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ORIGINAL ARTICLE

Safety and efficacy of mesenchymal stromal cell therapy for multi-drug-resistant acute and late-acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation

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Abstract

Graft versus host disease (GvHD) remains a significant risk for mortality and morbidity following allogeneic hematopoietic stem cell transplantation (HSCT). A growing literature supports successful applications of mesenchymal stromal cells (MSCs) for the treatment of steroid-refractory acute GvHD (aGvHD). However, there is limited knowledge about the effects of MSC treatment on late-acute GvHD (late aGvHD). In this article, we present our multicenter study on the safety and efficacy of MSC therapy for patients with steroid-refractory late aGvHD in comparison to those with aGvHD. The outcome measures include non-relapse mortality (NRM) and survival probability over a 2-year follow-up. The study includes a total of 76 patients with grades III-IV aGvHD ($n=46$) or late aGvHD ($n=30$), who had been treated with at least two lines of steroid-containing immunosuppressive therapy. Patients received weekly adipose or umbilical cord-derived MSC infusions at a dose of median 1.55 (ranging from 0.84 to 2.56) $\times 10^6$/kg in the aGvHD group, and 1.64 (ranging from 0.85 to 2.58) $\times 10^6$/kg in the late aGvHD group. This was an add-on treatment to ongoing conventional pharmaceutical management. In the aGvHD group, 23 patients received one or two infusions, 20 patients had 3–4, and three had ≥ 5. Likewise, in the late aGvHD group, 20 patients received one or two infusions, nine patients had 3–4, and one had ≥ 5. MSC was safe without acute or late adverse effects in 76 patients receiving over 190 infusions. In aGvHD group, 10.9% of the patients had a complete response (CR), 23.9% had a partial response (PR), and 65.2% had no response (NR). On the other hand, in the late aGvHD group, 23.3% of the patients had CR, 36.7% had PR, and the remaining 40% had NR. These findings were statistically significant ($p=0.031$). Also, at the 2-year follow-up, the cumulative incidence of NRM was significantly lower in patients with late aGvHD than in patients with aGvHD at 40% (95% CI, 25–62%) versus 71% (95% CI, 59–86%), respectively ($p=0.032$). In addition, the probability of survival at 2 years was significantly higher in patients with late aGvHD than in the aGvHD group at 59% (95% CI, 37–74%) versus 28% (95% CI, 13–40%), respectively ($p=0.002$). To our knowledge, our study is the first to compare the safety and efficacy of MSC infusion(s) for the treatment of steroid-resistant late aGVHD and aGVHD. There were no infusion-related adverse effects in either group. The response rate to MSC therapy was significantly higher in the late aGvHD group than in the aGvHD group. In addition, at the 2-year follow-up, the survival and NRM rates were more favorable in patients with late aGVHD than in those with aGVHD. Thus, the results are encouraging and warrant further studies to optimize MSC-based treatment for late aGVHD.

Keywords Allogeneic hematopoietic stem cell transplantation · Graft-versus-host disease · Mesenchymal stromal cells

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been shown to cure over 40 different oncologic and non-oncologic illnesses in the last five decades [1]. However, its application is still limited due to the potential of graft versus host disease (GvHD), which can occur in up to 40–80% of

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recipients. The incidence of GvHD is related to several factors, including the degree of HLA mismatch between the donor and recipient, the type of conditioning regimen, and the properties of donor cells used in the transplant. GvHD is divided into acute and chronic forms. Based on the severity and extent of organ involvement, acute GvHD (aGvHD) is categorized into four types, i.e., types I, II, III, and IV, for mild, moderate, severe, and very severe cases, respectively. Similarly, chronic GvHD (cGvHD) is categorized into limited cGvHD and extensive cGvHD based on the severity. As suggested by the 2014 NIH consensus, cGvHD can also be viewed as late aGvHD or cGvHD based on the clinical presentation and time of onset post transplant [2]. Acute GvHD most commonly targets the skin, gastrointestinal system (GIS), and liver [3, 4]. Skin findings include maculopapular rash, and in severe cases, bullous and ulcerative changes. The GI involvement almost always manifests with diarrhea that can be severe with over 2 L a day stool output. The inflammatory changes in the liver lead to jaundice or isolated choleostasis at varying severities that correlates with blood total bilirubin levels. In general, the clinical presentation of de novo late aGvHD is similar to aGvHD with predominant involvement of skin, GIS, and liver tissues [5]. Although there is a large body of literature on management of acute and chronic GvHD, treatment of late aGvHD remains poorly defined [6, 7]. While steroids remain the first-line treatment for all GvHD types, it is well established that 30–50% cases fail to respond. There have been trials of many agents for the treatment of steroid-refractory acute and late aGvHD; however, the response rates and treatment outcomes remain less than optimal [5, 8–11]. This enforces urgency on the unmet need for the development of new preventive measures and therapeutical agents against GvHD [12, 13].

