## Original Article

# Pathological changes in primary cilia: A novel mechanism of graft cholangiopathy caused by prolonged cold preservation in a rat model of orthotopic liver transplantation

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**Summary**      **To study the impairment of cholangiocyte primary cilia caused by prolonged cold preservation and its correlation with graft cholangiopathy after orthotopic liver transplantation (OLT). Subjects were 60 male Wistar rats that were divided into 2 groups: a control group ($n = 30$) receiving a donor liver preserved for 1 h and a study group ($n = 30$) receiving a donor liver preserved for 12 h. A two-cuff method was used to establish the OLT model, and the hepatic artery and bile ducts were reconstructed using stents. Samples were collected 2, 8, and 16 weeks after surgery, and 5 samples were collected from each group at each time point. Serum biochemical indicators were measured, morphological changes in intrahepatic bile ducts and cholangiocyte primary cilia were observed using an optical microscope and scanning electronic microscope, respectively, and the ciliary marker (α-tubulin) and membrane proteins (PC-1, TRPV4, and P2Y$_{12}$) were detected using immunofluorescence analysis and Western blotting. In the study group, phlogocytes infiltrated around bile ducts and bile ducts proliferated markedly at 8 weeks. At 16 weeks, the biliary structures were indistinct and some bile ducts disappeared, a large amount of collagen was deposited, numerous phlogocytes infiltrated around ducts, some biliary epithelial cells (BECs) were deformed or dead, and primary cilia disappeared. In the control group, the intrahepatic bile ducts and BECs were nearly intact and the primary cilia were present. In the study group, the expression of α-tubulin, polycystin-1 (PC-1), TRPV4, and P2Y$_{12}$ in bile ducts disappeared completely after 8 weeks. In the control group, expression of the marker and proteins decreased at 2 weeks and increased slightly after 8 weeks. These results suggest that the study group had dysfunctional primary cilia at the start of OLT and that this dysfunction was irreversible. In the control group, the primary cilia defects and subsequent biliary injury were temporary. Thus, prolonged cold preservation of a donor liver may cause graft cholangiopathy by altering the integrity and functions of cholangiocyte primary cilia.**

*Keywords:* Liver transplantation, cold preservation, primary cilium, graft cholangiopathy

## 1. Introduction

Graft cholangiopathy (GC) produces non-anastomotic strictures and intrahepatic bile duct dilation in the biliary tree of a graft and may be associated with the formation of biliary sludge or gallstones. Primary pathological manifestations of GC in the biliary tree are bile duct loss and cholestasis. Major risk factors for GC are ischemia/reperfusion injury, immune response, and cytotoxic injury (*1,2*).

Biliary epithelial cell (BEC) defects caused by cold preservation are the direct cause of biliary complications after liver transplantation (*3*). The pathophysiological modification of BECs lining the biliary tree is the pathologic basis of GC. Each intrahepatic BEC contains

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a primary cilium (*4*). The primary cilia that extend from the apical plasma membrane of BECs into the bile duct lumen are ideally positioned to detect changes in bile flow, composition, and osmolality, i.e., to be sensory organelles that may control cholangiocyte functions such as ductal bile formation (*5-7*). The primary cilia can sense a traumatic physical or chemical stimulus and they participate in the process of repairing injury (*8*). The primary cilia function as a result of membrane proteins such as polycystin-1 (PC-1), PC-2, TRPV4, $P2Y_{12}$, and fibrocystin (*9,10*).

An OLT model was created to verify whether or not prolonged cold preservation would cause GC by damaging the structures and functions of primary cilia.

## 2. Materials and Methods

### 2.1. *Animal model*

Male inbred Wistar rats (6-8 weeks old, 220-250 g, Vital River Laboratory Animal Technology Co. Ltd, Beijing, China) were used as donors or recipients, and care was provided according to the guidelines of the Institutional Animal Care and Use Committee. All animals were kept in a temperature-controlled environment with a 12-h light-dark cycle and were allowed free access to food and water 12 or 4 h before surgery.

