# A Novel MSC-Based Immune Induction Strategy in ABO-Incompatible Liver Transplantation: A Phase I/II Randomized, Open-Labeled, Controlled Trial

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**Research**

**Keywords:** Mesenchymal stem cells, ABO-incompatible liver transplantation, Severe hepatic failure, Rituximab

**Posted Date:** November 20th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-111230/v1



**Version of Record:** A version of this preprint was published at Stem Cell Research & Therapy on April 16th, 2021. See the published version at https://doi.org/10.1186/s13287-021-02246-4.

## Abstract

**Background:** ABO-incompatible liver transplantation (ABO-i LT) has become a rescue therapeutic option for severe hepatic failure patients. Although the use of rituximab greatly reduces the morbidity of antibody-mediated rejection (AMR), severe adverse effects, such as infection and biliary complications still seriously threatening the survival of the transplant recipients. The aim of this study is to evaluate the safety and feasibility of using mesenchymal stem cells (MSCs) to replace rituximab in ABO-i LT.

**Methods:** 22 severe hepatic failure patients undergoing ABO-i LT were enrolled and randomly divided into two groups, including the MSC group and Rituximab group. The safety of the application of MSCs and the incidence of allograft rejection, including antibody-mediated rejection (AMR) and acute cellular rejection (ACR) were evaluated in both groups at 2-year follow-up period as primary endpoints. Recipients and graft survivals and other postoperative complications were compared as secondary endpoints.

**Results:** No severe adverse effects occurred in the MSC group related to the MSC infusion. MSCs treatment obtained comparable, if not better, results to rituximab for decreasing the incidence of acute rejection, especially AMR (9.1% vs 27.3%). Inspiringly, compared to the Rituximab group, biliary complications (0% vs 45.5%) and infection (9.1% vs 81.8%), were significantly decreased in MSCs group. In addition, there were no significant difference in 2-year graft and recipient survivals in the two groups (81.8% vs 72.7%).

**Conclusions:** Our data shows that MSCs transfusion is comparable to rituximab treatment for AMR prophylaxis following ABO-i LT. Additionally, the results indicate that MSC is more beneficial to the prevention of infection and biliary complications, which may be introduced as a novel immunosuppressive approach for ABO-i LT.

**Trial registration:** Trial registration: chictr.org.cn, ChiCTR2000037732. Registered 31 August 2020- Retrospectively registered, http://www.chictr.org.cn/showproj.aspx?proj=57074.

## Background

Severe hepatic failure is a life-threatening illness with high mortality and morbidity, which is pathologically characterized by sudden and severe hepatocellular necrosis and clinically characterized by coagulopathy, jaundice and hepatic encephalopathy[1]. Emergency liver transplantation (LT) is, so far, still identified as the only durable effective therapeutic approach. Unfortunately, the implementation of ABO-compatible LT is limited, sometimes unavailable, due to globally donor shortage[2]. Therefore, ABO-incompatible (ABO-i) graft becomes an alternative option for LT. The first ABO-i LT was conducted and reported from Starzl in 1972, has tended to occur at higher risks of hepatic artery thrombosis, bile duct complications, antibody-mediated rejection (AMR), infection and poor graft and recipient survival. According to the study from Guggenheim et al, graft function at first two years after LT in ABO-i group (30%) was significantly lower than that in ABO-compatible group (76%)[3]. In order to overcome these disadvantages, various novel therapeutic strategies have been introduced over the past two decades, comprising anti-CD20 monoclonal antibody (rituximab), intravenous immunoglobulin (IVIG), splenectomy, immunoadsorption and plasma exchange. To date, similar prognostic outcomes and recipient survival rates of ABO-c LT was observed in the ABO-i LT population when union used with rituximab and desensitization[4]. And the further multivariate analysis demonstrated that the absence of rituximab administration was an independent risk factor for AMR[5]. However, the large sample-size retrospective research also reported that the incidence of bile duct injury still high after treated with rituximab[6].

With the properties of immunomodulation and regeneration, mesenchymal stem cells (MSCs) are emerging as a promising approach for many diseases, including acute-on-chronic hepatic failure, rheumatoid arthritis and inflammatory bowel disease. Of all, as the important roles in modulating the function of macrophage, natural killer (NK), T and B cells as well as further inducing the translation of Treg and Breg cells, MSCs are believed to prevent post-operative complications after transplantation and reduce the side effects of pharmacologic immunosuppression[7, 8]. In previous trial, Tan et al. shown that autologous MSCs administration exhibited lower incidence of acute rejection, lower risk of opportunistic infection, better renal function restores after kidney transplantation, compared to anti-IL-2 receptor monoclonal antibody treatment[9]. Detry once reported the use of MSCs treatment for liver transplant recipients and shown that there were no toxic and severe side-effects after a single-dose MSC transfusion[10]. Wang et al. shown that umbilical cords-derived MSC transfusion is feasible for inhibiting acute graft rejection after LT via increasing the percentage of Treg cells and the Treg/Th17 ratio[11]. Our previous study also revealed the beneficial effect of MSCs on attenuating ischemia-type biliary lesions (ITLBs) after LT[12]. However, clinical research of MSCs administration in ABO-i LT has not been conducted.

To our knowledge, this was the first, prospective, rituximab controlled, clinical phase I/II study to focus on the safety, tolerability and feasibility of intravenous transfusion of multi-doses allogeneic MSCs in severe hepatic failure patients underwent ABO-i LT. Potential side effects of MSCs administration, post-operative complications especially acute rejection, biliary complications and infection, over 2 years after operation were investigated as the primary endpoints. The secondary endpoints were set to clinically compare the recipients and graft survivals between MSCs and Rituximab groups.

## Methods

# Study design and participants

The current study was a prospective, monocentric, open-label, randomized, rituximab controlled, phase I/II clinical trial that featured a totally 2-year follow-up period. This trial was approved by the local ethics committee on clinical trials of the Third Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), which piloted conforming to the ethics guideline of the 1975 Declaration of Helsinki and registered in chictr.org.cn (ChiCTR2000037732). The target

population involved adults with severe hepatic failure, who were hospitalized for an emergency ABO-i LT. The detailed inclusion and exclusion criteria are listed in Fig. 1.

All participants were recruited in the Department of Liver Transplantation of the Third Affiliated Hospital of Sun Yat-sen University. Between August 2016 and August 2018, 22 patients were enrolled, which were screened from a total of 47 patients receiving ABO-i LT in our center, and randomly assigned equally to the MSC or Rituximab group. All patients in this trial provided written informed consent.

All enrolled patients in these two groups received standard immunosuppressive regimen treatment according to previous described, including steroids, baliximab, tacrolimus, mycophenolate mofetil and intravenous immunoglobulin (IVIG)[13]. In detail, 1 g steroid was administrated during operation, then 500 mg on day 1, 180 mg on day 2 tapered by 40 mg/d to 40 mg/d, subsequently steroid 48 mg/d p.o. tapered by 8 mg per three days to 8 mg/d and then maintain 8 mg/d for more than 1 year. IVIG (10 g/d) was used in the first seven days after LT. Tacrolimus (2.0 mg/d) and mycophenolate mofetil were initially administrated in the third day after ABO-i LT and have been taking to maintain blood concentrations of the drugs. Finally, baliximab was given twice, once during surgery and the other on the fourth day after transplantation. (Fig. 2)

## Liver transplant procedures

The detailed information of enrolled deceased liver graft donor was prospectively recorded: gender, age, donor after circulatory or brain death (DCD or DBD), cause of death, body mass index (BMI), ABO blood type, terminal serum sodium level, terminal hepatic and renal function tests, sojourn time of intensive care unit (ICU), the parameters of ventilator settings and need for vasopressors.

The ABO-i LT procedures, Piggy-back LT, were standardly performed in the authors' single center, which were specified in detail previously[14]. The recipients' characteristics were collected: gender, age, liver and renal function tests, coagulation function tests, Model for End-Stage Liver Disease (MELD) score and the titer of specific antibodies at hospitalizing for LT. During transplantation, operation time, cold graft ischemic time, intraoperative blood loss and blood transfusion volume were recorded.

## Preparation, culture and identification of allogeneic MSCs

## Umbilical cord donors

Human umbilical cords were obtained from healthy donors who understood the study, met the inclusion and exclusion criteria and signed written informed consent. Before prepared MSCs, umbilical cords were initiatively confirmed negative for cytomegalovirus (CMV) antigen, anti-human T lymphotrophic virus (HTLV) I/II antibody, anti-hepatitis A virus (HAV) IgM antibody, hepatitis B virus (HBV) antigen, anti-hepatitis C virus (HCV) antibody, hepatitis D virus (HDV) antigen, anti-hepatitis E virus (HEV) IgM/IgG antibodies, syphilis, anti-HIV-1/2 antibodies, fungi and bacteria. In addition, we limited the age of the donor ranking from 18 to 35.

