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STANDARDS, PROTOCOLS, POLICIES, AND REGULATIONS FOR CELL-BASED THERAPIES

STEM CELLS TRANSLATIONAL MEDICINE

A study of human leukocyte antigen-haploidentical hematopoietic stem cells transplantation combined with allogenic mesenchymal stem cell infusion for treatment of severe aplastic anemia in pediatric and adolescent patients

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Abstract

The clinical applications of human leukocyte antigen (HLA) haploidentical hematopoietic stem cells transplantation (haplo-HSCT) have offered most of the young severe aplastic anemia (SAA) patients an opportunity to accept curative therapy at the early stage of bone marrow lesions. However, the outcome of juvenile SAA patients received haplo-HSCT remain to be improved due to high incidence of graft failure and graft vs host disease (GVHD). Mesenchymal stem cells (MSCs) have been characterized by their hematopoiesis-supporting and immunomodulatory properties. In the current study, we designed a combination of haplo-HSCT with allogenic MSC for treatment of SAA in pediatric and adolescent patients and evaluated its effects. Juvenile patients (<18 years) with SAA (n = 103) were given HLA-haploidentical HSC combined with allogenic MSC after a conditioning regimen consisting of busulfan, cyclophosphamide, fludarabine, and antithymocyte globulin and an intensive GVHD prophylaxis, including cyclosporine, short-term methotrexate, mycophenolate mofetil, and basiliximab. Neutrophil engraftment was achieved in 102 of 103 patients in a median time of 14.3 days (range 9-25 days). The median time of platelet engraftment was 25.42 days (range 8-93 days). The cumulative incidence of II-IV acute GVHD at day +100 was 26.32% ± 0.19% and III-IV acute GVHD was 6.79% ± 0.06% at day +100, respectively. The cumulative incidence of chronic GVHD was 25.56% ± 0.26%.

The overall survival was 87.15% ± 3.3% at a median follow-up of 40 (1.3-98) months. Our data suggest that cotransplantation of HLA-haploidentical HSC and allogenic mesenchymal stem cell may provide an effective and safe treatment for children and adolescents with SAA who lack matched donors.

KEYWORDS

bone marrow niche, graft vs host disease, HLA-haploidentical HSCT, mesenchymal stem cells, pediatric and adolescent patients, severe aplastic anemia

1 | INTRODUCTION

Severe aplastic anemia (SAA) is a serious hematological disease characterized by extreme bone marrow hypocellularity and peripheral cytopenia.[1-3] Drugs, toxic agents, infections, and dysregulated T-cell repolarization are considered responsible for hematopoietic stem cell injury and lead to the pathogenesis of SAA.[1-3] In addition, an increasing number of studies have reported impaired components and disordered function of the bone marrow microenvironment in patients with SAA.[4-6] The fatty bone marrows of SAA patients usually contained fewer stromal cells and their progenitors. Numerous factors, including inflammation, proved contribute to the injuries of hematopoietic supporting cells.[7,8] Furthermore, the strongest evidence for an immune-mediated mechanism is the improvement of blood counts in SAA patients after immunosuppressive therapy (IST). However, most patients could not be cured by IST. The previous studies showed that only 25% remain long-term good-quality responders, and 10% to 15% even show clonal evolution to myelodysplastic syndrome or acute myeloid leukemia.[9-12]

HLA-matched hematopoietic stem cell transplantation (HSCT) has become the first-line treatment option for SAA treatment. By removing the clonal autoimmune T cells and restoring normal hematopoiesis, HSCT is a curative therapy for SAA patients.[13-15] In the absence of an HLA-matched HSC, haploidentical family donors offer the advantage of an acceptable HSC dose and immediate availability for almost any patient, which greatly improves the outcome of juvenile SAA patients by stopping irreversible pathological progress early and avoiding life-threatening complications.[16,17] Notably, the bidirectional effect is that HLA-haploidentical HSCT (haplo-HSCT) results in higher incidence of graft failure and graft vs host disease (GVHD) compared to HLA-matched HSCT.[18,19]

MSC and their progeny are important supporting cells in the bone marrow microenvironment.[20-22] By expressing secretory factors and cell surface adhesion molecules, they contribute to the building of bone marrow architecture, support HSC engraftment and proliferation, and their differentiation into multiple lineages of mature blood cells. To explore the clinical effects of MSC in HSCT, Lazarus et al performed the pilot works of cotransplanting HLA-identical sibling culture-expanded MSC with HSC for hematologic malignancy patients.[23] They reported no immediate infusional or late MSC-associated toxicities. Ball et al subsequently reported that 14/14 patients with hematologic malignancies or nonmalignant disorders showed sustained hematopoietic engraftment without any adverse reaction post transplantation of HLA-disparate $CD34^+$ cells and donor bone marrow MSC.[24] Afterwards, the data from two pediatric patients with refractory SAA indicated that cotransplanting haploidentical MSC with HLA-identical sibling-matched HSC may be helpful to overcome graft failure.[25] At almost the same time, we reported that infusion of patient-father-derived haploidentical MSC and HSC yielded rapid hematopoietic recovery in a 3-year-old child with SAA.[26]

In addition to the advantage of hematopoiesis-supporting capacity, MSC have attracted increasing attention due to their immunomodulatory properties. The accumulated studies and our previous works have demonstrated that MSC can suppress the maturation and activation of numerous immune cells, which contribute to the therapeutic effects of MSC for settlement of immune-disorders.[27-32] Because autoimmune factors are closely involved in SAA pathology, MSC may alleviate immune reaction-induced bone marrow lesions. In addition, MSC have been proved useful to prevent and treat GVHD.[32]

Significance statement

Haploidentical hematopoietic stem cells (HSCs) transplantation provides an immediate curative option for almost all newly diagnosed juvenile severe aplastic anemia (SAA) patients, but it presents a higher incidence of graft failure and graft vs host disease (GVHD). Mesenchymal stem cells (MSCs) were introduced here by virtue of their support of hematopoiesis and immunomodulatory capacities. The promising data based on a cohort of 103 cases have demonstrated that the protocol discussed in this study yielded high engraftment, rapid hematopoietic reconstitution, and a low incidence of acute and chronic GVHD. These findings indicate the possibility of using haploidentical HSC and allogenic MSC to treat SAA when diagnosed at an early stage.