Sixty rats were randomly divided into 2 groups. In the study group (*n* = 30), the donor livers were preserved for 12 h. In the control group (*n* = 30), the donor livers were preserved for 1 h. The donor liver was preserved with University of Wisconsin (UW) solution at 4°C after *in situ* perfusion. After hepatectomy of the recipient's liver, the donor liver was implanted in an orthotopic position. The anhepatic time in the recipient was kept to less than 15 min. After completion of the end-to-end anastomosis between the suprahepatic inferior vena cava of the recipient and the donor, the portal vein was reconstructed with the cuff technique, the liver was reperfused, circulation to the intrahepatic inferior vena cava was re-established with another cuff, and the hepatic artery and bile ducts were reconstructed using stents. All samples were collected 2, 8, and 16 weeks after surgery, and 5 samples were collected from each group at each time point.

### 2.2. *Serum biochemical testing*

Total bilirubin (TBIL), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (GGT) are considered to be markers of BEC injury after liver transplantation (*11*). Serum concentrations of TBIL, ALP, and GGT were measured with an automatic biochemistry analyzer (Glamour 6000, UV-VIS METROLAB S.A., Buenos Aries, Argentina).

### 2.3. *Histopathological observation*

Liver tissues were fixed in formaldehyde and embedded in paraffin and then cut into 3-5-μm sections. After deparaffinization, the tissues were stained with hematoxylin and eosin (HE) for histopathologic evaluation using an optical microscope (Olympus BX41, Tokyo, Japan).

### 2.4. *Scanning electron microscopy (SEM)*

Samples for SEM were fixed in 2.5% glutaraldehyde for 24 h, dehydrated with a graded ethanol series, and critical-point dried in $CO_2$. The dried samples were mounted on aluminum and sputter-coated with gold and they were then examined under a scanning electron microscope (Hitachi S-4300, Tokyo, Japan).

### 2.5. *Immunofluorescence analysis*

Immunofluorescence microscopy was performed with a Zeiss LSM 510 confocal microscope with a 100- Pan-Apochromat 1.4-nm oil objective (Carl Zeiss, Inc., Thornwood, NY, USA). Liver samples were frozen at –20°C and cut into 8-μm sections. Samples were then fixed in acetone for 10 min, washed 3 times in phosphate-buffered saline with 0.05% Triton-X, and incubated in 1% bovine serum albumin for 10 min at room temperature. After samples were washed 3 times in phosphate-buffered saline, they were incubated overnight at 4°C with antibodies against acetylated α-tubulin, PC-1, TRPV4, and $P2Y_{12}$ (1:50, Santa Cruz Biotechnology, Inc.). Afterwards, they were incubated for 1 hour with secondary antibodies (1:50) (Santa Cruz Biotechnology, Inc.). Nuclei were stained with 4-,6-diamidino-2-phenylindole.

### 2.6. *Western blotting of α-tubulin, PC-1, TPPV4, and $P2Y_{12}$*

Samples of the intrahepatic bile ducts were lysed in 1-mL lysis buffer. Equal amounts of sample were separated using SDS-PAGE. Gels were electroblotted with Sartoblot onto polyvinylidene difluoride membranes. The membranes were blocked in Carnation non-fat milk and probed with a 1:200 dilution of the α-tubulin, PC-1, TRPV4, $P2Y_{12}$ and β-actin antibodies (Santa Cruz Biotechnology, Inc.) and a 1:1,000 dilution of the second antibody (Rabbit anti-rabbit lgG, Santa Cruz Biotechnology, Inc.). Antigen/antibody complexes were visualized with a chemiluminescence system (Amersham Biosciences, Piscataway, NY, USA) and scanned into images. The relative densities of the bands were analyzed using NIH Image (version 1.61; National Institute of Health, Bethesda, MD, USA). The ratio of target protein to β-actin served as an index for statistical analysis.

### 2.7. *Statistical analysis*

Statistical analyses were performed using SPSS version 13.0 system for Windows (SPSS Inc. Chicago, IL, USA). Data are expressed as the mean and standard deviations (SD). Differences were analyzed using analysis of variance (ANOVA). Results were considered significant at $p < 0.05$.

## 3. Results

### 3.1. *Prolonged cold preservation injured cholangiocytes*

The control group exhibited inflammation and swelling in cholangiocytes with an increase in serum TBIL, ALP, and GGT at 2 weeks and a gradual return to normal after 8 weeks. In the study group, the cholangiocytes displayed sloughing and necrosis at 2 weeks, and this was accompanied by increasing levels of TBIL, ALP,

and GGT. These changes worsened over time (Figure 1, Table 1).