## Preparation, culture and identification of MSCs

The third-party MSCs preparation has been proved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University. Following the good manufacturing practice (GMP), the processes of cell preparation and culture were performed under standardized and aseptic condition in the Stem Cell Laboratory Facility of Biotherapy Center in our hospital[12]. Fresh UCs were obtained from the healthy pregnant women and immersed in 4℃ phosphate-buffered saline (PBS). Umbilical cords was twice with PBS to remove the remnant blood until it become white, cut into 10 $mm^3$/piece in 0.1% type I collagenase with $CaCl_2$ (3 mM) containing 0.1% hyaluronidase (Invitrogen, USA) and, then, incubated on a shaker (220 rpm) at 37℃ for 4 h digestion. Subsequently, the isolated cells were cultured in low-sugar Dulbecco's modified Eagle's medium (1 g/L DMEM, Gibco, Life, Austria) with 10% fetal bovine serum (FBS, Gibco, Life, Austria) in the humidified atmosphere, 5% $CO_2$, 37℃. Medium was refreshed every three days to remove nonadherent cells. To assess the phenotype on cells surface, flow cytometric analysis was performed, including CD105, CD73, CD44, CD90, CD45, CD34, CD166 and CD29. The differential potential was detected according to the 2006 International Society of Cellular Therapy's criteria, which investigated their ability to differentiate into osteocytes and adipocytes[15]. At 70%-80% confluence, MSCs were passaged by trypsin treatment. The MSCs were collected and clinically used at passage 3rd -5th. Before injection, cells were tested again with negative for HBV, HCV, HIV, syphilis, mycoplasma, fungus and endotoxin.

## Allogeneic MSCs transfusion

Allogeneic MSCs were collected and suspended in 100 ml 0.9% NaCl at the density of $1.0 \times 10^6$ cells/kg body weight. The enrolled patients received nine doses of MSCs infusion, including the first time, which were treated with 10% MSCs through portal vein after graft reperfusion and 90% transfused through peripheral vein during LT, and the subsequent 8 times (1 w, 2 w, 4 w, 8 w, 12 w, 16 w, 20 w and 24 w after operation) which were intravenously transfused via the vein of forearm. During the infusion, the MSCs suspension was transfused within 30 min and was swung gently every 3 min to avoid cell deposition. All patients were received 2 h observation after the transfusion to screen any adverse event.

## Primary and secondary endpoints

The primary endpoints of the investigation comprised the safety and tolerability of multi-doses MSCs administration in study subjects with the assessments of MSC-related adverse events (fever, headache, rash, vomiting, diarrhea and carcinogenesis) and the incidence of allograft rejection, including antibody-mediated rejection (AMR) and acute cellular rejection (ACR) at 2-year follow-up period. The secondary endpoints were preliminarily observed their efficacy on patients underwent ABO-i LT, compared with rituximab, including (1) the evaluation of graft and recipient survivals; (2) the incidence of postoperative complications, including biliary complications and specific infections.

## Graft biopsy and immunohistochemistry

At week 2 and month 6, surveillance biopsies were performed and 4 μm formalin-fixed, paraffin-embedded sections were prepared. Firstly, hematoxylin and eosin (H&E) staining was performed to describe characteristics of graft rejection following to the Banff criteria by double-blind scoring[16]. In addition, the sections also underwent cytokeratin 19 (CK19) staining to observe biliary formation, C4d staining to indirectly assess the severity of AMR and immunostaining with antibodies against human CD4, CD8 and CD20 to count these immune cells infiltration. The three most microscopic fields (400 × magnification) from each patient were randomly selected to calculate the mean number of positive cells. Archival tonsil sections were used as positive controls. And negative controls were considered as the sections only treating with secondary antibody and diaminobenzidine without the primary antibody. All of the primary antibodies were purchased from Abcam (USA), and secondary antibody and diaminobenzidine were from DAKO (USA).

## Definitions

Acute cellular rejection (ACR) was scored using Banff criteria. AMR was serologically diagnosed by significantly increased titer of specific antibodies and histologically diagnosed by the C4d staining[17]. Hepatic arterial stenosis was defined by visualizing Doppler ultrasonography and computed tomographic (CT) angiography. Biliary leakage was suspected with the persistent drainage of bile from the abdominal cavity and diagnosed by the postoperative cholangiography. Bile duct anastomotic stenosis was diagnosed by magnetic resonance cholangiopancreatography (MRCP). ITBL was suspected based on laboratory examinations, elevated levels of serum ALP and γ-GGT, and finally defined by contrast-enhanced ultrasonography (CEUS) and MRCP[18, 19]. Posttransplant septic shock was defined as patients that suffered from severe sepsis, which was a positive culture of pathogenic form of bacteria or fungi, with hyperlactatemia and obvious hemodynamic change requiring vasopressor therapy.

## Statistical analysis

As appropriate, summary data with continuous variables were presented as the descriptive statistics, including n, mean ± standard deviation (SD), median/interquartile or maximum/minimum, whereas the categorical variables were summarized using frequency and percentages. A mixed model (repeated measures) was used to analyze the outcomes from multiple follow-up times to compare the efficacy between these two groups, whereas comparisons within the same group were performed using the model-estimated contrasts. If the outcomes were highly skewed, a Wilcoxon Signed Rank Sum test was used to perform the comparisons from each intragroup follow-up effects by covariance adjusting of baseline. In addition, categorical variables were analyzed by Fisher's exact tests. All data from this study were analyzed by SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) or GraphPad Software version 7.0 (CA, USA), as appropriate. Two-sided $p < 0.05$ was considered as the statistical significance level.

# Results

## Baseline characteristics of recipients and donors

The baseline characteristics of the recipients and donors are listed in Table 1. From Aug. 2016 to Aug. 2018, 22 severe hepatic failure patients were enrolled to receive ABO-i LT in the Third Hospital of Sun Yat-sen University, which were divided into two groups randomly, including the Rituximab group (n = 11) and the MSC group (n = 11) (Fig. 1). Up to Aug. 2020, the median follow-up period was 32 months in the MSC group (4 days-48 months) and 37 months in the Rituximab group (2–47 months). Of all the patients, the most common primary disease (20/22) was hepatitis B virus-related acute-on-chronic liver failure. Receipt age, pre-operation WBC count and CPS scores were comparable between the rituximab and MSC group. There was no statistical difference of MELD score between the MSC and Rituximab groups (p = 0.217). The data of the blood type combinations between donor and recipient were provided with the isoagglutinin titer in Table 2. The most common combination of the ABO type of donor to recipient was AB to A (9/22). Both initial IgM and IgG isoagglutinin titers were ≤ 1:64. And no statistical differences of isoagglutinin titer were detected in pre-transplantation period. In addition, donor characteristics, especially cold ischemic time, as well as duration of transplant surgery and transfusions were comparable between MSCs and rituximab-treated recipients.

Table 1
Clinical and biochemical index of the patients at baseline

| | MSC(N = 11) | Rituximab(N = 11) | P value |
|---|---|---|---|
| Gender(male)[%] | 9(81.8%) | 10(90.9%) | 1.000 |
| Age(years) | 48.64 ± 7.94 | 43.64 ± 13.25 | 0.296 |
| WBC($\times 10^9$) | 7.32(3.86–11.60) | 9.29(6.69–15.66) | 0.365 |
| PLT($\times 10^9$/L) | 71.00(47.00–90.00) | 104.00(75.00-201.00) | 0.056 |
| AST(U/L) | 69.00(21.00-111.00) | 97.00(53.00-180.00) | 0.217 |
| ALT(U/L) | 30.00(9.00–94.00) | 99.00(18.00-233.00) | 0.171 |
| ALB(g/L) | 32.20(30.80–35.80) | 34.20(33.20–36.20) | 0.773 |
| TBIL(umol/L) | 420.73(38.45-541.73) | 590.07(202.35-702.68) | 0.116 |
| ALP(U/L) | 99.00(95.00-124.00) | 99.00(71.00-116.00) | 0.478 |
| GGT(U/L) | 49.00(33.00–77.00) | 51.00(42.00–75.00) | 1.000 |
| PT(sec) | 25.60(20.60–37.60) | 33.20(29.10–38.80) | 0.251 |
| INR | 2.31(1.75–3.67) | 3.17(2.71–3.98) | 0.270 |
| CREAT(umol/L) | 76.00(45.00-114.00) | 71.00(56.00-174.00) | 0.748 |
| BUN(mmol/L) | 7.73(5.01–9.16) | 5.35(3.18–15.44) | 0.699 |
| IgM | 8.00(8.00–32.00) | 8.00(8.00–16.00) | 0.898 |
| IgG | 16.00(8.00–32.00) | 32.00(2.00–64.00) | 0.478 |
| CPS Score | 11.82 ± 1.66 | 11.46 ± 1.57 | 0.604 |
| MELD Score | 31.00(30.00–40.00) | 40.00(32.00–40.00) | 0.217 |
| Donor age(years) | 41.09 ± 15.78 | 42.73 ± 8.63 | 0.767 |
| Cold ischemia time(h) | 6.00(6.00-7.50) | 6.00(4.72-7.00) | 0.270 |
| Operation time(min) | 443.64 ± 41.65 | 425.64 ± 60.23 | 0.425 |
| Anhepatic phase time (min) | 45.00(42.00–55.00) | 43.00(40.00–55.00) | 0.847 |
| Intraoperative blood loss(mL) | 1500.00(1500.00-2400.00) | 1500.00(1000.00-2000.00) | 0.365 |
| Blood transfusion volume | | | |
| RBC(U) | 12.00(6.00–16.00) | 9.50(7.50–16.00) | 0.797 |
| FFP(mL) | 2400.00(2100.00-3600.00) | 3050.00(2000.00-4000.00) | 0.606 |
| Cryo(U) | 28.00 ± 15.28 | 36.05 ± 8.08 | 0.138 |