However, clinical MSC infusions have been observed to suppress graft vs leukemia effect and we previously found that it even resulted in higher incidence of relapse in the treatment of hematologic malignancy.[33] In the contrast, it was at low risk in treating nonmalignant disorders, such as SAA, by MSC.

In the past two decades, we have been applying halpo-HSCT in the treatment of severe hematopoietic disorders in our transplant unit.[34-40] Based on our clinical observations and our understanding of stem cell biological properties, we have developed a novel protocol for SAA, especially for pediatric and adolescent SAA patients in our unit.[26,41-43] Due to the high risk of graft failure or rejection of haplo-HSCT, fludarabine was used in an intensive conditioning regimen. Additionally, MSC were routinely used in our protocol for hematopoietic promotion and immunomodulation. In the current study, we report promising clinical outcomes of a cohort including 103 juvenile patients (<18 years) with SSA.

2 | MATERIALS AND METHODS

2.1 | Patients

Between February 2012 and August 2019, 103 children with acquired SAA/VSAA (very severe aplastic anemia) who underwent haplo-HSCT at our transplant unit were enrolled in this study. All patients and donors provided written informed consent for this protocol. The study was approved by the Ethics Committee of the Air Force General Hospital. All patients met the following inclusion criteria: (a) diagnosed with SAA/VSAA according to the International Aplastic Anemia Study Group; (b) age < 18 years old; (c) no HLA-matched donors; (d) no active infection or severe disease of vital treatment; (e) and no response to previous immunosuppressive therapy, including CsA + andriol ± corticosteroid regimen (74 patients), CsA + ATG + andriol ± corticosteroid regimen (27 patients), and MSD + CsA + andriol ± corticosteroid regimen (two patients). The median time from diagnosis to transplantation was 24.70 (ranging from 1 to 96) months. The CsA was given orally (5-10 mg/kg/d) and the Cmin was maintained at 150 to 250 μg/L. The ATG was intravenous given (3-5 mg/kg/d) for 5 days. The patients, which accepted ATG therapy lasting more than 6 months and were still transfusion-dependent, were taken as ATG therapy failure cases.

2.2 | Conditioning regimen

Patients received the following conditioning regimen: busulfan (0.8 mg/kg every 6 hours on days −10 and − 9, total dose 6.4 mg/kg); fludarabine (30 mg/m^2/day on days −8 to −5, total dose 120 mg/m^2); cyclophosphamide (60 mg/kg/day on days −4 to −3, total dose 120 mg/kg); and ATG (rabbit, SangStat, Lyon, France; 2.5 mg/kg/day on days −4 to −1, total dose 10 mg/kg, n = 91) or ALG (porcine, Wuhan Institute of Biological Products, China; 25 mg/kg/day on days −4 to −1, total dose 100 mg/kg, n = 12).

2.3 | Preparation of allogenic MSC

This study was approved by the ethics and technological committees of the Air Force General Hospital (2010-0182). Allogenic MSC were uniformly prepared from human umbilical cords (UC) and donor bone marrow samples in the GMP workshops of Stem Cell Center of Air Force General Hospital and the written informed consent was obtained.[44] Briefly, healthy human UC was collected from full-term cesarean section births and processed within 3 to 6 hours. UC tissues were diced into small fragments after umbilical arteries and veins were removed. The UC tissues were digested in 0.2% collagenase at 37°C overnight. The double volume of 0.05% trypsin was added next day and maintained for 1 hour at 37°C. The released cells were harvested and cultured in human MSC serum-free culture media (HangZhou Biowish Bio-tech Co., HangZhou, China) in plastic cell culture flasks. The cells at passage 3 were used for therapy. For bone marrow MSC preparation, bone marrow samples were aspirated from the iliac crest of healthy donors, and mononuclear cells were prepared by gradient centrifugation and cultured in human MSC serum-free culture media. The cells at passage 3 were harvested for therapy.[22] For MSC quality control, cell immuno-phenotypic analysis and differentiation assays were performed to evaluate the MSC properties. In addition, bacterial and fungal cultivation of the medium was performed 48 hours before cell harvesting.[44]

2.4 | Stem cell collection and infusion

All donors received human granulocyte colony-stimulating factor (G-CSF) at a dose of 5 to 10 μg/kg/day for 4 to 5 consecutive days. Bone marrow cells were collected on day 4. The volume of the harvests was 10 to 20 mL/kg of donor. In the cases of ABO-incompatibility, red blood cells were removed by sedimentation with Hespan (B. Braun Medical Inc., Irvine, California). Peripheral blood stem cells (PBSCs) were harvested on day 5. The total target mononuclear cells were ≥5 × 10^8/kg and CD34$^+$ cells ≥2 × 10^6/kg of the recipient weight. The UC-MSC (n = 75) or BM-MSC (n = 28) were intravenously given to patients with a uniform cell number (1 × 10^6/kg of the recipient weight) with donor bone marrows.

2.5 | GVHD prophylaxis

In the current protocol, acute GVHD (aGVHD) prophylaxis included cyclosporin A (CsA), short-term methotrexate (MTX), mycophenolate mofetil (MMF), and monoclonal antibody against human CD25 (basiliximab). IV CsA were administered up to 1.5 mg/kg from day −9 to day −2 and adjusted to 2.5 mg/kg/day on day −1. When the patient's gastroenteric function recovered, CsA was given orally at the dose of 5 mg/kg/day. MMF was administered orally at a dose of 250 to 1000 mg daily from day +7 to day +100. MTX was given intravenously at a dose of 15 mg/m^2 on day +1, and 10 mg/m^2 on days +3, +6, and + 11. Basiliximab was given at 20 mg/day on days +1 and + 4.

2.6 | Definitions and post-transplantation evaluations

Neutrophil engraftment was defined as the first day of an absolute peripheral neutrophil count >0.5 × 10^9/L for three consecutive days. Platelet engraftment was defined as the first day of the platelet count >20 × 10^9/L without transfusion support for seven consecutive days. Hematopoietic chimerism was evaluated by chromosome for sex-mismatched pairs or polymerase chain reaction (PCR) amplification of short tandem repeats (STR) for sex-matched pairs.