Since the early 2000s, there have been several studies on successful appliactions of mesenchymal stromal cells (MSCs) for the treatment of aGvHD [14–18]. MSCs are pleuropotent stem cells able to differentiate in vitro and in vivo into tissues of mesenchymal origin. It has been well established that these cells promote the growth, differentiation, and engraftment of hematopoietic cells [19]. Also, MSCs have been shown to have immune-modulatory properties in clinical and preclinical models through multifactorial mechanisms on inflamatory milieu and T cells. As a result, once infused into a patient, MSCs can downregulate immune-mediated damage against host target cells and tissues [20]. Several phase II and III trials have put forward growing evidence on the safety and efficacy of MSC treatment for aGvHD. Accordingly, 60–75% of aGvHD patients, refractory to conventional treatment, improved upon MSC treatment [21–25]. We also achieved similar treatment outcomes with MSC for acute and chronic GvHD [26]. Although there is considerable literature on MSCs for the treatment of aGvHD and prophylaxis against cGvHD, there remains a paucity of knowledge on MSCs for treatment of late aGvHD [18, 26, 27]. We now present our results comparing the safety and efficacy of MSC treatment between two patient groups who developed steroid-refractory aGVHD or late aGvHD following HSCT. In addition, we report the non-relapse mortality (NRM) rate and survival probability of these patients over a 2-year follow-up following MSC.

Materials and methods

Regulatory approvals

This is a multicenter prospective study, approved by the Ethics Committee of Erciyes University and the National Stem Cell Council of the Turkish Ministry of Health, for MSC treatment of GvHD. The study was conducted after obtaining signed informed consent from the donors and patients, or their legal guardians. The study was conducted at two medical centers using MSC provided by two independent stem cell laboratories.

Patient characteristics and transplantation procedures

The study involved 83 subjects with steroid-refractory grade III-IV aGvHD or late aGvHD treated with MSCs between September 2016 and May 2021 at Erciyes Transplantation Center, Kayseri and Medstar Hospital, Antalya. Prior to enrollment, all patients were under standard of care for conventional therapeutics as well as assessment of treatment response and diagnosis of steroid resistance for GvHD per current literature [18, 28].

Pre-HSCT conditioning

In transplantations from fully matched donors, the patients received either myeloablative conditioning (MAC) or reduced intensity conditioning (RIC) regimens. The MAC regimen was based on intravenously (i.v.) cyclophosphamide (60 mg/kg/day on days − 8 and − 7), combined mainly with busulfan (3.2 mg/kg/day on days − 5 to − 2), melphalan (90 mg/m^2 on day − 2), and thiotepa (5 mg/kg/day on days − 4 and − 3). The RIC regimen was based on i.v. fludarabine (30 mg/kg/day on days − 6 to − 3), combined with busulfan (3.2 mg/kg/day on days − 6 to − 3), rabbit anti-thymocyte globulin (r ATG; 5 mg/kg/day on days − 2 and − 1).

In haploidentical transplantations, again the conditioning regiments included MAC and RIC. Accordingly, the MAC regimen was based on i.v. fludarabine (30 mg/kg/day on days − 7 to − 5) combined with cyclophosphamide (50 mg/kg/day on days + 3 and + 4), and 12 Gy TBI. Finally, the RIC regimen

was based on i.v. fludarabine (30 mg/kg/day on days − 6 to − 2) combined with cyclophosphamide (14.5 mg/kg/day on days − 6 and − 5), and 2 Gy total body irradiation (TBI).

GvHD prophylaxis

All patients who underwent matched HSCT were treated with cyclosporine A (CsA) and methotrexate (MTX). The CsA was given at 3.0 mg/kg/day infused i.v. on day − 1 followed by 5 mg/kg/day by mouth until day + 180 with dose adjustments based on the serum through levels. The dose of MTX was 15 mg/m^2 i.v. on days + 1, then 10 mg/m^2 i.v. on days + 3 and + 6 after transplantation.