Abbreviations: MSC, mesenchymal stem cell; WBC, white Blood Cell; PLT, platelet; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALB, albumin; TBIL, total bilirubin; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; PT, prothrombin time; INR, International Normalized Ratio; CREAT, creatinine; BUN, blood urea nitrogen; CPS, Child-Pugh score; MELD, Model For End Stage Liver Disease; RBC, red blood cell; FFP, fresh frozen plasma; Cryo, cryoprecipitate.

Table 2
Blood type combinations between donor and recipient with the isoagglutinin titer.

| Patient number | Donor blood type | Recipient blood type | Isoagglutinin titer IgM | Isoagglutinin titer IgG |
|---|---|---|---|---|
| 1 | AB | A | 1:8 | 1:32 |
| 2 | AB | A | 1:64 | 1:2 |
| 3 | A | O | 1:32 | 1:64 |
| 4 | B | O | 1:8 | 1:32 |
| 5 | AB | A | 1:8 | 1:8 |
| 6 | AB | A | 1:32 | 1:16 |
| 7 | AB | A | 1:4 | 1:16 |
| 8 | AB | A | 1:8 | 1:8 |
| 9 | B | A | 1:8 | 1:4 |
| 10 | A | B | 1:4 | 1:8 |
| 11 | A | O | 1:8 | 1:64 |
| 12 | B | O | 1:16 | 1:64 |
| 13 | AB | A | 1:8 | 1:2 |
| 14 | B | O | 1:8 | 1:2 |
| 15 | AB | O | 1:32 | 1:32 |
| 16 | B | O | 1:16 | 1:64 |
| 17 | B | O | 1:8 | 1:64 |
| 18 | AB | B | 1:4 | 1:2 |
| 19 | AB | A | 1:8 | 1:16 |
| 20 | AB | A | 1:8 | 1:32 |
| 21 | B | O | 1:64 | 1:64 |
| 22 | A | B | 1:4 | 1:32 |

# Primary Outcomes:

# Safety of MSCs Infusions in ABO-I Liver Transplant Recipients

MSCs infusions were performed according to design scheme shown in Fig. 2. Most of the observed adverse events were of grades I/II and transient, including fever, headache, rash, vomiting and diarrhea, which appeared in close association with the administration of MSCs (Table 3). No significant variations in vital parameters (such as blood pressure, heart rate and $SpO_2$) were detected during and after MSCs infusion. Moreover, no patients in the MSC group developed *de novo* cancerous complications (including post-transplant lymphoproliferative disease) during the 2-year follow-up period.

Table 3
Side effects after MSC infusion

| | No. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Adverse Event | Time 2 (n = 3) | Time 3 (n = 4) | Time 4 (n = 0) | Time 5 (n = 2) | Time 6 (n = 2) | Time 7 (n = 3) | Time 8 (n = 5) | Time 9 (n = 3) | Total (n = 25) |
| Fever | 2 | 2 | 0 | 1 | 1 | 2 | 3 | 1 | 13 |
| Headache | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 3 |
| Rash | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 2 |
| Vomiting | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 4 |
| Diarrhea | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 3 |

# The incidence of AMR and ACR

Several studies had demonstrated that the incidences of antibody-mediated rejection (AMR) and acute cellular rejection (ACR) are much more frequent in ABO-i recipients than in ABO-compatible recipients. As illustrated in Table 4, our study showed that three patients in the Rituximab group developed acute liver rejection reaction at day 20, day 43 and day 78, respectively; While only one episode in the MSC group at day 25. MSCs therapy significantly decreased the

rate of acute rejection in the MSC group compared with Rituximab group in the follow-up period (9.1% vs. 27.3%, p = 0.586). Immunohistochemistry of CD4 and CD8 demonstrated that T lymphocytes were strikingly infiltrated into hepatic tissue of acute allograft rejection (Fig. 3A-B). Meanwhile, C4d staining shown that there was one episode of AMR in each group (Fig. 3B). The symptoms could be relieved by increasing the dosage of current immunosuppressive agents.

Table 4
Prognosis in 2 years after liver transplantation

| | MSC(N = 11) | Rituximab(N = 11) | P value |
|---|---|---|---|
| Survival rate No. (%) | 9(81.8%) | 8(72.7%) | 1.000 |
| Graft survival rate No. (%) | 9(81.8%) | 8(72.7%) | 1.000 |
| Acute rejection No. (%) | 1(9.1%) | 3(27.3%) | 0.586 |
| Biliary complications No. (%) | 0(0%) | 5(45.5%) | 0.035[a] |
| Ischemia type biliary lesion No. (%) | 0(0%) | 4(36.4%) | 0.090 |
| Bile duct anastomotic stenosis No. (%) | 0(0%) | 3(27.3%) | 0.214 |
| Bile leakage No. (%) | 0(0%) | 1(9.1%) | 1.000 |
| Septic shock No. (%) | 1(9.1%) | 9(81.8%) | 0.002[b] |
| Pulmonary infection No. (%) | 0(0%) | 8(72.7%) | 0.001[b] |
| Biliary infection No. (%) | 0(0%) | 1(9.1%) | 1.000 |
| Splenic abscess No. (%) | 1(9.1%) | 0(0%) | 1.000 |
| Arteriostenosis No. (%) | 1(8.3%) | 3(27.3%) | 0.586 |
| De novo tumor No. (%) | 0(0%) | 1(9.1%) | 1.000 |
| [a] $P < 0.05$, [b] $P < 0.01$. | | | |
| Abbreviations: MSC, mesenchymal stem cell. | | | |

# Secondary Outcomes:

## The Effects of MSCs on Patient and Graft Survival

Five recipients died during the follow-up period. In detail, two patients in the MSC group died of abdominal hemorrhage (n = 1) and hepatic failure caused by compressed inferior vena cava (n = 1). Three patients in the rituximab group died of sepsis-associated multiple organ failure, including two patients died due to severe pulmonary (n = 1) and biliary tract infection (n = 1) within 3 months after transplantation, as well as one patient at sixteenth month from pulmonary infection induced septic shock and refractory anemia. Two-year graft and recipient survivals were 81.8% (9/11) and 72.7% (8/11) in the MSC and the rituximab group, respectively.

## The Effects of MSCs on Liver Graft function

As shown in Fig. 4 and Table 5, to investigate the impact of MSCs on liver function, we measured the Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Albumin (ALB), Total Bilirubin (TBIL), Alkaline Phosphatase (ALP), Gamma Glutamyl Transferase (GGT), Serum Creatinine (CREAT) and Blood Urea Nitrogen (BUN) in all patients throughout the follow-up period. Both ALT and AST reached the peak at first day, then decreased close to normal levels. While no significant differences were detected in each time points of both groups. ALB showed upwards trends after MSCs infusions, especially much significantly higher than that in Rituximab group at week 4; however, there were no statistical differences between the MSC and Rituximab groups. TBIL were decreased significantly after MSCs infusions, which was compared with the Rituximab group mainly within first week. Although ALP, GGT, CREAT and BUN were shown no significant differences between two groups, the fluctuation range in the MSC group was much milder than that in the Rituximab group.