### 3.2. *Prolonged cold preservation injured bile ducts*

In the control group, the biliary structures in portal area and interlobular ducts were almost normal and bile duct proliferation and phlogocyte infiltration were marked at 2 weeks. These manifestations slowly disappeared prior to 16 weeks. In the study group, numerous phlogocytes infiltrated around bile ducts and bile ducts proliferated markedly at 2 weeks. These manifestations became more marked at 8 weeks. At 16 weeks, the biliary structure was indistinct; some bile ducts in the portal area disappeared and a large amount of collagen was deposited around ducts (Figure 2).

### 3.3. *Prolonged cold preservation injured primary cilia*

In the control group, some cholangiocytes lost their primary cilia while other cholangiocytes had shortened cilia. Expression of the ciliary marker (α-tubulin) and membrane proteins (PC-1, TRPV4, and $P2Y_{12}$) decreased at 2 weeks and increased slightly after 8 weeks. In the study group, the primary cilia were sparse and shorter. Moreover, the primary cilia disappeared
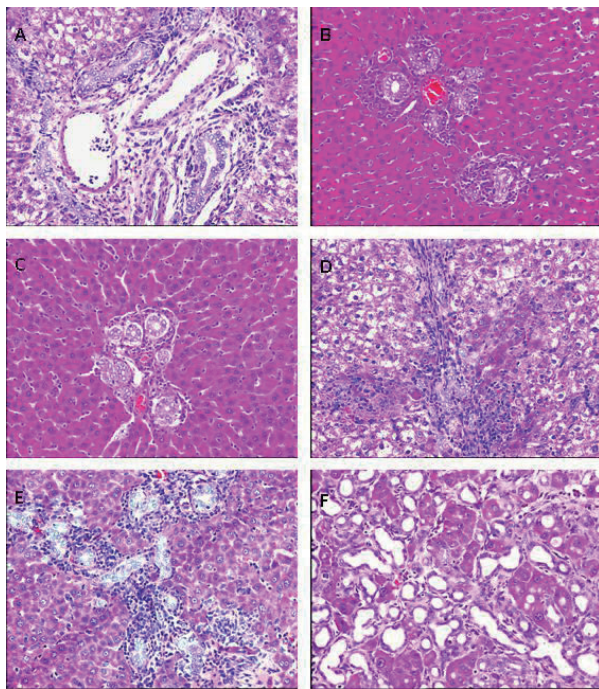


**Figure 1. Cholangiocyte expression. Control group:** There was marked bile duct edema, bile duct proliferation, and phlogocyte infiltration around ducts at 2 weeks **(A)**. Bile duct edema resolved and phlogocyte infiltration decreased while bile duct proliferation remained the same at 8 **(B)** and 16 weeks **(C)**. **Study group:** Cholangiocytes swelled and some died, many phlogocytes infiltrated around bile ducts, and bile ducts proliferated at 2 weeks **(D)**. These manifestations became more apparent at 8 weeks **(E)**. Numerous cholangiocytes died, the biliary structure was indistinct, and some bile ducts disappeared at 16 weeks **(F)**.
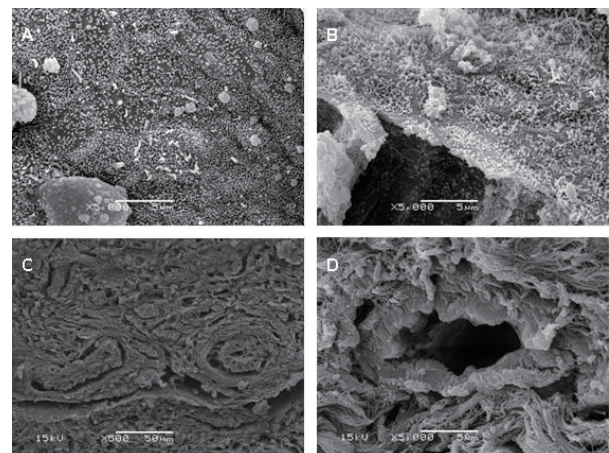


**Figure 2. Bile duct expression (scanning electron microscopy). Control group:** The primary cilia shortened (×5,000, **A**). **Study group:** Most cholangiocytes lost primary cilia. Cholangiocytes with primary cilia had sparse and shorter cilia at 2 weeks (×5,000, **B**). Bile ducts proliferated markedly at 8 weeks (×2,000, **C**). Cholangiocytes died and were sloughed off and collagen was deposited around ducts at 16 weeks (×5,000, **D**).