Primary graft failure (PGF) was defined as failure to achieve neutrophil engraftment until 28 days post-haplo-HSCT. Secondary GF was defined as graft loss after initial engraftment, which means the recipients experienced pancytopenia and hypocellular BM without moderate to severe aGVHD. aGVHD was defined according to the criteria described by the 1994 Consensus Conference on aGVHD Grading. Chronic GVHD (cGVHD) was defined according to the National Institutes of Health Consensus Conference on cGVHD Project.

Diagnosis of CMV infection, CMV pneumonia, EBV infection, and EBV-associated post-transplant lymphoproliferative disorders (PTLDs) were based on standard clinical criteria.

Overall survival (OS) was defined as the time from the date of transplantation to the date of death or last follow-up. Failure-free survival (FFS) was defined as the time from the date of transplantation to failure or last follow-up. Death without disease progression was defined as transplantation-related mortality (TRM).

2.7 | Supportive care and prevention of other complications

All patients entered into a laminar air-flow room after receiving medicated baths and skin preparation. Cefuroxime was taken to prevent bacterial infection and posaconazole or voriconazole was taken to prevent fungal infection. The prophylaxis of virus included ganciclovir from day −9 to day −2 and cyclovir from day 1 to month 12. All patients received G-CSF at a dose of 5 to 10 μg/kg/day from day +7 until myeloid recovery. Heparin were administered to prevent veno-occlusive disease. Human immunoglobulin was administered IV at a dose of 2.5 to 5 g three times a week from day 0 to month 3 post-transplantation.

2.8 | Statistical analysis

TRM and OS were estimated by the Kaplan-Meier method and compared using the log-rank test. The cumulative incidence (CI) was used to calculate the incidence of aGVHD and cGVHD with death and GF as competing risks by the competing risk model in the R package "cmprsk." Statistical analyses were conducted based on data available on August 31, 2019. Statistical analyses were performed using the SPSS 22.0 and GraphPad Prism 6 and R version 4.0.2. *P* values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Patient characteristics

A total of 103 juvenile patients were enrolled in this study. Details are shown in Table 1. The clinical data included 56 male patients and 47 female patients. The median age was 8.82 years (range 2-18). All the patients received haplo-HSCT as salvage therapy. Among these patients, two received MSD HSCT previously, 27 received ATG treatment, and 74 patients previously received CsA ± andrio ± corticosteroid treatment. The median age of the donors was 35 (8-45)

TABLE 1 Patient, donor, and graft characteristics

Variable	N = 103
Patient median age, year (range)	8.82 (2-18)
Patient sex, no. (%)	
Male	56 (55%)
Female	47 (46%)
Previous treatment	
CsA ± andrio ± corticosteroid	74 (72%)
ATG + CsA ± andrio ± corticosteroid	27 (26%)
MSD + CsA ± andrio ± corticosteroid	2 (2%)
Donor median age, year (range)	35 (8–45)
Donor/patient sex, no. (%)	
Male/male	43 (41%)
Female/female	9 (9%)
Male/female	36 (35%)
Female/male	15 (15%)
Donor/patient relationship, no. (%)	
Father	75 (73%)
Mother	22 (21%)
Sibling	6 (6%)
ABO blood types match, no. (%)	
Matched	61 (60%)
Minor mismatched	24 (23%)
Major mismatched	12 (11%)
Major and minor	6 (6%)
Conditioning regimen, no. (%)	
BU/Cy + FU	103 (100%)
Allo-MSC (1 × 10^6/kg)	1.09 (0.93–1.43)
Source of graft, no. (%)	
BM + PB	98 (95%)
BM	5 (5%)
Median BM/PB MNC, ×10^8/kg (range)	13.18 (6.26-27.50)
Median BM/PB $CD34^+$ cells, ×10^6/kg (range)	6.83 (1.73-18.70)

Abbreviations: Allo-MSC, allogenic mesenchymal stem cell; ATG, antithymocyte; BM, bone marrow; Bu, busulfan; CsA, cyclosporine A; Cy, cyclophosphamide; FU, fludarabine; HSCT, hematopoietic stem cell transplantation; MNC, mononuclear cell; PB, peripheral blood; SAA, severe aplastic anemia.

years. The median nucleated cells and $CD34^+$ cells were 13.18×10^8/kg (range $6.26\text{-}27.50 \times 10^8$/kg) and 6.83×10^6/kg (range $1.73\text{-}18.70 \times 10^6$/kg), respectively. The median allogenic MSC cells infused were 1.09×10^6/kg (range $0.93\text{-}1.43 \times 10^6$/kg).

3.2 | Engraftment

One hundred two juvenile patients achieved myeloid engraftment after haplo-HSCT (Table 2). One patient died of primary GF (a 17 years old female patient, the donor was her mother and had unmatched blood type, she died at 35 days post HSCT because of septicemia). The median time for myeloid engraftment was 14.30d (range 9-25). Cumulative incidence of neutrophil engraftment was 93.20% ± 2.48% at *D* + 21 and 99.03% ± 0.97% at *D* + 28. The median time for platelet engraftment was 25.42 days (range 8-93). The cumulative incidence of platelet engraftment was 73.78% ± 4.33% at *D* + 28 and 95.89% ± 2.00% at *D* + 100.

One patient experienced secondary graft failure because of soft tissue infection. This patient was a 9-year-old male. The donor was his father and had unmatched blood type. He died at 2 months post-HSCT because of infection.

Two patients demonstrated platelet graft failure. They both had received ATG treatment previously. The donor was their father and had unmatched blood type. They died at 2 months and 11 months post-HSCT because of infection, respectively.

3.3 | Graft vs host disease

Among the 102 patients with primary engraftment, 27 patients experienced aGVHD (Table 2). Twenty cases had II aGVHD, five cases had III aGVHD, and two cases had IV aGVHD. The cumulative incidence of grades II-IV aGVHD and of grades III-IV aGVHD were 26.32% ± 0.19%, and 6.79% ± 0.06% on day +100 (Figure 1A) The cumulative incidences of grades II-IV aGVHD for infusing the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 18.82% ± 6.80% and 32.43% ± 0.38%, respectively(P = .11) (Figure 1B). The cumulative incidences of grades III-IV aGVHD for or the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 4.44% ± 0.09% and 8.62% ± 0.13%, respectively (P = .28) (Figure 1C).