Table 5
Levels of AST,ALT、ALB、TBIL、ALP、GGT、CREAT and BUN in the two groups at baseline、day1、week1、2、4、8 and 12

| | AST | | | ALT | | | ALB | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rituximab | MSC | P | Rituximab | MSC | P | Rituximab | MSC | P |
| **Baseline** | 97.00(53.00-180.00) | 69.00(21.00-111.00) | 0.217 | 99(18–233) | 30(9–94) | 0.171 | 34.20(33.20–36.20) | 32.20(30.80–35.80) | 0.773 |
| **Day1** | 794.00(342.00-1032.00) | 782.00(308.00-2120.00) | 0.748 | 355.00(148.00-852.00) | 380.00(259.00-935.00) | 0.652 | 35.10(31.50–37.00) | 33.90(30.90–35.40) | 0.411 |
| **Week1** | 45.00(20.00–88.00) | 34.00(24.00–34.00) | 0.898 | 106.00(24.00-217.00) | 79.00(58.00-219.00) | 0.652 | 35.50(31.90–40.40) | 34.30(31.10–36.30) | 0.263 |
| **Week2** | 33.00(15.25–60.25) | 18.50(12.50-37.25) | 0.387 | 67.50(19.50-176.25) | 36.00(17.50–116.00) | 0.426 | 36.10(33.40–38.70) | 36.90(34.73–40.48) | 0.644 |
| **Week4** | 38.00(21.00-124.00) | 28.00(17.00–36.00) | 0.261 | 49.00(26.00-158.00) | 28.00(17.50–55.50) | 0.295 | 39.10(36.60–42.60) | 45.40(40.90-47.45) | 0.01* |
| **Week8** | 43.00(19.00–67.00) | 18.00(14.50–39.00) | 0.112 | 30.00(16.00-158.00) | 28.00(15.50–91.50) | 0.766 | 43.20(37.50–44.50) | 44.20(41.60–47.50) | 0.152 |
| **Week12** | 28.00(20.00–32.00) | 21.00(18.00–40.00) | 0.489 | 20.00(13.00-50.50) | 21.00(13.50–96.50) | 0.73 | 40.70(38.85–45.65) | 42.60(41.85–46.30) | 0.245 |

Table 5
(Continued)

| | ALP | | | GGT | | | CREAT | | |
|---|---|---|---|---|---|---|---|---|---|
| | Rituximab | MSC | P | Rituximab | MSC | P | Rituximab | MSC | P |
| **Baseline** | 99.00(71.00-116.00) | 99.00(95.00-124.00) | 0.478 | 51.00(42.00–75.00) | 49.00(33.00–77.00) | 1 | 71.00(56.00-174.00) | 76.00(45.00-114.00) | 0.748 |
| **Day1** | 63.00(45.00–89.00) | 65.00(50.00–73.00) | 0.478 | 51.00(22.00–62.00) | 45.00(34.00–51.00) | 1 | 105.00(72.00-183.00) | 92.00(79.00-201.00) | 1 |
| **Week1** | 68.00(59.00-181.00) | 82.00(59.00-103.00) | 0.949 | 78.00(39.00-279.00) | 149.00(64.00-203.00) | 0.949 | 54.00(44.00-124.00) | 60.00(48.00–84.00) | 0.898 |
| **Week2** | 99.50(70.50-135.75) | 67.00(47.50–95.50) | 0.24 | 103.50(76.00-215.25) | 61.50(35.75–139.00) | 0.705 | 68.50(48.50-112.50) | 49.50(45.00-56.25) | 0.282 |
| **Week4** | 105.00(75.00-208.00) | 82.00(64.50–97.50) | 0.112 | 78.00(30.00-218.00) | 67.00(36.00-132.00) | 0.656 | 66.00(47.00–90.00) | 62.00(58.50–73.00) | 0.656 |
| **Week8** | 125.00(102.00-226.00) | 90.00(73.00-117.00) | 0.056 | 95.00(50.00-361.00) | 55.00(28.50–60.00) | 0.031* | 83.00(56.00-177.00) | 68.00(57.50–95.00) | 0.261 |
| **Week12** | 86.00(69.00-111.50) | 87.00(62.50-130.50) | 1 | 78.00(42.00-272.00) | 35.00(21.00-92.50) | 0.094 | 80.00(73.50–127.00) | 77.00(62.50–95.00) | 0.436 |

* $P < 0.05$.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALB, albumin; TBIL, total bilirubin; ALP, alkaline phosphatase; GGT, gamma-g
BUN, blood urea nitrogen. MSC, mesenchymal stem cell.

## Post-transplant complications: infection

Due to the immunosuppressive effect of rituximab, opportunistic and severe infection is another common complication after ABO-i LT, which might progress to septic shock, even multiple organ failure. Thus, we investigated the impact of MSCs on incidence of infection during the 2-year follow-up period (Table 4). Nine patients (9/11) developed pulmonary (n = 8) and biliary tract (n = 1) infection, two of which died of severe and refractory pulmonary infection within 3 months after transplantation. On the other hand, only one patient (1/11) was diagnosed splenic abscess in the MSC group, indicating that MSCs treatment significantly decreased the rate of septic shock in the MSC group compared with the Rituximab group (9.1% vs. 81.8%, p = 0.002).

## Post-transplant complications: Biliary tract complications

Biliary tract complications including ischemic-type biliary lesions (ITBL), biliary constriction and fistula are the major complications after ABO-i LT using rituximab. In our study, the overall rate of biliary tract complications occurred in the MSC group was significantly lower than that in the Rituximab group (0% vs. 45.5%, p = 0.035) (Table 4). Our previous research had proved the therapeutic effect of MSCs on biliary tract complications, especially ITBL. In this study, four patients in Rituximab group developed ITBL within 2 months after LT, the incidence rate was much higher than that in the MSC group (36.4% vs. 0%, p = 0.09) (Table 4). Except for magnetic resonance cholangiopancreatography (MRCP) or contrast-enhanced ultrasonography (CEUS), the diagnosis of ITBL was also confirmed with immunohistochemistry of CK19 (Fig. 5). Adjustment of immunosuppressive agents with medication (n = 3) and interventional therapy (n = 1) were given to treat ITBL. In addition, biliary fistula was only found in one patient of Rituximab group, which progressed to septic shock.

## Post-transplant complications: others

Besides, as shown in Table 4, the arterial complications were comparable between two groups (8.3% vs. 27.3%, p = 0.586). To our surprise, there was one patient in Rituximab group developing hepatocellular carcinoma, which was confirmed by pathological diagnosis. Thereafter, this patient was received TACE treatment and still alive now.

## Discussion

To our knowledge, we firstly conducted a randomized, open-labelled clinical trial to evaluate the safety of intravenous transfusion of allogeneic MSCs and compared the preliminary outcomes of MSCs and rituximab in recipients with severe hepatic failure receiving emergent ABO-i LT. Overall, this phase I/II study shown that multi-doses of MSCs administration were safe and well-tolerance in this population. Furthermore, we also provided that as an adjunct treatment, transfusion of MSCs not only has comparable ability of rituximab in preventing AMR, but also has lower incidences of post-operative severe infection and biliary complications after ABO-i LT.

With the incredible capabilities of immunomodulation and organ protection, MSCs have been gradually praised as a promising therapy, and increasing clinical trials were allowed to administrate for various diseases, which have shown encouraging outcomes. However, several issues still exist to be discussed, and the source of MSCs are firstly worth considered. Olivier et al. preferred to the transfusion of BM-MSCs for patients after LT[10]. Besides, Wang et al. exhibited the role of MSCs in attenuating acute graft rejection after LT via affecting the percentage of Tregs and Treg/Th17 ratio[11]. In this study, we also used MSCs deriving from umbilical cord for the patients after ABO-i LT, as umbilical cord obtain are more available without any ethical problems[20]. The process of MSCs harvest in our center was performed at the Stem Cell Laboratory Facility of Biotherapy Center of the Third Affiliated Hospital of Sun Yat-sen University and abided by the standardized, aseptic requirement. Secondly, the doses and times of MSCs administration are also controversy. It was believed that the minimum effective cell dosage was $1 \times 10^7$ cells/kg, and high rate of mortality was occurred when single-dose was more than $21 \times 10^7$/kg[21, 22]. However, due to combined utilization of multiple immunosuppressants, we adopted $1 \times 10^6$ cells/kg of single-dose infusion of MSCs in this study, which was similar to the suggestion in the previous experience of MSCs administration in organ transplantation fields[10, 23]. In addition, a single-dose and multiple-dose administration were both reported respectively in the past researches. Our previous clinical study shown the hepatoprotective effects of repeated doses of MSCs transfusion for improving ITBL after LT without any severe side-effects[12]. Thus, we continued to adopt this strategy in the current study and used eight doses of MSCs in the postoperative period. Furthermore, as hyperacute rejection reaction, theoretically, occurs immediately after graft reperfusion, we added a dose in this period during operation, including 10% MSCs administration through portal vein after graft reperfusion and 90% transfused through peripheral vein[24].

As this is the first report about the administration of stem cell-based therapy for preventing postoperative complications after ABO-i LT, safety issue remains the primary concern for our observation. Due to their characterization, MSCs liable to embolize pulmonary circulation in animal experiments when they were transfused through peripheral or central vein so that increased the burden of pulmonary exchange[25]. In addition, with the abilities of immunosuppression and multi-lineage differentiation, MSCs theoretically exist a potential risk of carcinogenesis[26]. Fortunately, the results from this trial shown that our eleven recipients receiving MSCs therapy did not arise severe infusional toxicity, including allergic reaction and did not develop any signs of pulmonary dysfunction and malignant transformation after 2-year follow-up. Only a few patients were noted limiting fever and completely recover 3 h after the infusions without any special treatment. In generally, our data indicated that allogeneic MSCs administration for these patients was safe, which is adherence to the investigations of our previous study of treating patients with ITBL after LT[12].