**Table 1. Comparison of biochemical indicators in two groups (mean ± s)**

| Items | Control group | | | Study group | | |
|---|---|---|---|---|---|---|
| | 2 weeks ($n = 5$) | 8 weeks ($n = 5$) | 16 weeks ($n = 5$) | 2 weeks ($n = 5$) | 8 weeks ($n = 5$) | 16 weeks ($n = 5$) |
| TBIL (μmol/L) | 14.28 ± 7.9 | 3.96 ± 1.13[c] | 2.37 ± 0.86[c] | 17.75 ± 8.34[a,b] | 24.53 ± 7.95[a,b] | 41.62 ± 14.95[a,b] |
| ALP (U/L) | 320.64 ± 36.75 | 77.35 ± 15.92[c] | 68.63 ± 4.02[c] | 383.67 ± 53.23[a,b] | 435.87 ± 86.34[a,b] | 574.56 ± 106.63[a,b] |
| GGT (U/L) | 34.72 ± 9.36 | 5.90 ± 2.58[c] | 6.73 ± 1.60[c] | 46.75 ± 4.93[a,b] | 61.45 ± 10.52[a,b] | 78.22 ± 12.05[a,b] |

[a] $p < 0.05$, within the same group over time; [b] $p < 0.05$, *vs.* control group at the same time point; [c] $p < 0.05$, *vs.* 2 weeks for control group.

and the expression of α-tubulin, PC-1, TRPV4 and P2Y$_{12}$ was completely absent after 8 weeks (Figures 2, 3, and 4; Table 2).

## 4. Discussion

The vascular plexus around bile ducts is a unique blood supply for BECs, so they are susceptible to damage as a result of ischemia/reperfusion and immune injury (*12,13*). That said, BECs are powerful cells because they produce 40% of bile even though they only account for about 4% of all cells in the liver (*14,15*).

During liver transplantation, BECs inevitably suffer ischemia/reperfusion injury and exhibit immunological



**Figure 3. The expression of α-tubulin, PC-1, TRPV4, and P2Y$_{12}$. Control group:** The expression of α-tubulin **(A)**, PC-1 **(B)**, TRPV4 **(C)**, and P2Y$_{12}$ **(D)** decreased at 8 weeks. **Study group:** Expression of α-tubulin **(E)**, PC-1 **(F)**, TRPV4 **(G)**, and P2Y$_{12}$ **(H)** was absent. Immunofluorescence ×200.

rejection (*16,17*). Cold ischemia and reperfusion injury (CIRI) is crucial to estimating graft function after cadaveric donor liver transplantation. However, some marginal donor livers that have been preserved in the cold for a prolonged period must be used because of the scarcity of donors. Use of these livers is associated with a number of biliary problems, such as delayed biliary recovery, ischemic bile duct lesions, and biliary strictures after liver transplantation (*18*). During transplantation, the bile ducts are susceptible to warm ischemia before they are subsequently preserved, rewarmed, and reperfused (*19*). Cholangiocytes lining the bile duct wall are weakened by depletion of energy stores. Cholangiocytes are susceptible to CIRI because of the following factors: an increase in oxygen free radicals and depletion of glutathione in cellular stores, the toxic effects of hydrophobic bile salts, and the infiltration of inflammatory cells into the basement membrane and release of inflammatory mediators (*20*). Therefore, the number of cholangiocytes that detach from the underlining basement membrane and enter bile is related to the duration of cold ischemia (*21*). Sufficient cellular repair or regeneration to replace the lost cells damaged by CIRI is generally considered necessary for bile duct recovery after liver transplantation and the extent of the cholangiocyte regenerative response is considered to correlate with the degree of injury. The primary cilia may, as sensory organelles, participate in bile formation and bile duct recovery after CIRI.