The cumulative incidence of cGVHD was 25.56% ± 0.26%, and the cumulative incidence of moderate-severe cGVHD was 7.57% ± 0.11% on month +98 (Figure 1D). The cumulative incidences of cGVHD for the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 25.48% ± 0.64% and 24.47% ± 0.41% (P = .51), respectively (Figure 1E). The cumulative incidences of moderate cGVHD for the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 4.05% ± 0.16% and 10.62% ± 0.28% on month +98, respectively (P = .21) (Figure 1E,F). During the follow-up period, the five patients with moderate cGVHD are still receiving CsA/tacrolimus and/or steroids.

TABLE 2 Clinical outcomes after HST

Variable	N = 103
Median neutrophil recovery, days (range)	14.30 (9-25)
Median platelet recovery, days (range)	25.42 (8-93)
Primary graft failure, no. (%)	1 (0.98%)
Secondary graft failure, no. (%)	1 (0.98%)
Platelet graft failure, no. (%)	2 (1.96%)
Infection, no. (%)	
Pulmonary infections	25 (24.27%)
Septicemia	6 (5.82%)
Mucositis/stomatitis	9 (8.74%)
Liver infection	5 (4.85%)
Soft tissue infection	6 (5.82%)
Viremia	
CMV	93 (90.29%)
EBV	93 (90.29%)
EBV-associated PTLD	6 (5.82%)
Acute GVHD	
II	20 (19.42%)
III	5 (4.85%)
IV	2 (1.94%)
Chronic GVHD	
Mild	27 (26.21%)
Moderate	5 (4.85%)
Severe	0 (0%)
TRM	
Causes of death, no. (%)	
Secondary graft failure	1 (0.98%)
GVHD	3 (2.91%)
Infection	9 (8.74%)
Thrombotic microangiopathy	1 (0.98%)
CNS demyelinating disease	1 (0.98%)
Median follow-up time among living patients, months (range)	40 (1.3-98)

Abbreviations: CMV, cytomegalovirus; CNS, central nervous system; GVHD, graft-vs-host disease; M, bone marrow; PB, peripheral blood; PTLD, post-transplant lymphoproliferative disorder; TRM, transplantation-related mortality.

3.4 | Infectious complications

Among 103 SAA children, 51 patients (49.51%) experienced infections (Table 2). Twenty-five patients suffered from pulmonary infections, six patients suffered from septicemia, nine patients suffered from