As another primary outcome, we additionally compared the incidence of allograft rejection between the Rituximab group and the MSC group. Traditionally, AR is divided into AMR and ACR. During ABO-i LT, high titer of ABO antibodies may lead to a high risk of AMR. Rituximab is an immune-chimeric monoclonal antibody that specifically points to the transmembrane protein CD20 molecule, which expressed on the majority of B cells, but not on antibody-producing plasma cells, to deplete B cells. Rituximab is also approved for the application in transplantation field, especially in the ABO-i organ transplantation. In 2003, Monteiro et al. firstly reported the administration rituximab for the recipients receiving ABO-i LT, and since then several studies have demonstrated that rituximab obviously reduced graft loss rates and is crucial to prevent the risk of AMR after ABO-i LT[27, 28]. In this setting, we found that MSCs treatment resulted in the lower risk of AMR was comparable to that of rituximab following ABO-i LT. This encourage result also indicated that MSCs might replace rituximab to modulate the functions of B cells.

As the secondary outcome, we prospectively assessed the therapeutic effects of MSCs on ABO-i LT recipients by comparison with the Rituximab group. No difference could be investigated between rituximab and MSCs treatment on the changes of the levels of ALB, ALP, AST, γ-GGT, BUN, CREAT and TBIL during the follow-up periods. Interesting, of these results, we found that a more distinct rise of post-transplant ALB level was observed in the MSC group compared with the Rituximab group, which might speculate that MSCs play an important role in repairing liver function[29]. In addition, we also compared the rate of opportunistic infections between these two groups, due to both are immunosuppressive. Previous study shown that along with the effect on preventing AMR, treatment with rituximab increased the incidence of severe infections[30]. Again, a large-sample size trial of multiple sclerosis shown that the rate of serious infections after used rituximab was higher than that in the control group[31]. And other study showed that MSCs treatment for living-related kidney transplant recipients oppositely exhibited lower rate of infectious complications[9]. In the present study, higher rate of serious infection was observed in the Rituximab group than that in the MSC group. Of all, three cases died from infection after treated with rituximab, and it did not happen in the MSC group. Through liver biopsy, we also detected that compared with MSCs, CD20-postive B cells were obviously depleted in the liver tissue by rituximab, which may explain why rituximab treatment susceptible to opportunistic infection. Other indicators, including survival rate, graft survival rate, acute rejection and arteriostenosis, were also no significant difference from these two groups except biliary complications. As large-size retrospective study by Song et al demonstrated that biliary complication in the only concern in ABO-i LT after treated with rituximab[6]. Likely, high rate of ITBL in the Rituximab group was observed in this study. Consistent with our previous studies, MSCs also exhibited ability in protecting biliary structure following ABO-i LT[12]. To explain this issue, we referred the past researches, and found that after ABO-i LT kidney transplantation, the graft vascular endothelium markedly expressed ABO blood group antigens that is the target of attack[32]. Lacob et al. revealed that the presence of anti-human leucocyte antigen (HLA) class II antibodies was closely associated with biliary

injury after LT[33]. And we speculated that MSCs might exhibit critical roles in regulating the level of DSA and affecting the membranous expression of MHC-II of bile duct epithelium following ABO-i LT to improve biliary injury.

There are several important shortcomings in this study which should be acknowledged for improving further investigation. First, this phase I/II trial is the first study only enrolled 11 ABO-i LT recipients in both the MSC group and the Rituximab group. Therefore, a well-designed study with a large sample size, multicenter and long-term investigation is required to further ascertain these outcomes. Second, due to the characteristics of complications of ABO-i LT, we administrated repetitive infusions of MSCs both during and post operation via portal vein and peripheral vein. However, the timing, the dose and the route of MSCs administration remain deserve to deliberation and should be further evaluated. Furthermore, this is an open-labeled study, and neither recipients nor observers were blinded to the therapeutic strategy. Thus, bias was inevitable in the interpretation of adverse events. Finally, although we have detected the changes of several serum cytokines in this two groups during the follow-up period, the deep mechanism of the effect of MSCs in ABO-i LT should be further observed with evaluating the change of immune cell subpopulation in peripheral circulation.

## Conclusions

The present study reported the first prospective, controlled clinical study evaluating the feasibility and safety of allogeneic MSCs transfusion in a series of severe hepatic failure patients receiving ABO-i LT. In this study, no severe side-effects of MSCs transfusion after operation could be found. In addition, besides AMR prevention, MSCs administration effectively reduced the incidence of opportunistic infection and biliary complications, compared with rituximab. Although this is a small cohort study, the know which we have gained regarding the biological effect of MSCs in ABO-i LT allowed us to conduct a large and randomized study to further confirm the feasibility of MSCs treatment for preventing post-operative complications after ABO-i LT (Chictr.org.cn: ChiCTR2000037732).

## Abbreviations

ABO-i LT: ABO-incompatible liver transplantation; SHF: Severe hepatic failure; AMR: Antibody-mediated rejection; MSC: Mesenchymal stem cell; IVIG: Intravenous immunoglobulin; IBD: Inflammatory bowel disease; NK: natural killer; ITLBs: Ischemia-type biliary lesions; MMF: Mycophenolate mofetil; DCD: Donor after circulatory death; DBD: Donor after brain death; ICU: Intensive care unit; MELD: Model for End-Stage Liver Disease; CMV: cytomegalovirus; HTLV: Human T lymphotrophic virus; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; GMP: Good manufacturing practice; PBS: Phosphate-buffered saline; FBS: Fetal bovine serum; AR: Allograft rejection; AMR: Antibody-mediated rejection; ACR: Acute cellular rejection; H&E: Hematoxylin and eosin; CK19: Cytokeratin 19; CT: Computed tomographic; MRCP: Magnetic resonance cholangiopancreatography; CEUS: Contrast-enhanced ultrasonography; HLA: Human leucocyte antigen; WBC: White Blood Cell; PLT: Platelet; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALB: Albumin; TBIL: Total bilirubin; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; PT: Prothrombin time; INR: International Normalized Ratio; CREAT: Creatinine; BUN: Blood urea nitrogen; CPS: Child-Pugh score; RBC: Red blood cell; FFP: Fresh frozen plasma; Cryo: Cryoprecipitate.

## Declarations

### Ethics approval and consent to participate

The study design was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. All included patients were informed about the nature of the study and gave their written informed consent.

### Consent for publication

All patients signed a consent form for their data to be used for research or publication.

### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.


### Funding

This work was supported by the grants from the National Key R&D Program of China (2017YFA0104304)，National Natural Science Foundation of China (81770648, 81802402, 81870449, 81900597, 81970567), National Key R&D Program of Guangdong Province (2019B020236003), Guangdong Natural Science Foundation (2017A030311034, 2018A03031032，2018A030313043), Medical Scientific Research Foundation of Guangdong Province (A2018130), Sci-tech Research Development Program of Guangzhou city (158100076), Young Teacher Development Program of Sun Yat-Sen University (17ykpy57, 19ykpy35, 20ykpy41), China Postdoctoral Science Foundation (2019M653199) and Academician Shusen Lanjuan Talent foundation.


### Author contributions

Y.C.Z., S.H.Y., Q.Z., A.P.X. and Y.Y.: conception and design, financial support, administrative support and final approval of manuscript; Y.C.Z., J.B.Z., H.M.Y., J.Z., J.Y.C. and W.J.C.: collection and assembly of data, data analysis and interpretation, and manuscript writing; T.Y.L. and L.C.: collection and/or assembly of

data and data analysis and interpretation; C.D., W.J.C., A.P.X. and Q.Z.: provision of MSCs and its quality control; Y.C.Z., H.M.Y., J.R.L., J.Y., H.Z., G.Y.W., B.S.F., T.Z., J.Z., G.S.W., H.L., G.H.C., S.H.Y. and Y.Y.: provision of patients, patient management and patient follow-up. All authors read and approved the final manuscript

#### Acknowledgements

We are grateful to all clinical investigators and the patients included.