All patients who underwent haploidentical HSCT were treated with a combination of CsA and mycophenolate mofetil (MMF). CsA prophylaxis was the same as described above except it was started on day + 4 following HSCT. In this group, the dose of MMF was + 15 mg/kg TID by mouth (max 3 g/day) between day + 5 and day + 35 following transplantation, and 15 mg/kg BID between day + 4 and day + 35 following transplantation for those treated with MAC regimen and those treated with RIC regimen, respectively.

In addition, all patients received ursodeoxycholic acid for prophylaxis against liver GvHD. Routine antimicrobial prophylaxis included moxifloxacin or levofloxacin against bacterial infection, trimethoprim/sulfamethoxazole against *Pneumocystis jiroveci*, acyclovir or valacyclovir against viral infections, and fluconazole against fungal infection.

Diagnosis and grading of GvHD

Acute and late aGvHD were diagnosed and graded according to recent international criteria [2, 29, 30] as follows: aGvHD was defined as features of aGvHD with onset before day + 100 after HSCT, and late aGvHD was defined as features of aGvHD observed after day + 100. Also, late aGvHD was classified as de novo if the new onset of symptoms and signs of aGvHD were seen after day 100; recurrent, if there was a recurrence of previously resolved aGvHD after day 100; or persistent, if persistent symptoms and signs of aGvHD were seen after day 100 without prior resolution. The patients who had a diagnosis of acute and de novo late aGVHD were involved in the study.

Tissue biopsy

If clinically indicated, biopsy samples from the gastrointestinal tract, skin, and liver were obtained in selected patients for diagnostic or prognostic purposes. Samples were processed per routine and reviewed by staff pathologists. Apoptosis in the basal layer of the epidermis, apoptotic enterocytes in the crypt, and degenerative changes in interlobular bile ducts were used as the minimal diagnostic criteria for skin, gastrointestinal, and liver GvHD, respectively, according to NIH consensus criteria [31]. The presence of extensive destruction epithelium, crypts, ducts, and severe inflammation were accounted for severe GvHD.

Treatment of GvHD prior to MSC administration

Patients diagnosed with acute or de novo late aGvHD were treated with oral prednisone (2 mg/kg daily, or 60 mg/m^2/day or methylprednisolone i.v. equivalent) as the initial therapy for 2 weeks, which was tapered over the next 8 weeks. In accordance with the literature, all patients received at least two lines of steroid-containing immunosuppressive therapy before MSC administration, and the treatment was continued during MSC therapy [18, 28]. Steroid refractoriness was defined as no response during 5 days or progression within 3 days after treatment with at least 1 mg/kg body weight of methylprednisolone equivalent. First-line treatment is defined as the addition of steroids to the ongoing prophylactic immunosuppressive regimen, and second-line treatment is defined as the addition of MMF, tacrolimus, and/or extracorporeal photochemotherapy. Lastly, third-line treatment is defined as the addition of ruxolitinib or imatinib. No-response treatment was defined as non-improvement or getting worse in the GvHD phase and/or its symptoms.

MSC isolation and characterization

All procedures involved in donor tissue collection, manufacturing, and testing of MSCs were carried out according to good manufacture practice (GMP) protocols authorized by the Turkish Ministry of Health. MSCs were derived from two sources: umbilical cord or adipose tissue from unrelated, HLA-mismatched adult donors at the Genome and Stem Cell Center of Erciyes University (GENKOK), Kayseri, Turkey and ATIGEN-CELL, Antalya, Turkey. MSCs were isolated and cultured as previously described [26]. Briefly, MSCs were isolated by enzymatical digestion and cultured in human MSC growth medium (consisting of alpha-modified Eagle's medium with 1% penicillin–streptomycin, 1% L glutamine, and 10% human serum). Adherent cells were further cultured with media changes every 3 days. When they were 70–80% confluent, cells were detached by trypsin–EDTA and passaged at a ratio of 1:3. Third-passage MSCs were used for all patients. For release testing, MSCs were assessed for cell appearance, viability, identification, purity, and potency and were screened for contamination. Flow cytometry analyses were performed using Navios (Beckman Coulter, USA) and analyzed with the KALUZA software (Beckman Coulter). The culture-expanded cells expressed CD44, CD73, CD90, and CD105, but not CD11a, CD34, or CD45 (BD Stem Flow hMSC kit, BD). Aliquats

of cells were stored in − 80 nitrogen tanks until thawing on the same day of treatment.