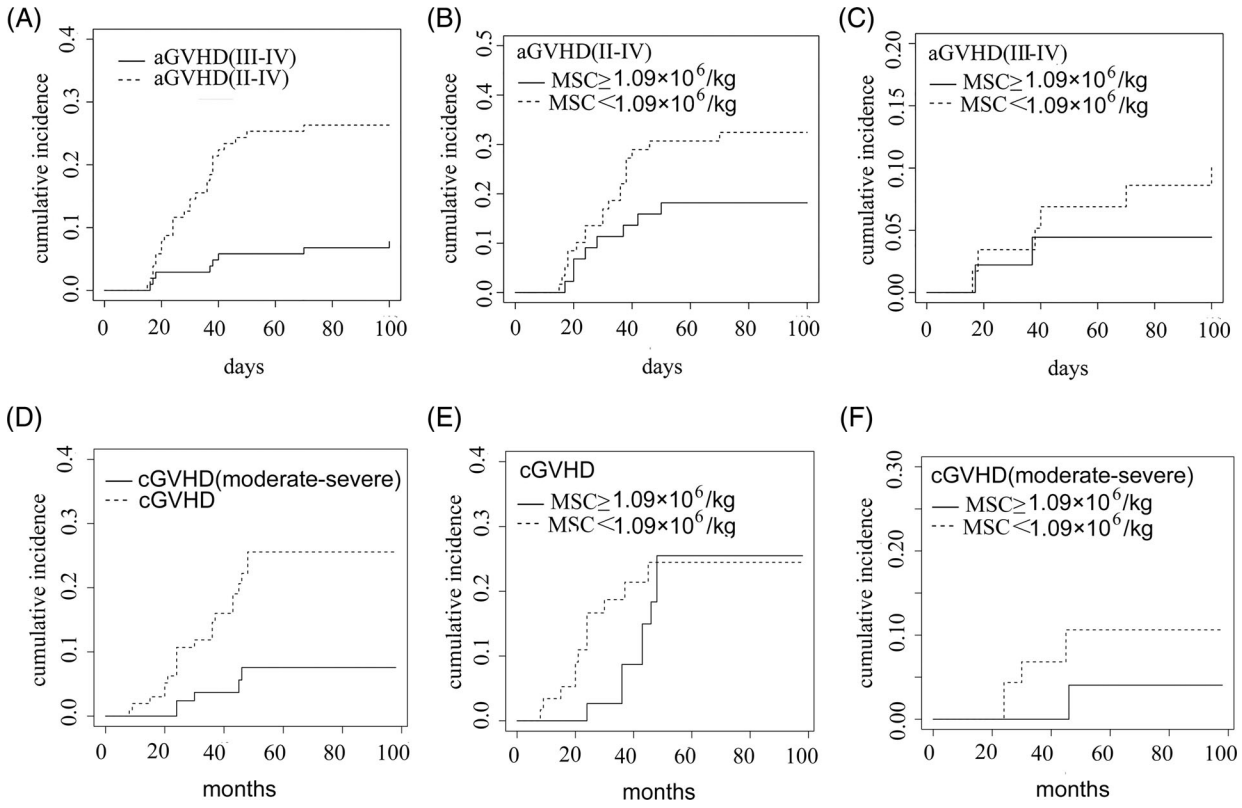

FIGURE 1 Cumulative incidences of aGVHD and cGVHD post the cotransplantation of allogenic MSCs and haploidentical HSC. A, The cumulative incidence of grades II-IV aGVHD and of grades III-IV aGVHD were 26.32% ± 0.19%, and 6.79% ± 0.06% on day +100. B, The cumulative incidences of grades II-IV aGVHD for infusing the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 18.82% ± 6.80% and 32.43% ± 0.38%, respectively (P = .11). C, The cumulative incidences of grades III-IV aGVHD for or the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 4.44% ± 0.09% and 8.62% ± 0.13%, respectively (P = .28). D, The cumulative incidence of cGVHD was 25.56% ± 0.26%, and the cumulative incidence of moderate-severe cGVHD was 7.57% ± 0.11% on month +10. E, The cumulative incidences of cGVHD for the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 25.48% ± 0.64% and 24.47% ± 0.41% (P = .51), respectively. F, The cumulative incidences of moderate cGVHD for the volume of MSC ≥ 1.09×10^6/kg and MSC < 1.09×10^6/kg were 4.05% ± 0.16% and 10.62% ± 0.28% on month +100, respectively (P = .21). aGVHD, acute graft vs host disease. cGVHD, chronic graft vs host disease

mucositis/stomatitis, five patients suffered from liver infection, and six patients suffered from soft tissue infection.

Ninety-three patients (90.29%) experienced CMV reactivation post-transplantation, but no patient developed CMV disease. Ninety-three patients (90.29%) experienced occurred Epstein-Barr virus (EBV) reactivation, and six patients progressed to EBV-associated PTLD (Table 2). All the patients received rituximab treatment and recovered finally.

3.5 | OS and TRM

There are 15 patients died by the August 2019. The major causes of deaths included infection and GHVD (Table 2). The TRM rate was 14.72% ± 3.51%. The causes of TRM included infection in nine cases, GVHD in three cases, GF in one case, CNS demyelinating disease in one case, and thrombotic microangiopathy in one case. A total of 103 patients have survived with an OS was 87.15% ± 3.33% at the follow-up of 40 (1.3-98) months (Figure 2A). The OS for the volume of MSC≥1.09×10^6/kg and MSC < 1.09×10^6/kg were 93.12% ± 3.83% and 81.03% ± 5.16% (P = .08), respectively. There is no difference of OS in patients in the dose range of infused MSC. EFS is as same as OS (Figure 2B).

3.6 | The effects of MSC from different resource on the engraftment, incidence of GVHD, OS, and TRM

To clarify the potential effects of MSC from different sources, the engraftment time, IC of aGVHD, cGVHD, OS, and TRM, separately in group of UC-derived MSC(UC-MSC) and BM-derived MSC (BM-MSC), were analyzed.

The median time of myeloid engraftment in UC-MSC group and BM-MSC group were 14.09 ± 0.36 days and 14.29 ± 0.71 days (P = .79), and the median time of platelet engraftment in UC-MSC group and BM-MSC group were 25.14 ± 2.44 and 30.22 ± 5.60 (P = .33). In addition, the IC of myeloid engraftment (UC-MSC: 96.42% ± 0.17% vs BM-MSC: 93.24% ± 0.09%, P = .81) at D + 21

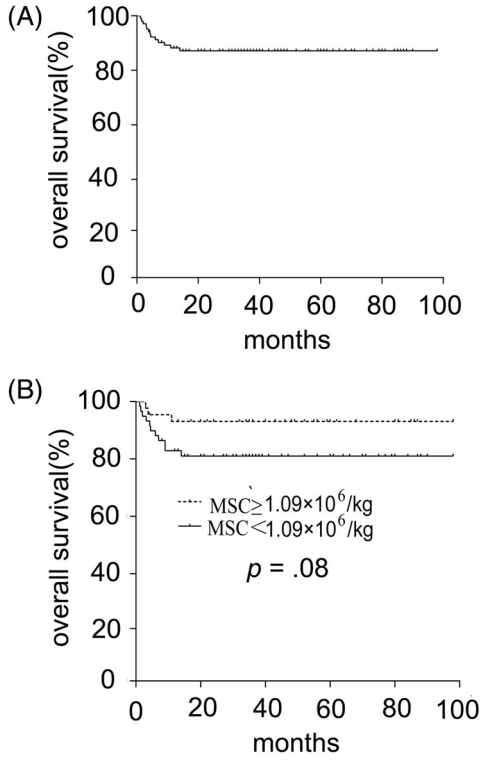

FIGURE 2 OS and TRM mortality post the cotransplantation of allogenic MSCs and haploidentical HSC. A, The TRM rate was 14.72% ± 3.51%. A total of 103 patients have survived with an OS was 87.15% ± 3.3% at the follow-up of 40 (1.3-98) months. B, The OS for the volume of MSC ≥ 1.09 × 10^6/kg and MSC < 1.09 × 10^6/kg were 93.12% ± 3.83% and 81.03% ± 5.16% (*P* = .08), respectively

(Figure 3A), and IC of platelet engraftment (UC-MSC: 97.26% ± 0.04% vs BM-MSC:96.29% ± 0.22%, *P* = .57) in UC-MSC group and BM-MSC group were comparable at D + 100 (Figure 3B).

Furthermore, no significant differences were observed in the IC of aGVHD (II-IV aGVHD, UC-MSC: 28.42% ± 0.27% vs BM-MSC: 21.42% ± 0.62%, *P* = .55; III-IV aGVHD, UC-MSC: 8.07% ± 0.10% vs BM-MSC: 3.57% ± 0.12%, *P* = .43) (Figure 4A,B). and the IC of cGVHD (total cGVHD, UC-MSC: 25.29% ± 0.27% vs BM-MSC: 15.02% ± 017%, *P* = .06; moderate-severe cGVHD, UC-MSC: 6.03% ± 0.08% vs BM-MSC: 4.20% ± 0.19%, *P* = .67) (Figure 4C,D).

Moreover, the OS in UC-MSC group and BM-MSC group patients were 83.88% ± 4.26% and 92.86% ± 4.86% (*P* = .29) (Figure 5A), and the TRM in UC-MSC group and BM-MSC group patients were 22.81% ±1.65% and 14.28% ± 0.86% (*P* = .52) (Figure 5B).