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## Figures

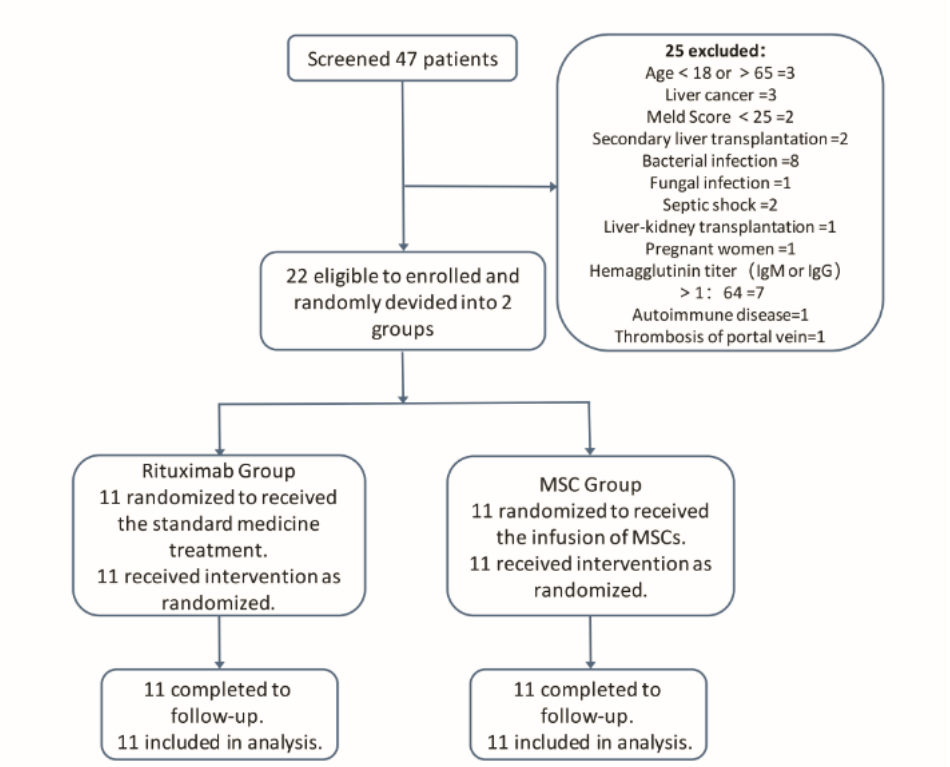
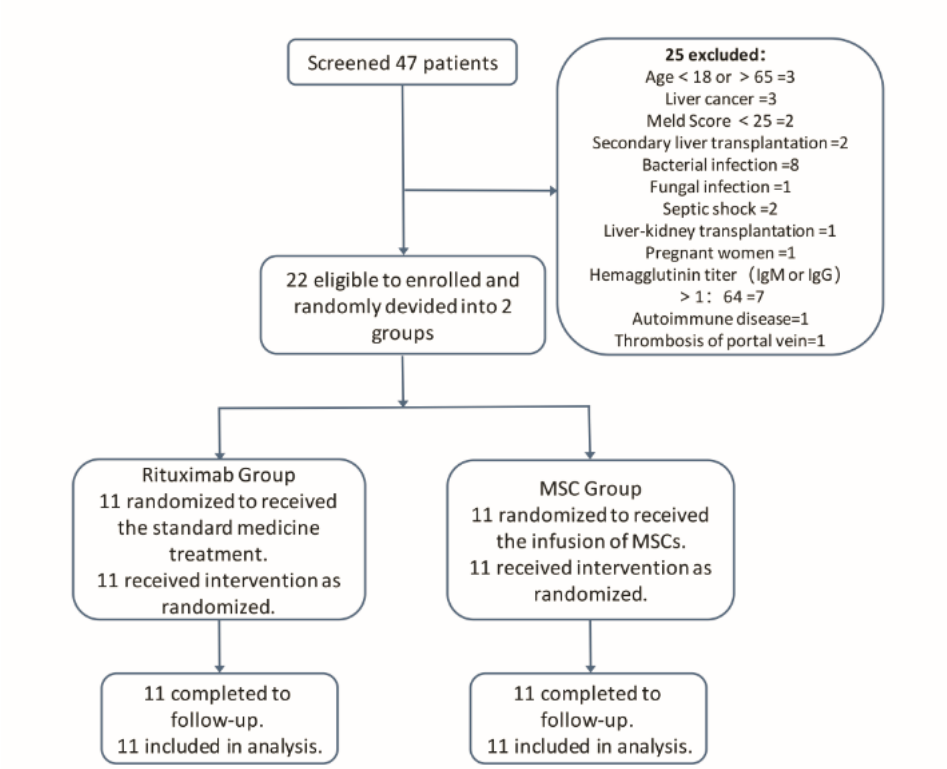



**Figure 1**

Study flow chart. Enrollment, randomization and follow-up of patients in the MSCs and rituximab trial. Between August 2016 and August 2018, a total of 47 severe hepatic failure patients receiving ABO-i LT were screened. 22 participants were enrolled for the present study and randomly divided into 2 groups (the MSC group=11, the Rituximab group=11). At last, 11 participants in the MSC group and 11 in the Rituximab group were completed to follow-up and included in analysis.

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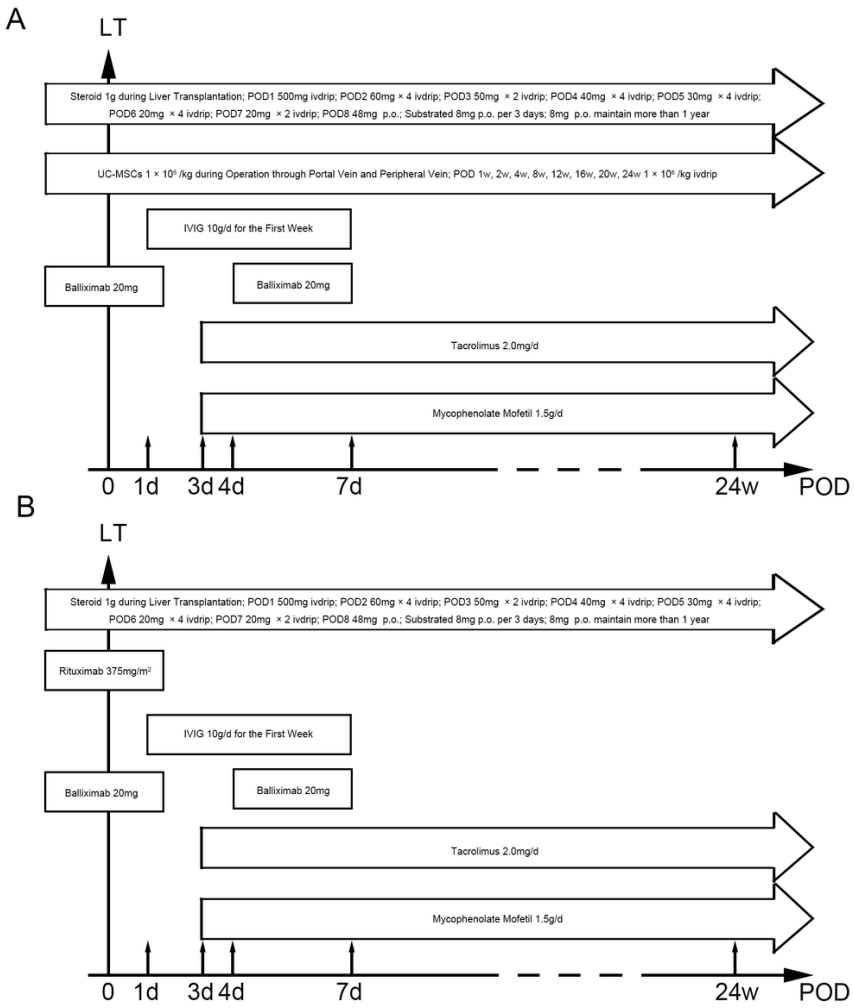
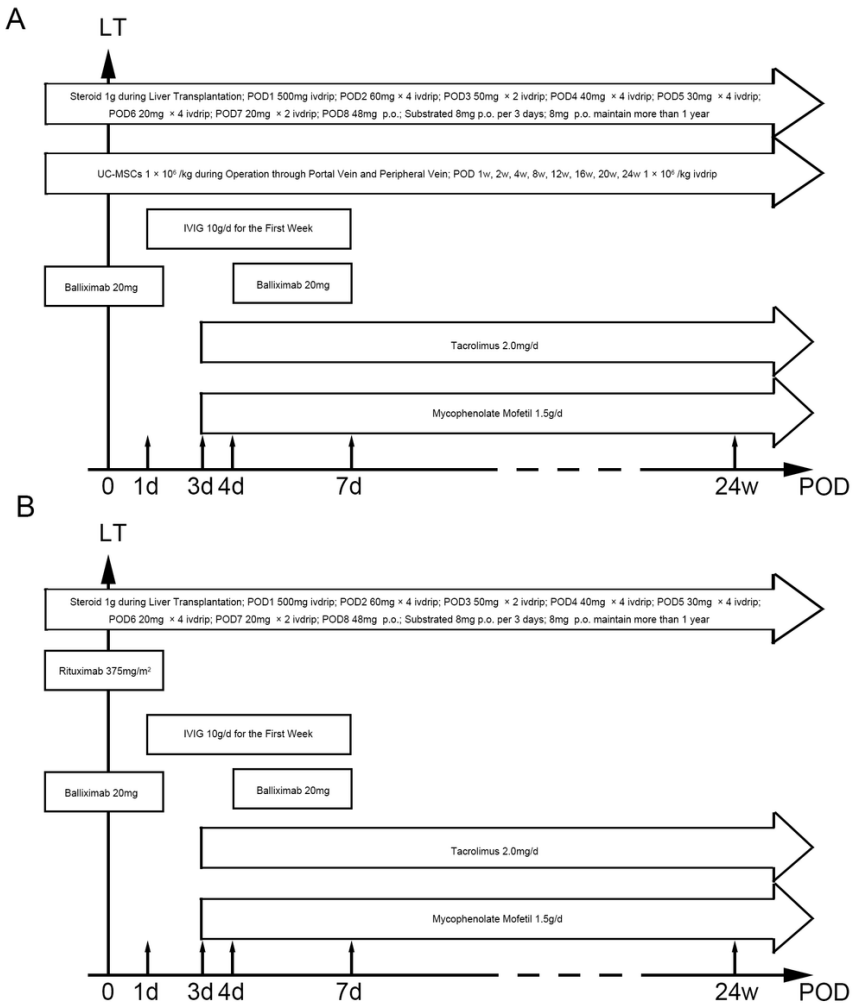



**Figure 2**

Immunosuppression protocol for ABO-i LT in the MSCs and rituximab trial. LT, liver transplantation; IVIG, intravenous immunoglobulin.