Treatment of GvHD with MSCs

As stated above, all patients received at least two lines of steroid-containing immunosuppressive therapy before the initiation of weekly MSC infusion as an add-on treatment. Decisions on the length and number of MSC infusions were personalized by the medical team based on the patient's response and clinical condition.

MSC infusion protocol included pretreatment with H1-receptor and H2-receptor antagonists. Single-cell suspension of MSCs (viability > 95%) in 50 mL isotonic sodium chloride solution was infused i.v. over 5–10 min under close observation. Patients remained under continuous monitoring for 2 h following treatment.

Assessment of MSC treatment for safety

Patients were evaluated for safety until the time point of death, withdrawal, or 90-day follow-up from the first MSC infusion for adverse events and serious adverse events (AEs and SAEs) per Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. (https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_8.5x11.pdf).

Assessment of MSC treatment for efficacy

For patients with acute and late-acute GvHD, the response to MSC treatment was defined according to the published guidances [14, 33]: For aGvHD, complete response (CR) was defined as the absence of GvHD signs. Partial response (PR) was defined as at least one-grade decrease in aGvHD symptoms and signs when compared with day 0. No response (NR) was defined as no change in aGvHD grade. For late aGvHD, CR was defined as the resolution of all clinical manifestations of late aGvHD in all the involved organs, except the irreversible injury. PR was defined as global assessment improvement by at least one point or at least a 50% improvement of clinical manifestations but without CR, and NR was defined as no improvement or deterioration of all affected organs.

Statistical analysis

Histogram, q-q plots, and Shapiro–Wilk's test were applied to assess the data normality. The Levene test was used to test variance homogeneity. To compare the inter-group differences, independent sample *t*-test or Mann–Whitney *U* tests were performed for continuous variables, while Pearson chi-square tests or Fisher exact tests were performed for categorical variables. The Kaplan–Meier method was used to estimate the survival probabilities between acute and late-onset groups, as well as between responders and non-responders. The log-rank test was used to compare these survival probabilities between groups. The Pepe and Mori test was used to compare acute and late-onset groups while taking into account the competing risks using the cumulative incidence rates. The cumulative incidence of NRM was calculated using relapse or disease progression of the underlying malignancy as competing risk. Analyses were performed using the TURCOSA (Turcosa Analytics Ltd. Co., www.turcosa.com.tr) and R 4.0.1 (www.r-project.org) statistical software. A *p*-value less than 5% was considered statistically significant.

Results

Baseline demographics and transplantation characteristics

This is a multicenter, prospective study on steroid-refractory grade III-IV aGvHD or late aGvHD. A total of 83 patients were screened: Among those, 37 patients had late aGvHD. This included three persistent and four recurrent late aGvHD patients; to prevent confusion on diagnosis, these seven patients were excluded. As a result, only 30 patients with de novo late GvHD were included in the study. Thus, our study cohort was composed of a total of 76 patients: 46 with aGvHD and 30 with late aGvHD.

Table 1 describes the demographics and transplantation characteristics of patients. The median age was 40 years (ranging from 18 to 69) in the aGvHD group and 39 years (ranging from 19 to 69) in the late aGvHD group. There were 25 (54.3%) female in the aGvHD group, and 14 (46.7%) in the late aGvHD group. Among patients with aGvHD, 28 (60.9%) had undergone matched related HSCT, 11 (23.9%) haploidentical HSCT, and 7 (15.2%) matched unrelated HSCT. Among patients with late aGvHD, 23 (76.7%) had undergone matched related HSCT, 4 (13.3%) haploidentical HSCT, and 3 (10%) matched unrelated HSCT. The source of hematopoietic stem cells was either peripheral blood (PBSC), which was used for 87% and 93.3% of aGvHD and late aGvHD patients, respectively, or bone marrow, which was used for 13% and 6.7% of aGvHD and late aGvHD, respectively. Overall, in the aGvDH group, 33 (71.7%) patients received MAC and 13 (28.3%) received RIC regimen, and in the late aGvHD group, 25 (83.3%) patients received MAC and 5 (16.7%) received RIC regimen (Table 1). In both groups, GvHD prophylaxis mostly comprised CsA plus MTX. No major differences were seen in the baseline or HSCT characteristics of the two groups.

