, IBM, USA). The univariate analysis used a t test and a rank sum test, and the multivariate analysis used a logistic regression model to obtain the relevant risk factors, with $P < 0.05$ considered as statistically significant.

Results

Incidence of ITBL between the NITBL and ITBL groups

Among the 50 patients, a total of 8 patients had ITBL, with the incidence rate as 16%, including mild ITBL in 3 cases, moderate ITBL in 3 cases, and severe ITBL in 2 cases. Two patients with mild ITBL improved after biliary support therapy, and their liver function returned normal after we removed the drainage tube, but another patient underwent sustained biliary support therapy for more than one year and showed no significant liver functional abnormality or cholangitis. The patients with moderate ITBL underwent sustained biliary support therapy for more than one year, and 1 patient died of tumor recurrence, and 1 patient had intermittent attacks of cholangitis and abnormal liver function. The two patients with severe ITBL suffered ITBL-caused liver failure but achieved health after re-transplantation. The 1-year survival rate of ITBL patients was 87.5% (7/8), and the 1-year survival rate of the grafts was 62.5% (5/8) (Figure 1). There was no statistical significance in the survival rate ($P = 0.599$) or the graft survival rate ($P = 0.073$) between the two groups.

Relationship between different risk factors of ITBL and typing

The results showed that there was no statistical significance in the anhepatic time or the intraoperative use of erythrocytes and plasma between the ITBL and NITBL groups. There was statistical significance in the cold ischemic...
Comparison of HE staining results between the NITBL and ITBL groups

The bile duct tissue underwent HE staining and light microscopy (×100), and the results showed that the epithelium in the NITBL group was arranged neatly and had no obvious shedding of the bile duct epithelium (Figure 2A); the ITBL group showed sparsely arranged bile duct epithelia and obvious shedding (Figure 2B). The TUNEL staining results (×200) showed that the epithelia in the NITBL group were neatly arranged, and the biliary epithelial cells had no brown staining, namely the TUNEL staining was negative (Figure 3A). The ITBL group showed sparsely arranged bile duct epithelia and obvious shedding; the nuclei of a large number of biliary epithelial cells were stained brown, namely the TUNEL staining was positive (Figure 3B).

Eletron microscopy analysis between the NITBL and ITBL groups

The results of electron microscopy showed (Figure 4) that the biliary epithelial microvilli in the NITBL group was arranged neatly (Figure 4A) with complete inter-cell junctions (Figure 4C), homogeneously distributed chromatins in the nuclei (Figure 4E), and minor vacuolated mitochondria (Figure 4H). The microvilli in Group ITBL disappeared (Figure 4B) with ambiguous inter-cell junctions (Figure 4D), chromatin margination (Figure 4F) and pycnosis (Figure 4G) in the nuclei, and a large amount of mitochondrial vacular degeneration (Figure 4I).

Multi-factor analysis group NITBL and ITBL

The cold ischemic time, the anhepatic time, and the intraoperative usage of erythrocytes and plasma were included in the multivariate analysis, and the results showed that the cold ischemic time and the intraoperative usage of erythrocyte showed the strongest correlation with ITBL than the other factors, but there was no statistical significance. No independent risk factor for ITBL was found (Table 2).

Table 1. The relationships among different risk factors for ITBL and typing

<table>
<thead>
<tr>
<th>Group</th>
<th>ITBL (n = 8)</th>
<th>NITBL (n = 42)</th>
<th>Statistical value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhepatic time (min)</td>
<td>49.13±14.00</td>
<td>44.12±11.23</td>
<td>t = 1.235</td>
<td>0.272</td>
</tr>
<tr>
<td>v (units)</td>
<td>25.75±25.55</td>
<td>13.83±9.67</td>
<td>Z = -1.502</td>
<td>0.133</td>
</tr>
<tr>
<td>Frozen plasma (ml)</td>
<td>4250.0±3412.2</td>
<td>2466.7±1277.7</td>
<td>Z = -1.588</td>
<td>0.112</td>
</tr>
<tr>
<td>Cold ischemic time (h)</td>
<td>10.75±3.012</td>
<td>8.50±2.587</td>
<td>T = 4.831</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Table 2. Multivariate analysis

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Statistical value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE</td>
</tr>
<tr>
<td>Cold ischemia</td>
<td>0.304</td>
<td>0.169</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>0.044</td>
<td>0.030</td>
</tr>
<tr>
<td>Constant</td>
<td>-5.362</td>
<td>1.838</td>
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</table>
ITBL after liver transplantation

Preservation injury, also known as ischemic injury, refers to the dysfunctional injury that occurs immediately after transplantation and can be caused by the surgery, vascular problems, immune factors, adverse drug reactions, infection, or exposure to toxins. The causes of preservation injury include hypotension of the donor or receptor and thermal ischemia/cold ischemia/reperfusion injury during organ preservation, among which cold ischemia is the most important [15].