4 | DISCUSSION

In the current study, we report a clinical study of cotransplantation of allogenic MSC and HLA-haploidentical HSC for juvenile SAA. HSCT from a matched related donor (MRD) has been taken as one of the first-line treatments for newly diagnosed young patients with SAA, which reduced the heavy blood transfusion and related rejection, avoided the complications caused by IST, and decreased the risk of hematopoietic malignancies resulting from SAA.[9,45,46] The data from the independent transplant units have demonstrated that halploidentical HSCT also exhibited promising outcome for juvenile patients in SAA. Yang et al compared the outcomes of frontline immunosuppressive therapy and frontline haplo-HSCT for children with

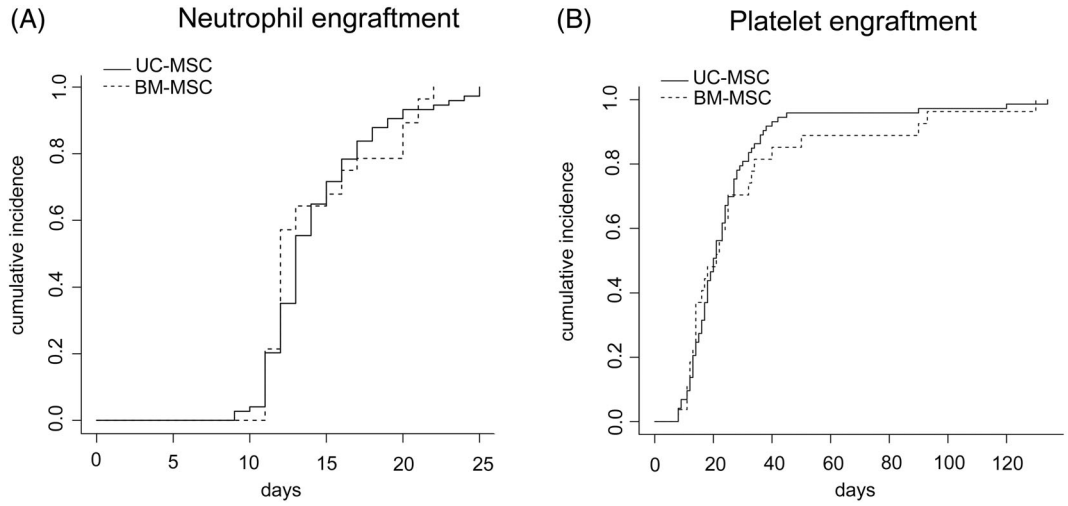

FIGURE 3 The effects of MSCs from different resource on hematopoietic engraftments. A, The median time of myeloid engraftment in UC-MSC group and BM-MSC group were 14.09 ± 0.36 days and 14.29 ± 0.71 days (*P* = .79), and the median time of platelet engraftment in UC-MSC group and BM-MSC group were 25.14 ± 2.44 and 30.22 ± 5.60 (*P* = .33). In addition, the IC of myeloid engraftment (UC-MSC: 96.42% ± 0.17% vs BM-MSC: 93.24% ± 0.09%, *P* = .81) at *D* + 21. B, IC of platelet engraftment (UC-MSC: 97.26% ± 0.04% vs BM-MSC:96.29% ± 0.22%, *P* = .57) in UC-MSC group and BM-MSC group were comparable at *D* + 100