Table 1 Baseline demographics and transplantation characteristics

Variable	Type of GvHD		*p*
	aGvHD (*n* = 46)	Late aGvHD (*n* = 30)	
Age (years)	40 (18–69)	39 (19–69)	0.890
Gender (female)	25 (54.3)	14 (46.7)	0.513
Diagnosis			0.089
Acute leukemia	36 (78.3)	26 (86.7)	
Chronic leukemia	2 (4.3)	0	
Malign lymphoma	1 (2.2)	3 (10)	
Other	7 (15.2)	1 (3.3)	
Donor age (years)	38 (18–63)	41 (20–61)	0.704
Donor gender (female)	12 (26.1)	14 (46.7)	0.065
Gender match	40 (86.9)	23 (76.6)	0.296
Donor type			0.449
HLA-matched sibling	28 (60.9)	23 (76.7)	
Haploidentical relative	11 (23.9)	4 (13.3)	
HLA-matched unrelated	7 (15.2)	3 (10)	
Graft type			0.287
PBSC	40 (87)	28 (93.3)	
Bone marrow	6 (13)	2 (6.7)	
CMV serostatus			0.244
Negative in donor and recipient	0	2 (6.7)	
Positive in donor and recipient	40 (87)	23 (76.7)	
Positive in donor	2 (4.3)	2 (6.7)	
Positive in recipient	4 (8.7)	3 (10)	
Conditioning regimen			0.245
MAC	33 (71.7)	25 (83.3)	
RIC	13 (28.3)	5 (16.7)	
GvHD prophylaxis			0.337
CsA + MTX	35 (76.1)	25 (83.3)	
CsA + MMF	6 (13)	3 (10)	
Ex vivoT cell depletion	5 (10.9)	2 (6.7)	

Data are presented as *n* (%), median (range). Abbreviations: *GvHD* graft-versus-host disease; *aGvHD* acute graft-versus-host disease; *HLA* human leukocyte antigen; *PBSC* peripheral blood stem cells; *CMV* cytomegalovirus; *MAC* myeloablative conditioning; *RIC* reduced intencity conditioning; *CsA* cyclosporine A; *MTX* methotrexate; *MMF* mycophenolate mofetil

Table 2 Graft-versus-host disease details and previous therapy counts

Variable	Type of GvHD		*p*
	aGvHD (*n* = 46)	Late aGvHD (*n* = 30)	
Skin GvHD total	27 (58.7)	15 (50.0)	0.456
Grade 3	4 (14.8)	0	
Grade 4	23 (85.2)	15 (100.0)	
Liver GvHD total	19 (41.3)	10 (33.3)	0.484
Grade 3	12 (63.2)	8 (80.0)	
Grade 4	7 (36.8)	2 (20.0)	
GIS GvHD total	32 (69.6)	17 (56.7)	0.251
Grade 3	11 (34.4)	5 (29.4)	
Grade 4	21 (65.6)	12 (70.6)	
Previous therapies			0.406
First line	46	30	
Second line	35	28	
Third line	10	7	

Data are presented as *n* (%). Abbreviations: *GvHD* graft-versus-host disease; *aGvHD* acute graft-versus-host disease; *GIS* gastrointestinal system

GvHD characteristics

The median time from HSCT to GvHD was 46 days (ranging from 13 to 96) in the aGvHD group, and 136 days (ranging from 100 to 572) in the late aGvHD group. Table 2 shows the organ involvement, the grade level, and the number of prior treatments for GvHD. In the aGvHD group, 27 (58.7%) patients had skin GvHD, 32 (69.6%) presented with GIS GvHD, and 19 (41.3%) had GvHD of the liver. In the late aGvHD group, 15 (50%) patients had skin GvHD, 17 (56.7%) patients presented with GIS GvHD, and 10 (33.3%) had GvHD of the liver. There was no statistically significant difference regarding organ involvement or grade between aGvHD and late aGvHD groups. Forty-five patients had biopsies for the evaluation of GvHD to yield a total of 72 tissue biopsies (22 liver biopsies, 22 colon biopsies, 21 skin biopsies, 4 duodenum biopsies, and 3 stomach biopsies). As shown in Fig. 1A, 1B, and 1C, the pathology review showed histologic features of GvHD in 22 out of the 72 (31%) samples. These included evidence of severe GvHD in 2/22 liver, 2/22 colon, 7/21 skin, and ¼ duodenal tissues reviewed (Fig. 1D).