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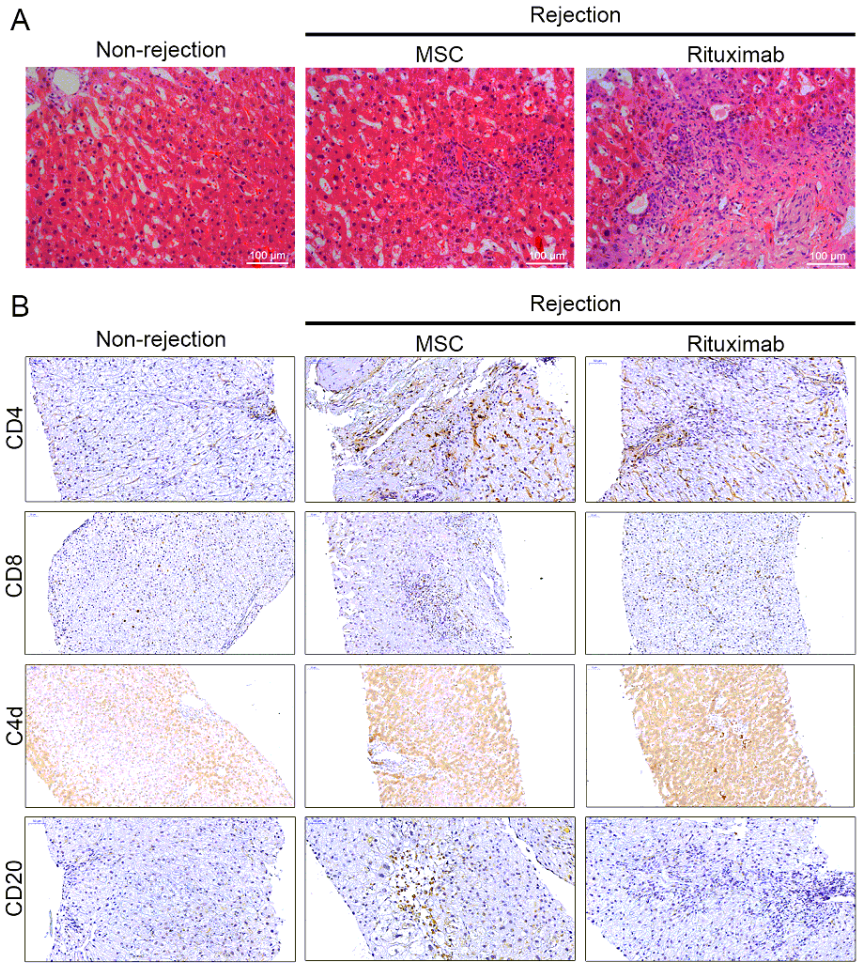
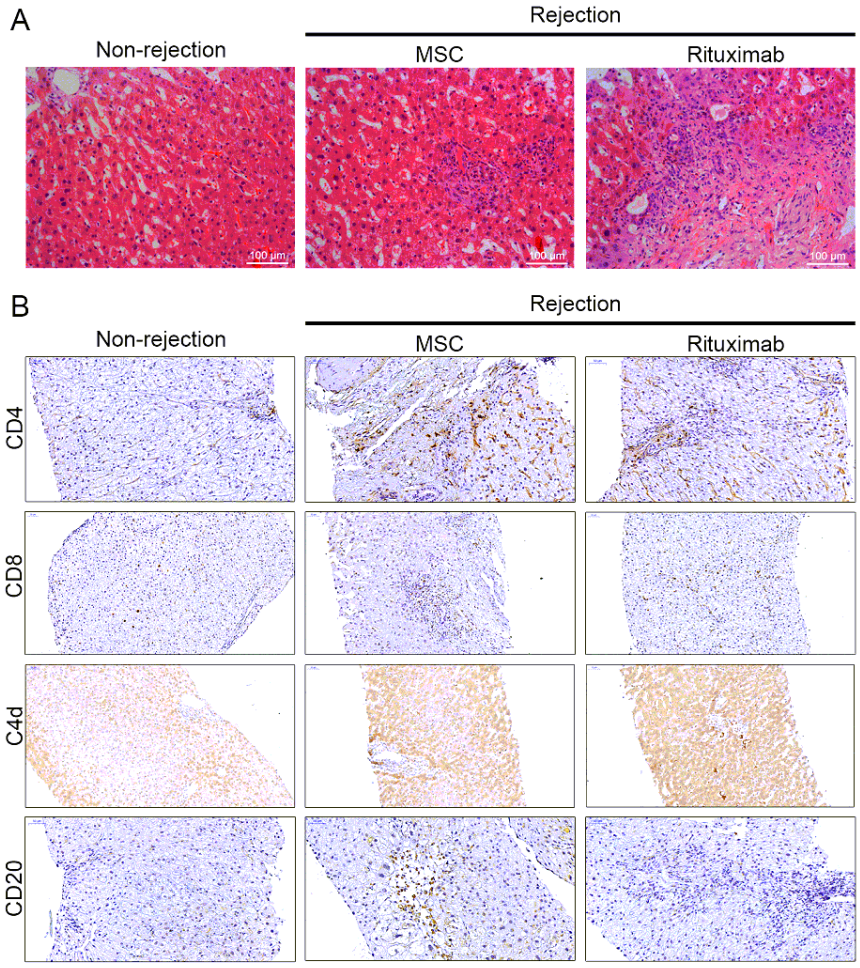



**Figure 3**

The sixth-month graft biopsies. A. Representative sections of liver stained with hematoxylin and eosin (H&E), including non-rejection and rejection during MSCs or rituximab treatment (magnification ×200). B. Representative IHC images of CD4, CD8, C4d and CD20 staining, including non-rejection and rejection during MSCs or rituximab treatment (magnification ×200).