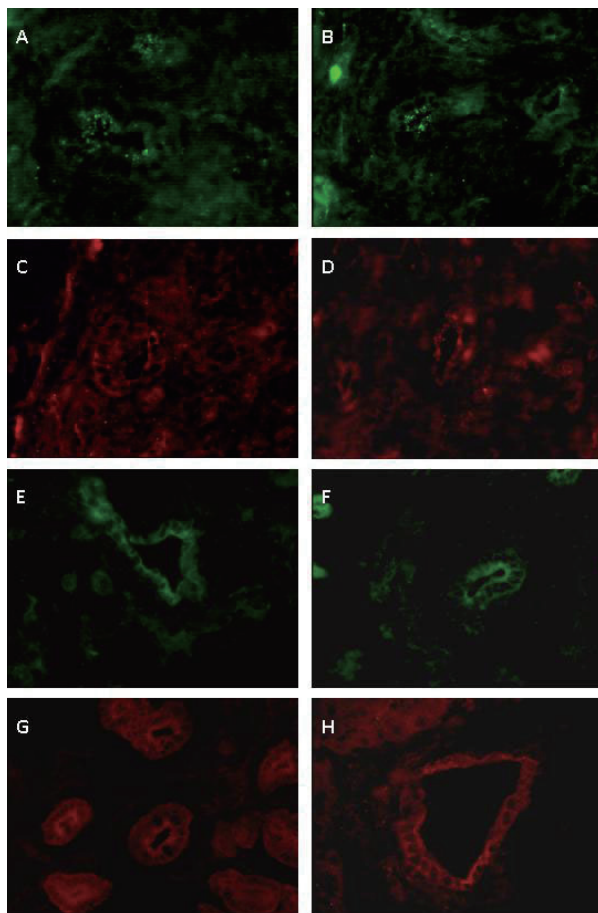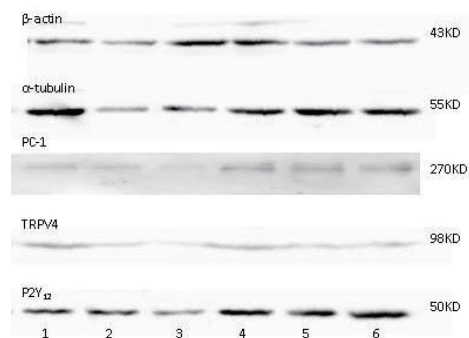In order to explore the effect of GC on primary cilia



**Figure 4. The expression of α-tubulin, PC-1, TRPV4, and P2Y$_{12}$ in intrahepatic bile ducts. Study group: 1** (2 weeks), **2** (8 weeks), **3** (16 weeks); **Control group: 4** (2 weeks), **5** (8 weeks), **6** (16 weeks).

**Table 2. Levels of expression of α-tubulin, PC-1, and TRPV4 in intrahepatic bile ducts (mean ± s)**

| Items | Control group | | | Study group | | |
|---|---|---|---|---|---|---|
| | 2 weeks ($n = 5$) | 8 weeks ($n = 5$) | 16 weeks ($n = 5$) | 2 weeks ($n = 5$) | 8 weeks ($n = 5$) | 16 weeks ($n = 5$) |
| α-tubulin/β-actin | 1.34 ± 0.58 | 1.79 ± 0.47 | 1.71 ± 0.62 | 0.86 ± 0.62[a,b] | 1.14 ± 0.43[a,b] | 0.69 ± 0.33[a,b] |
| PC-1/β-actin | 0.87 ± 0.23 | 0.73 ± 0.29 | 0.95 ± 0.32 | 0.42 ± 0.18[a,b] | 0.23 ± 0.11[a,b] | 0.08 ± 0.04[a,b] |
| TRPV4/β-actin | 0.48 ± 0.18 | 0.53 ± 0.22 | 0.64 ± 0.26 | 0.33 ± 0.11[a,b] | 0.17 ± 0.09[a,b] | 0.13 ± 0.06[a,b] |
| P2Y12/β-actin | 2.16 ± 0.74 | 2.53 ± 0.46 | 2.72 ± 1.14 | 1.53 ± 0.59[a,b] | 0.76 ± 0.48[a,b] | 0.64 ± 0.25[a,b] |