All patients were poor responders to the first-line (steroid) treatment that was started routinely upon the onset of aGvHD or late aGvHD. In the acute GvHD group, 35 patients had second-line treatment, and 10 patients had third-line treatment before the MSC therapy. Also, in the late aGvHD group, 28 patients had second-line, and 7 patients had third-line treatment. The second and third-line therapies varied based on the type of transplantation of patients, clinical presentation of the GvHD, and the prophylaxis medications. Tacrolimus or extracorporeal photochemotherapy, for instance, was added as second-line treatments for patients using MMF for prophylaxis. Tacrolimus or MMF was administered as a second-line treatment to patients who did not use MMF prophylactically. In the aGvHD group, ruxolitinib was administered to seven patients and imatinib to three patients as a third-line therapy. Six patients in the late aGvHD group received ruxolitinib, and one patient received imatinib as a third-line therapy. There was no statistically significant difference regarding to the pre-MSC treatment lines between the groups.

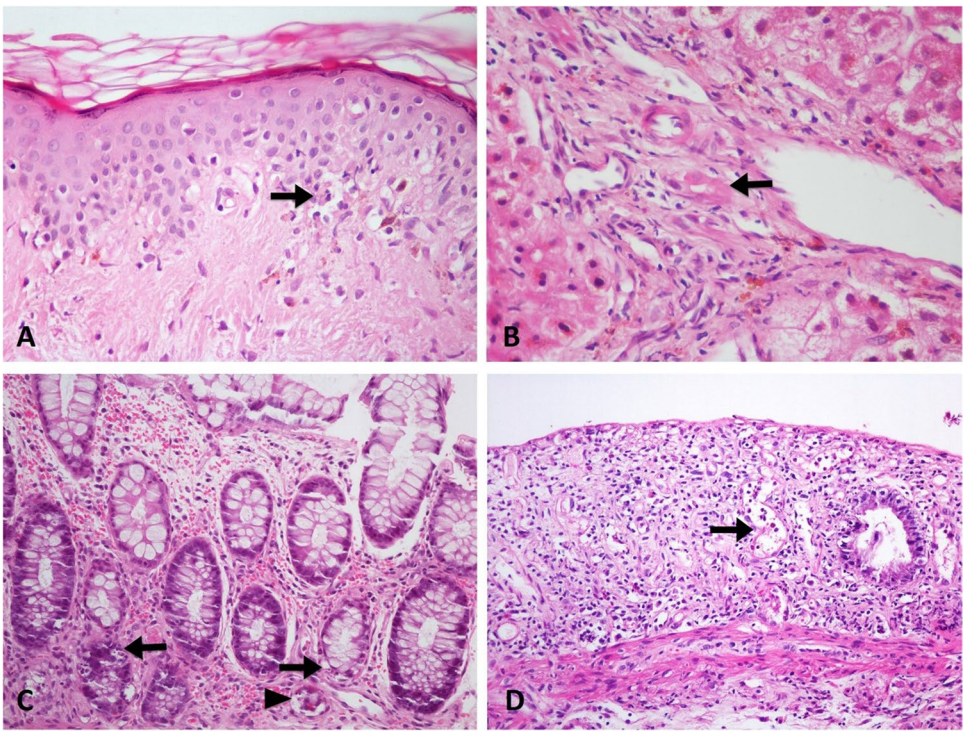

Fig. 1 Skin GvHD. Epidermis showing basal apoptotic bodies (arrow) and vacuolisation in basal layer (hematoxylin and eosin, × 100). **B** Hepatic GvHD. Bile duct showing destructive changes (arrow) with cytoplasmic eosinophilia and nuclear dispolarity (hematoxylin and eosin, × 100). **C** Gastrointestinal GvHD. Colon crytps showing apoptotic bodies (arrows) and crypt loss (arrowhead) (hematoxylin and eosin, × 100). **D** Severe gastrointestinal GvHD. Extensive crypt loss (arrow) in colonic mucosa (hematoxylin and eosin, × 100)

MSC treatment and response

Table 3 shows the determinants of the MSC treatment. In total, 194 MSC infusions were given, consisting of 135 adipose-derived MSCs (ASCs) (88 infusions for aGvHD and 47 for late aGvHD) and 59 umbilical cord MSCs (UC-MSCs) (42 infusions for aGvHD and 17 for late aGvHD). The median numbers of MSC infused were 1.55 (ranging from 0.84 to 2.56) × 10^6/kg/weekly in the aGvHD group, and 1.64 (ranging from 0.85 to 2.58) × 10^6/kg weekly in the late aGvHD group. Within the aGvHD group, 23 patients received one or two infusions, 20 had 3–4, and three had ≥ 5 infusions. Within the late aGvHD group, 20 patients received one or two infusions, nine had 3–4, and one had ≥ 5. No infusion-related adverse effect or toxicity was observed in any of the treated patients.