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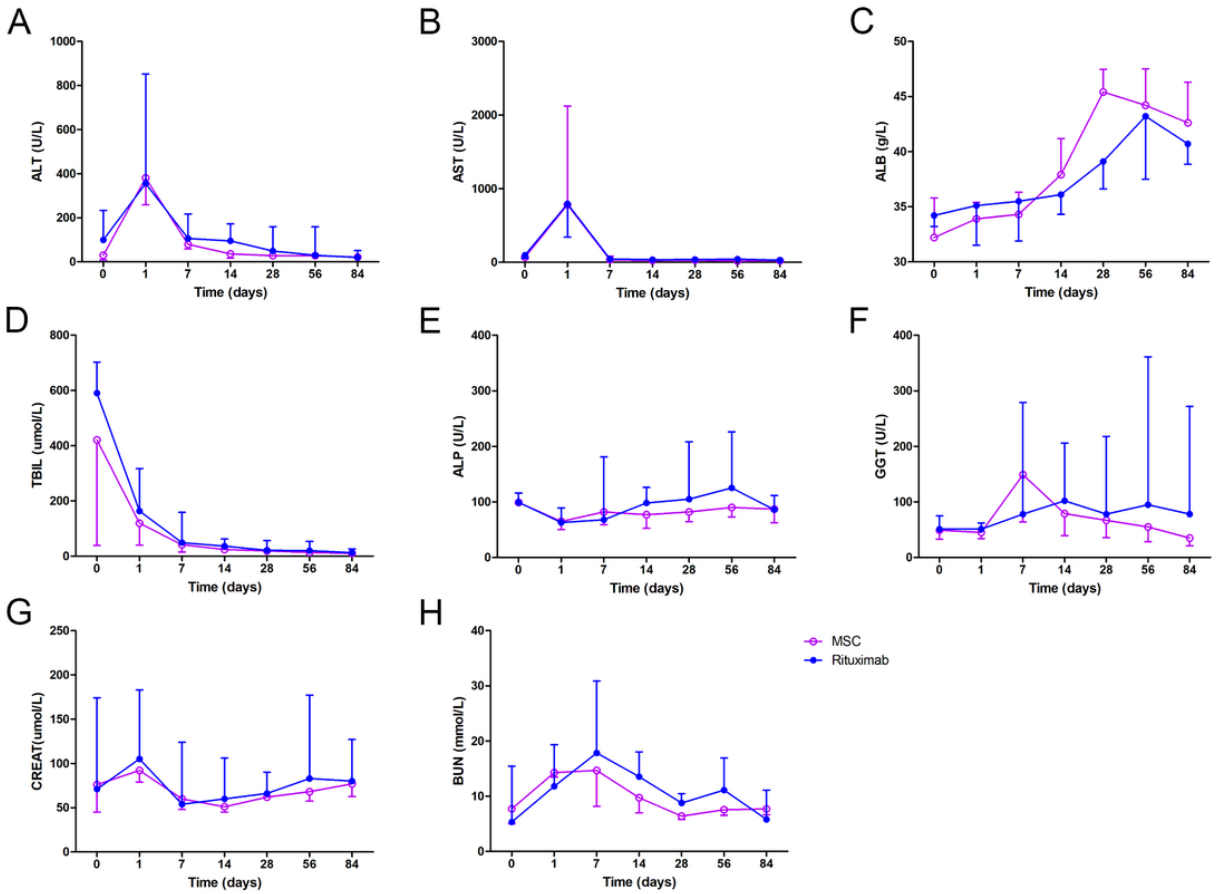
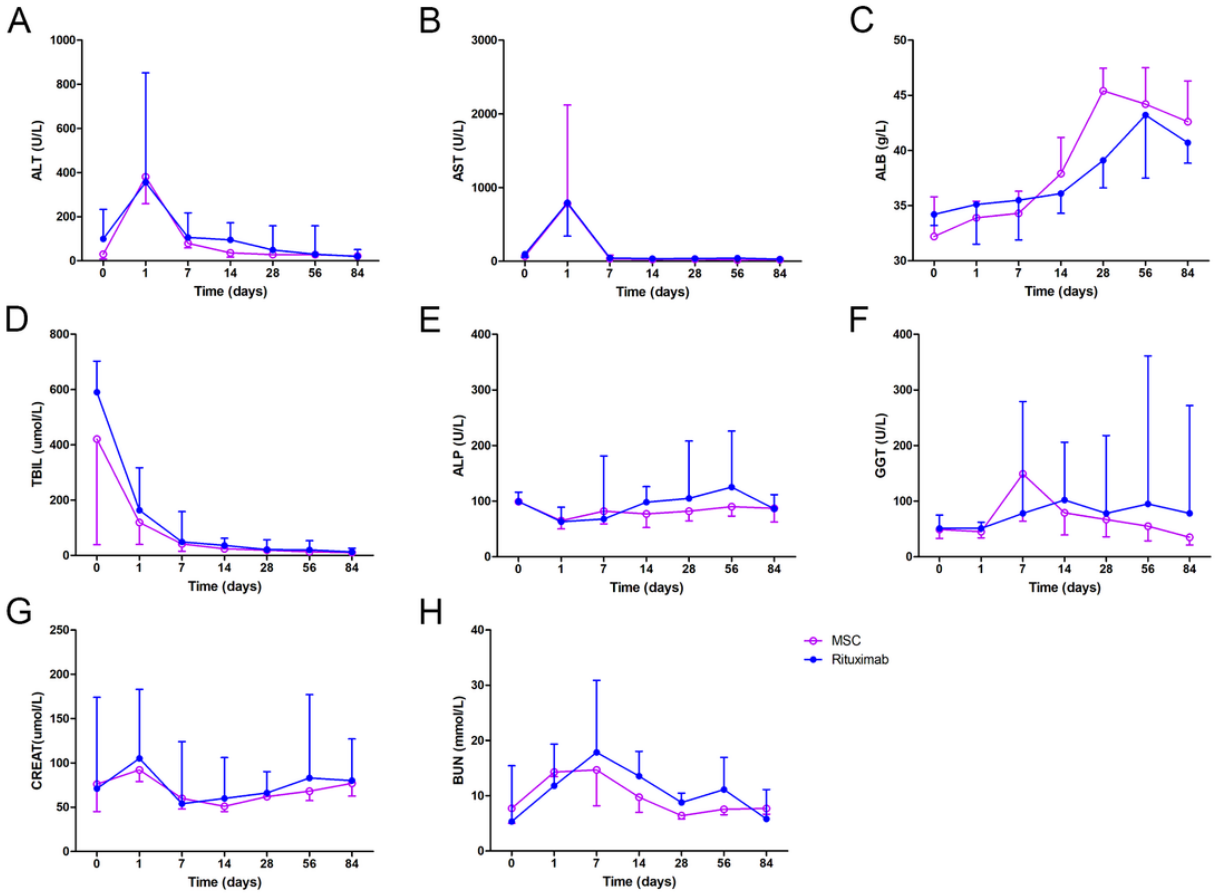



**Figure 4**

Postoperative laboratory tests Including ALT (U/L), AST (U/L), ALB (g/L), TBIL (umol/L), ALP (U/L), γ-GGT (U/L), CREAT (umol/L) and BUN (mmol/L). (Data are presented as mean±SEM; *, $p<0.05$).

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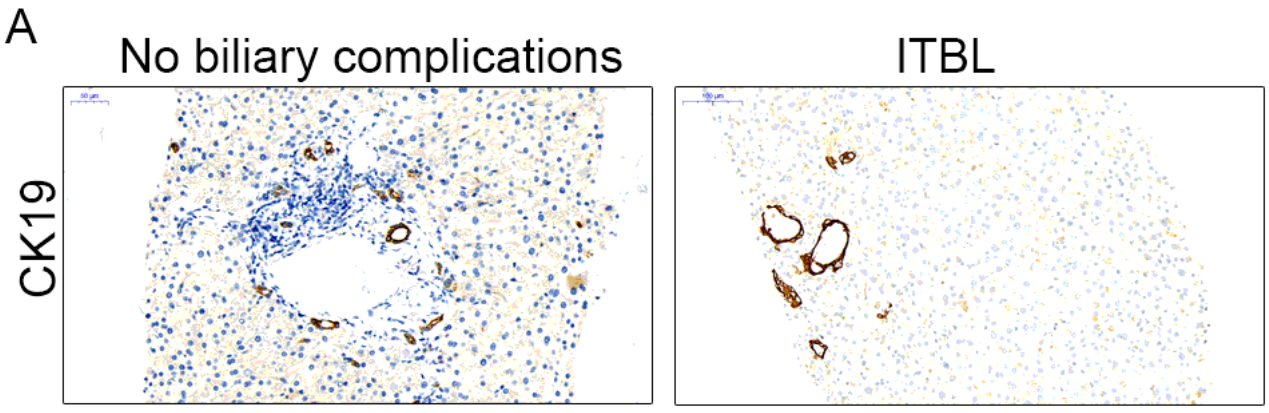


Figure 5

The sixth-month graft biopsies. A. Representative sections of liver stained with CK19, including no biliary complications and ITBL (magnification ×200).

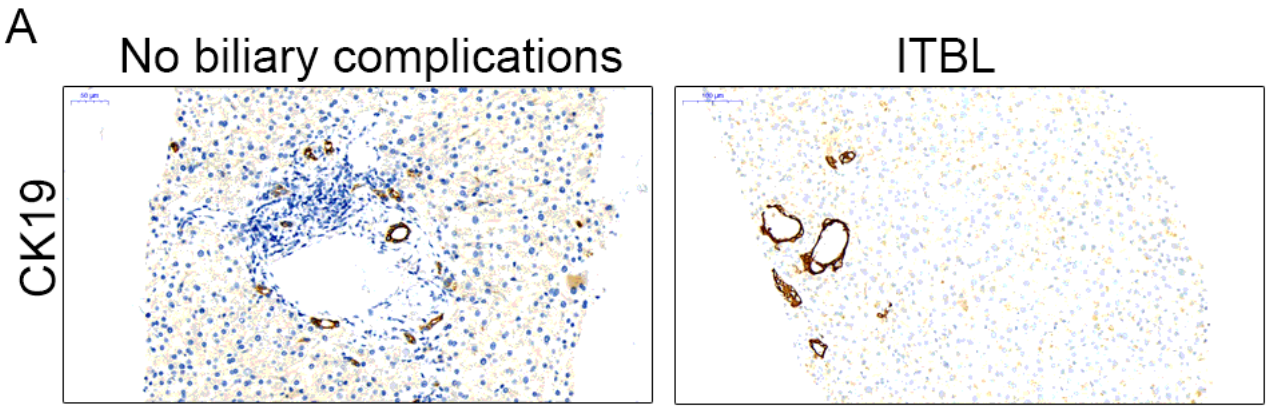


**Figure 5**

The sixth-month graft biopsies. A. Representative sections of liver stained with CK19, including no biliary complications and ITBL (magnification ×200).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ClinicalTrialProtocol.docx
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