[a] $p < 0.05$, within the same group; [b] $p < 0.05$, *vs.* control group at the same time.

as a result of prolonged cold preservation, the current study created a rat model of OLT after preserving the donor liver in UW solution at 4°C for 1 or 12 h. This study then observed several indicators over time. The morphology of intrahepatic bile ducts and BECs was markedly altered in the study group in comparison to the control group, although bile duct proliferation did proceed for up to 8 weeks. Cholangiocyte sloughing and necrosis were marked at 16 weeks. These manifestations were accompanied by an increase in serum TBIL, ALP, and GGT. These findings indicated that prolonged cold preservation was responsible for injury to bile ducts and BECs, and this injury was in turn closely associated with GC. However, the question of whether or not powerful organelles like the primary cilia affect GC must be studied further.

In the liver, only BECs have cilia. Liver disease caused by defects in ciliary structures and ciliary dysfunction is known as cholangiociliopathy. Cholangiociliopathy includes cystic and/or fibrotic liver diseases such as autosomal dominant polycystic kidney disease (ADPKD, caused by mutations in either *PKD1* or *PKD2*, genes that encode PC-1 and PC-2, respectively), autosomal recessive PKD (ARPKD, caused by mutations in a single gene, *PKHD1*, that encodes fibrocystin), nephronophthisis (NPHP, caused by mutations in the six *NPHP1-6* genes that encode nephrocystins 1-6), Bardet-Biedl syndrome (BBS, caused by mutations in the 12 *BBS* genes that encode BBS1-12), and Meckel-Gruber syndrome (MKS, caused by mutations in three *MKS1-3* genes that encode MKS1 and meckelin) (*22,23*). In addition, some forms of cholecystopathy such as primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) may also be forms of cholangiociliopathy. Abnormalities in cilia are involved in the development of some tumors, such as astrocytomas and glioblastomas (*24,25*). Understanding the key mechanisms that regulate sensation/transduction by cholangiocyte cilia or the phenotypic manifestations of these actions (*i.e.*, the concentrations of intracellular $Ca^{2+}$ and cAMP) should facilitate the development of targeted therapies to prevent or halt these diseases. Indeed, a study recently found that treating PCK rats (an animal model of ARPKD) with octreotide, the synthetic analog of somatostatin, decreased cAMP levels in cholangiocytes, it suppressed hepatic cyst growth, and it reduced hepatic fibrosis (*26*). Clinical trials are underway to assess this therapeutic approach in humans.

The current results indicated that cholangiocyte primary cilia in the study group were irreversibly damaged after surgery. These cilia were quite sparse at 2 weeks and nearly absent at 8 weeks. At 8 weeks, BECs had a normal morphology for the most part. BECs suffered irreversible injury at 16 weeks. In the study group, levels of α-tubulin, PC-1, TRPV4, and $P2Y_{12}$ expression in intrahepatic bile ducts decreased markedly starting at 2 weeks. This finding indicates that the primary cilia were dysfunctional at the start of OLT and that this dysfunction was irreversible. In the control group, primary cilia defects and subsequent biliary injury were temporary, suggesting that ciliary abnormalities are part of the pathophysiology of GC.

Genes and proteins that play a role in the structure or function of primary cilia may represent new targets for treatment of biliary lesions or reconditioning. Levels of biochemical indicators and bile duct morphology in the study group indicated that prolonged cold preservation of a donor liver can cause GC. Moreover, the current results suggested that prolonged cold preservation resulted in reduced expression of α-tubulin, PC-1, TRPV4, and $P2Y_{12}$ in intrahepatic bile ducts, and this was in turn closely associated with GC. The current results suggest that prolonged cold preservation of a donor liver may cause GC by altering the integrity and functions of cholangiocyte primary cilia.

This study focused on determining whether injury to the primary cilia is the determining factor for GC as a result of prolonged cold preservation. Although logistically difficult, examining abnormalities in primary cilia as a result of GC would prove interesting. There may be association between those abnormalities and GC, and such an approach would facilitate the study of the mechanisms of the disease and therapies to treat it. Nevertheless, whether or not the morphologically and functionally abnormal primary cilia that were observed lead to GC has yet to be determined.

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