There was no statistically significant difference between the groups in terms of MSC source, dose, or the number of infusions. Figure 2 shows the response rates to MSC treatment. In the aGvHD group, 10.9% of the patients had CR, 23.9% had PR, and 65.2% had NR. On the other hand, in the late aGvHD group, 23.3% of the patients had CR, 36.7% had PR, and the remaining 40% had NR. These findings were statistically significant ($p = 0.031$).

Table 3 Mesenchymal stem cell administration

Variable	Type of GvHD		*p*
	aGvHD ($n = 46$)	Late aGvHD ($n = 30$)	
MSC dose (× 10^6 cells/kg)	1.55 (0.84–2.56)	1.64 (0.85–2.58)	0.265
MSC source			0.382
Adipose tissue	31	22	
Umbilical cord	15	8	
Number of MSC infusions			0.343
1–2	23	20	
3–4	20	9	
≥ 5	3	1	

Data are presented as *n* (%), median (range). *MSCs* mesenchymal stem cells

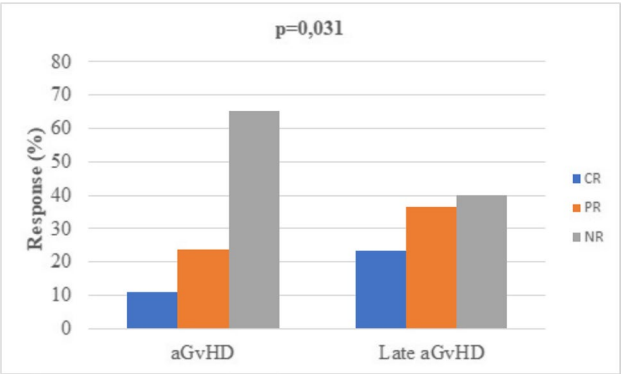

Fig. 2 Response rates to MSC treatment. CR, complete response; PR, partial response; NR, no response

NRM and survival analysis

As shown in Fig. 3 and Fig. 4, the long-term outcomes at the 2-year follow-up following MSC treatment were better for the late aGvHD than the aGvHD group. This was evident as the cumulative incidence of NRM was significantly lower in the late aGvHD group [40% (95% CI, 25–62%)] than that in the aGvHD group [71% (95% CI, 59–86%)] ($p = 0.032$) (Fig. 3). Similarly, the probability of survival was significantly higher among patients with late aGvHD [59% (95% CI, 37–74%)] than among patients with aGvHD [28% (95% CI, 13–40%)] ($p = 0.002$) (Fig. 4).

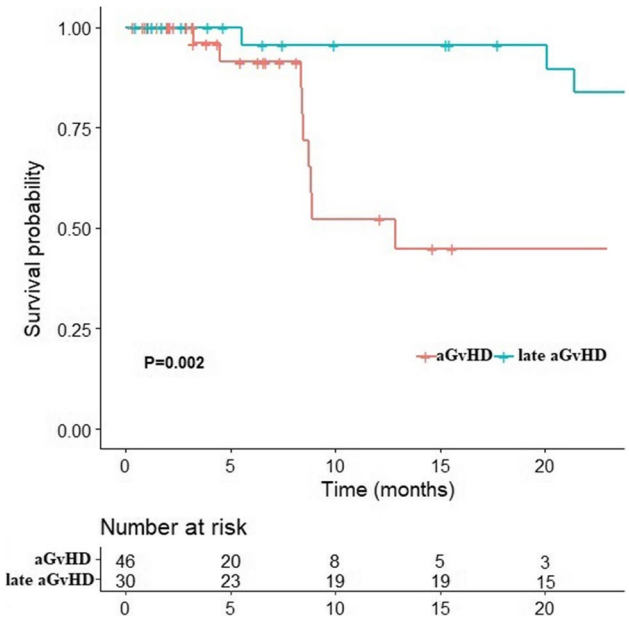

Fig. 4 Two-year survival estimates of patients. Survival probability was 59% (95% CI, 37–74%) for the late aGvHD group and 28% (95% CI, 13–40%) for the aGvHD group

Discussion

This study was conducted at two major bone marrow transplant centers in Turkey serving over 200 patients a year. All treatment regimens used before or after HSCT, including those for steroid-resistant GvHD, are standardized thourghout Turkey based on published international guidelines, under the premises of Turkish Ministry of Health. This allowed comparable screening, assessment, and monitoring to minimize inter-observer variability across the study sites.