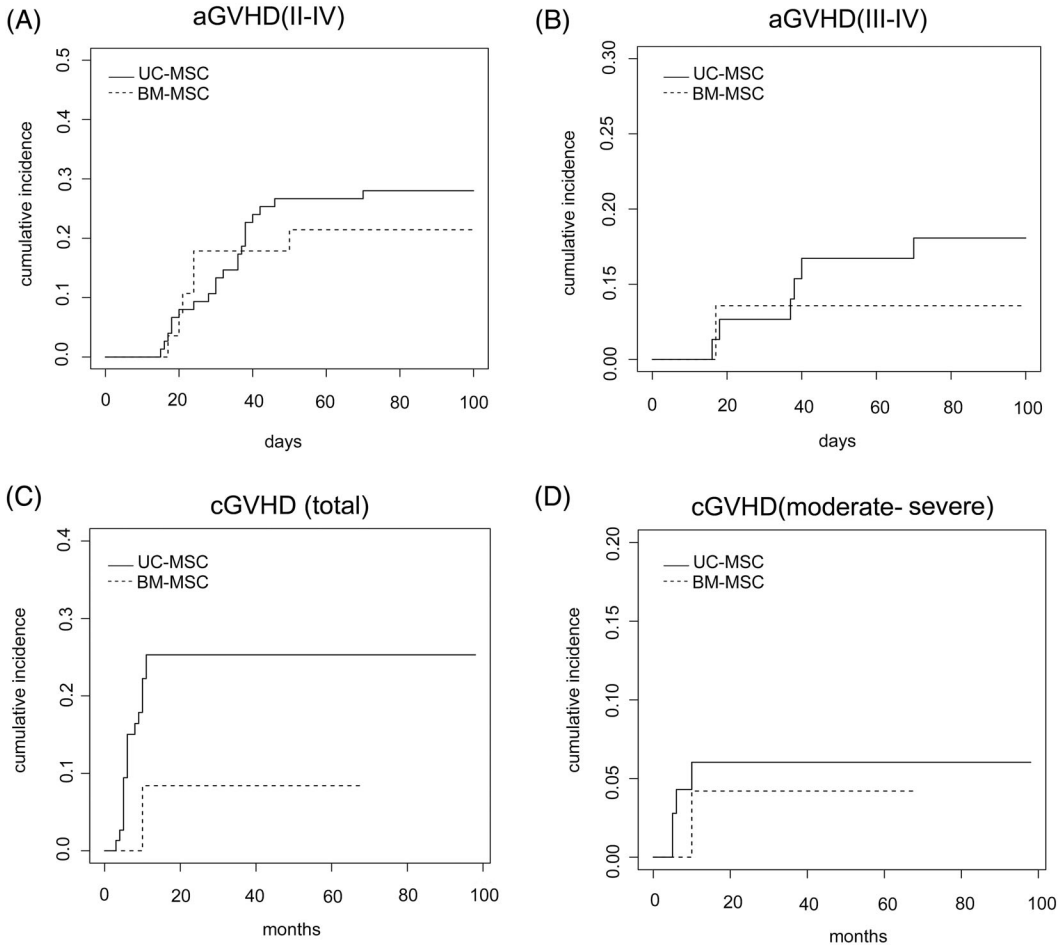

FIGURE 4 The effects of MSCs from different resource on IC of aGVHD and cGVHD. A and B, The IC of aGVHD (II-IV aGVHD, UC-MSC: 28.42% ± 0.27% vs BM-MSC: 21.42% ± 0.62%, *P* = .55; III-IV aGVHD, UC-MSC: 8.07% ± 0.10% vs BM-MSC: 3.57% ± 0.12%, *P* = .43) and C and D, the IC of cGVHD (total cGVHD, UC-MSC: 25.29% ± 0.27% vs BM-MSC: 15.02% ± 017%, *P* = .06; moderate-severe cGVHD, UC-MSC: 6.03% ± 0.08% vs BM-MSC: 4.20% ± 0.19%, *P* = .67) in UC-MSC group and BM-MSC group were comparable

SAA who lack an HLA-matched sibling donor. Based on the analysis of aGVHD, cGVHD, and severe infection of 49 patients with SAA who received frontline IST (n = 9) or frontline haplo-HSCT (n = 20), they suggested that haplo-HSCT may be a better treatment than IST for children and adolescents with SAA who lack an HLA age-matched familial donor.[45] The data is consistent with an independent study performed by Choi et al and of 42 patients with SAA who received frontline IST (n = 19) or frontline HSCT from an alternative donor (n = 23), which also demonstrated alternative donor HSCT may be a better treatment option than IST for children and adolescents with SAA who lack a HLA-matched familial donor.[46] In addition, unmanipulated haploidentical HSCT achieved similar outcomes as matched unrelated donor (MUD) HSCT in young SAA patients, which further identifying it as an effective and safe option for SAA.[18]

Although haplo-HSCT has been proven to have potential in the settlement of young SAA, the higher incidence of GF and GVHD limited its early applications in clinical trials. To overcome it, numerous haplo-HSCT protocols for SAA have been developed in different transplant units. We reported a novel protocol of haploidentical HSCT in treating children with SAA in 2010.[26] Our conditioning regimen consisted of fludarabine, cyclophosphamide and BU, and GVHD prophylaxis was performed by administration of anti-CD25 monoclonal antibody, cyclosporine A, methotrexate, mycophenolate mofetil and antithymocyte globulin. Fludarabine inhibits or eradicates recipient's T cells.[43,47] In addition, we further depleted T cells in vivo using CD25 mono-antibody.[26,41-43] Fludarabine and low doses of BU were used to facilitate donor engraftment in addition to CY/ATG. Most SAA patients who received haploidentical HSCT in our transplant unit achieved rapid hematopoietic engraftment and lower GVHD. Xu et al issued a protocol for SAA in 2012.[48] The conditioning regimen included BU, CY, and thymoglobulin. The GVHD prophylaxis included CsA, mycophenolate mofetil (MMF), and short-term MTX. The source of grafts was a combination of G-CSF-primed BM and G-CSF-mobilized peripheral blood stem cells. The protocol yielded a high engraftment rate and clinical outcome in the settlement of SAA. Recently, Kim et al optimized haploidentical transplantation using selective T cell depletion and conditioning regimens including low-dose total body irradiation for enhancing engraftment.[49] They found

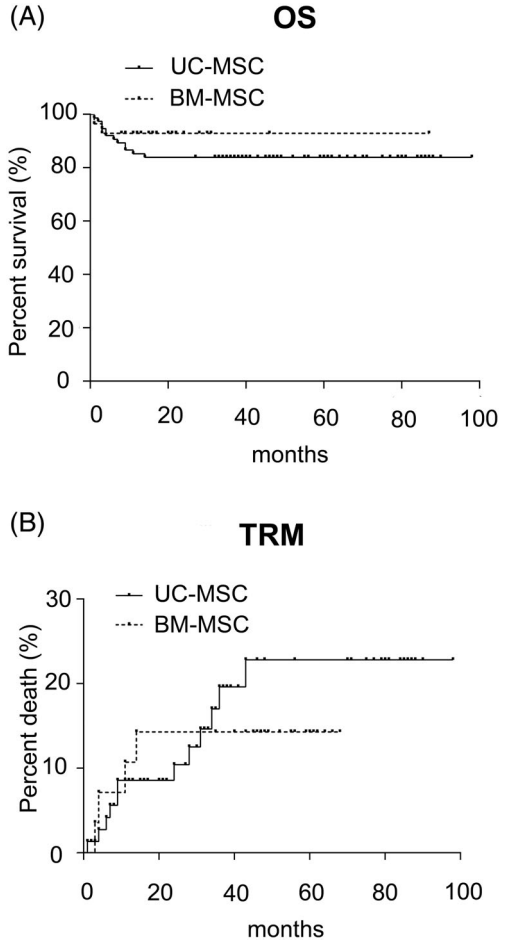

FIGURE 5 The effects of MSCs from different resource on OS and TRM. A, The OS in UC-MSC group and BM-MSC group patients were 83.88% ± 4.26% and 92.86% ± 4.86% (P = .29), and B, the TRM in UC-MSC group and BM-MSC group patients were 22.81% ±1.65% and 14.28% ± 0.86% (P = .52)

that haploidentical HSC could rapidly engraft and reconstitute the recipient hematopoiesis and suggested the protocol may be a realistic therapeutic option for pediatric patients with SAA. Moreover, Clay et al used uniform reduced-intensity conditioning with post graft high-dose cyclophosphamide for refractory SAA.[50] They used G-CSF mobilized PBSCs instead of bone marrow to achieve a high dose of infused $CD34^+$ cells for engraftment. The nonmyeloablative protocol has been met with widespread enthusiasm in Europe and the US.[50-53] However, there is no unified conditioning regimen for haploidentical-HSCT of SAA, and further basic and clinical studies are needed for protocol optimization.