The biliary cells are more susceptible to ischemic injury and reperfusion injury during cold preservation, including the toxic effects of anhydrous bile salts [16], especially when the biliary epithelium undergoes apoptosis or shedding. The biliary wall, which lacks certain protections, is more susceptible to the infringement of bile salts. Although the biliary branches were washed during the cold preservation, the residual bile may still damage the biliary epithelial cells. Long-term cold ischemia can result in the shedding of the transplanted biliary epithelium into the bile, which is likely to be manifest as early bile reduction and a continuous increase of serum lactate, obvious coagulation disorder, less bile, hypoglycemia, renal insufficiency, or encephalopathy. In the first few days after transplantation, the increased serum levels of ALT and AST may also indicate a serious injury [20, 21]. Unless the transplantation fails or other injury occurs, the transaminases usually return to normal in a few days. If the graft survives in the initial lesion, it may appear with a prolonged cholestasis, showing an increase of the total serum bilirubin and γ-GPT [16, 22-25]. Usually, the situation will gradually improve, but it may take months to restore the abnormal bilirubin and tissue structures back to normal. In the process of repeated injury and repair, scars may form, and biliary stricture lesions may finally occur and form ITBL; the portal area that lacks the nutrition from the liver's lobular sinus will become the site worst affected, as well as the site usually involved with the ITBL.

After a year’s follow-up of the 50 patients, 8 patients got ITBL, among whom 2 were retransplanted due to functional deprivations of the ITBL graft. ITBL became the only reason for the increased incidence of postoperative cholestasis syndromes in those marginal donors, noncardiac donors, or donors with long-term cold ischemia [17]. Long-term cold ischemia may prolong the cholestasis period of preservation/reperfusion injury and cause prolonged cholestasis-induced preservation/reperfusion injury syndrome, as well as ITBL over the long term [18, 19].

Preservation injury can occur immediately after an ischemia-reperfusion of the liver, lasting for 1 to 2 months after liver transplantation, and eventually lead to the occurrence of ITBL. Severe preservation injury mostly manifests as early bile reduction and a continuous increase of serum lactate, obvious coagulation disorder, less bile, hypoglycemia, renal insufficiency, or encephalopathy. In the first few days after transplantation, the increased serum levels of ALT and AST may also indicate a serious injury [20, 21]. Unless the transplantation fails or other injury occurs, the transaminases usually return to normal in a few days. If the graft survives in the initial lesion, it may appear with a prolonged cholestasis, showing an increase of the total serum bilirubin and γ-GPT [16, 22-25]. Usually, the situation will gradually improve, but it may take months to restore the abnormal bilirubin and tissue structures back to normal. In the process of repeated injury and repair, scars may form, and biliary stricture lesions may finally occur and form ITBL; the portal area that lacks the nutrition from the liver's lobular sinus will become the site worst affected, as well as the site usually involved with the ITBL.
re-transplantation in this study and the main cause of perioperative graft failure. Therefore, ITBL can be seen as a very obvious threat to the patients’ survival.

The single-factor analysis of the risk factors of ITBL shows that the cold ischemic time is a risk factor of ITBL. The multivariate analysis also reveals that, among all factors, the cold ischemic time of the graft is the most relevant factor associated with the occurrence of ITBL. There has been no retrospective analysis which evaluates that significant relevance, which may be because of the limited number of ITBL cases. Retrospective studies with sufficient cases can be more instructive.

In the hepatic hilar region, the endothelial cells in larger biliary duct also become the first target of cold ischemic injury [26]. This study observed that the gap among the epithelial cells increased, the cytoplasmic chromatin aggregated, and the cells exhibited vacuolar degeneration. Such phenomena as intracellular mitochondrial vacuolar changes, mitochondrial vacuolar changes in the fibrous cells, epithelial chromatin aggregation, cytoplasmic shedding/necrosis, and apoptosis/degeneration/shedding of a large number of epithelial cells are the result of cold preservation injuries in the hepatic hilar region. At the same time, we also observed that such injuries increase with the prolonging of the cold ischemic time.

This prospective study targeting the risk factors of ITBL summarized the clinical data of 50 patients, and the univariate analysis shows that there is statistical significance in the cold ischemic time between the ITBL and the NITBL groups. The multivariate analysis shows that cold ischemic time, the intraoperative amount of fresh frozen plasma, and anhepatic time are not independent risk factors for ITBL after liver transplantation. In contrast, due to the limited number of cases in the ITBL group, retrospective studies with sufficient cases can be more instructive. The results of light and electron microscopy reveal that the biliary epithelial cells in the ITBL group gradually appear to have more mitochondrial vacuolar degeneration, cell gap widening, more apoptotic cells, and final mucosal shedding. At the same time, the biliary fibroblasts and collagen cells also appear to have different degrees of apoptosis. The bile ducts that lose the mucosal protection will be further damaged by harmful substances
such as bile salts, which eventually will lead to ITBL after reperfusion.

Conclusions

In the patients who have had an orthotopic liver transplant, the cold ischemic time is a risk factor for epithelial injury in the biliary tract, and the prolongation of the cold ischemic time has an obvious correlation with donated bile duct tissue injury, so such patients are more likely to have ITBL.

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Disclosure of conflict of interest

None.

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