In addition to an intensive conditioning regimen, which includes fludarabine and anti-CD25 mono-antibody, MSC were used in our protocol to promote engraftment and preventing GVHD. MSC are one of pivotal cellular components in bone marrow niche and have been demonstrated useful for promote HSC engraftment in animal experiments and clinical trials. Abbuehl et al reported that cotransplantation of primary bone marrow MSC were capable of repairing niche damage and improved HSCT.[21] They showed that intra-bone transplantation of primary MSC quantitatively reconstituted stroma function in vivo. In addition, MSC cotransplantation doubled the number of functional, donor-derived HSCs and significantly reduced clinically relevant side effects associated with HSCT including neutropenia and humoral immunodeficiency. Wang et al reported clinical data that are consistent to experimental findings.[54] They cotransplanted culture-expanded MSC to 35 children with SAA undergoing haplo-HSCT and observed rapid hematopoietic engraftment (myeloid engraftment 14 days, ranged 10-22 days; platelet engraftment was 18 days, ranged 9-36 days). Beside hematopoietic promotion, the clinical application of MSC infusion have been considered as an effective treatment option to add efficiency and reducing complication partially by suppressing GVHD. Introna et al administered third-party bone marrow-derived MSC into 40 patients (15 children, 25 adults) experiencing steroid-resistant grade II-IV GVHD.[55] No acute toxicity was reported. The overall response rate was 67.5%. The complete response rate was significantly more frequent in patients exhibiting grade II GVHD compared with higher grades. Their report demonstrated that MSC could be safely administered on top of conventional immunosuppression for steroid-resistant GVHD treatment. The data from large-scale meta-analyses also suggest that infusion of MSC could be an acceptable treatment for patients with steroid-refractory acute GVHD. In addition to treating GVHD, MSC have been used taken as one of the GVHD prophylaxis components. Morata-Tarifa et al reported that patients infused with MSC for GVHD prophylaxis showed a 17% increased OS and a reduced incidence of acute GVHD grade IV and chronic GVHD compared with controls.[56] Zhao et al demonstrated that MSC therapy demonstrated substantial improvements in terms of complete response (CR) and OS for cGVHD.[57] The underlying mechanisms of MSC-mediated GVHD prevention remain incompletely understood. MSC exert their immunomodulatory effects on immune cells via direct cell-cell contact, the release of soluble factors, and production of extracellular vesicles.[58,59] Our previous works showed that MSC infusion increased the number of T lymphocytes and decreased the CCR7 expression and proportion of dendritic cells in the secondary lymphoid organs of aGVHD mice.[28] Consequently, MSC delay the development of GVHD by impairing the migration of T and DC to target organs. In addition, CCR7 guided migration of MSC to secondary lymphoid organs.[60] Despite the uncertainties and unanswered questions regarding their mechanism of action, MSC therapy is now considered second-line therapy for steroid-refractory GVHD in many units.

Furthermore, we analyzed the results from published similar study without MSC cotransfusion. In our study, neutrophil engraftment was achieved in 102 of 103 patients in a median time of 14.30 days (range 9-25 days). The median time of platelet engraftment was 25.42 days (range 8-93 days). The CI of II-IV aGVHD at day +100 was 26.32% ± 0.19% and III-IV aGVHD was 6.79% ± 0.06% at day +100, respectively. The CI of cGVHD was 25.56% ± 0.26%. Moreover, the OS was 87.15% ± 3.33% at a median follow-up of 40 (1.3-98) months. Xu et al reported a similar protocol for haplo-HSCT in the treatment of SAA of juvenile and adult patients without MSC infusion.[47] Accordingly, 19 patients achieved 100% donor myeloid engraftment: the

median time for neutrophil engraftment was 12 days (ranging from 10 to 29 days) and for platelets was 18 days (ranging from 8 to 180 days), respectively. However, the CI was 42.1% ± 11.3% for grade II-IV aGVHD and 56.2% ± 12.4% for cGVHD, which is higher than our data. In addition, the OS was 64.6% ± 12.4% with a median 746-day (90-1970) follow-up for surviving patients while the OS in our current study is 87.15% ± 3.3% at a median follow-up of 40 (1.3-98) months. In another study including 89 cases of haplo-HSCT for juvenile and adult SAA patients without MSC cotransplantation, the median time for myeloid engraftment was 12 (range 9-20). The CI of grades II-IV acute graft-vs-host disease (aGVHD) (30.3%), grades III-IV aGVHD (10.1%), and cGVHD (30.6%) are higher than those in our study. The three-year estimated overall survival (OS) rates were 86.1%.[61] The data based on similar but without MSC protocols prove that MSC might contribute to the improvement of haplo-HSCT for SAA. However, we are aware that MSC infusion is not a sufficient factor to yield better results. Numerous factors including patient age and heavy blood infusion might influence the clinical outcomes.

Nevertheless, we must acknowledge several limitations in our study. First, our data are from a single center, and multicenter and randomized studies are required to further confirm the benefits. Second, to determine the cellular and molecular mechanisms that contribute to the therapeutic effects of our protocol, GVHD animal models and high-throughput assays may be helpful to address this issue. Third, the number of SAA patients included in the current study is relatively small, and the clinical case size should be enlarged to reinforce the findings in further studies. Fourth, that quantity of infused MSC may influence CIs of GVHD, and so forth. Further investigations of the relevance of MSC dose and HSCT results should be performed in the future.

5 | CONCLUSIONS

In the present study, we reported the result of a protocol of cotransplantation of haploidentical HSCs and allogenic MSC for young SAA patients. Our study shows fast hematopoietic reconstitution and low incidence of GVHD. However, a comprehensive analysis of underlying mechanisms of the protocol in animal models and high-throughput assays is needed to further investigate and clarify these results.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

L.D.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript; D.-M.H.: provision of study material or patients, collection and/or assembly of data; X.-L.Z.: provision of study material or patients; H.-M.Y., M.X.: collection and/or assembly of data, data analysis and interpretation; J.L., L.Z., S.L.: collection and/or assembly of data; N.M., Z.-K.G., H.-M.N.: conception and design; H.-X.W.: conception and design, data analysis and interpretation, final approval of manuscript; H.Z.: conception and design, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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