There has been growing literature on the MSC treatment of aGvHD following the pioneering report by Le Blanc et al. in 2004 [17]. Since then, many studies have confirmed beneficial effects of MSCs with response rates reaching 60–75% among patients with severe aGvHD [18, 22, 32]. This inspired exploratory studies for the cell-based treatment of cGvHD, those often suffer from poor outcomes due to the lack of standard second-line or salvage therapy if they fail to improve on steroids [33]. Among the limited number of studies, Weng et al. reported 19 patients with cGvHD, who were treated with MSCs resulting in a response rate of 73.7% ($n = 14$; 4 CR and 10 PR) and a 2-year survival rate of 77.7% [27]. Likewise, Peng et al. reported that among 23 patients with refractory cGvHD, 20 achieved CR or PR after treatment with MSC [34]. We also reported similar findings following MSC treatment of steriod-resistant aGVHD and

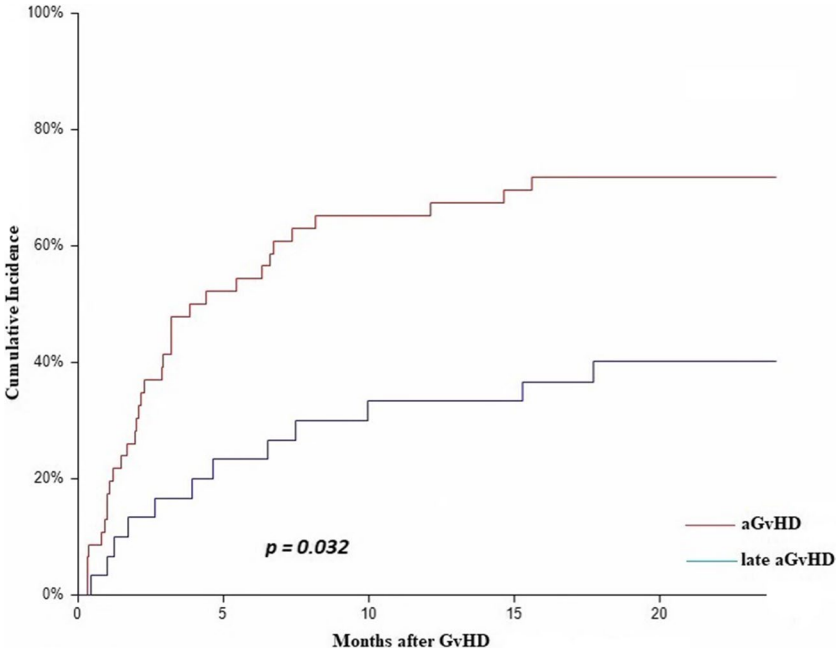

Fig. 3 Two-year cumulative incidence of NRM. NRM was 40% (95% CI, 25–62%) among the late aGvHD group and 71% (95% CI, 59–86%) among the aGvHD group

cGvHD; i.e., a response rate of 79% ($n=15$, 9 CR, 6 PR) and 80% ($n=4$, 2 CR, 2 PR), respectively [26]. None of these studies stratified data based on subtypes of cGvHD. Upon encouraging results, the current study was undertaken to address this knowledge gap for safety and efficacy of MSC-based treatment for late aGvHD.

The presented treatment outcomes showed a favorable impact of MSC treatment particularly on late aGvHD compared to aGvHD. This was evident for both short-term (CR, PR) and long-term (survival, NRM) outcomes. Currently, the insigth into the mechanisms of improvement by cell-based treatment is limited, but it is likely to center on multifactorial immune-modulatory, angiogenic, and tropic effects [35]. In aGvHD, activation of donor antigen-presenting cells, neutophils, and natural killer (NK) cells leads to T cell activation and perpetuation of proinflammatory cytokines, including tumor necrosis factor-alfa (TNF-α), interleukin (IL)-1, IL-2, IL-6, and interferon-ϒ (IFN-ϒ). This is counfounded with the involvement of somatic tissue factors (including ST2 and Reg3) and pattern recognition receptors (including damage-associated molecular patterns or DAMP) resulting in endothelial activation and inflammatory changes in target tissues. In contrast, there is limited literature on the immunopathogenesis of late aGvHD: It has been shown that these patients develop increased numbers of unswitched memory B cells and NK cells, along with decreased numbers of cytotoxic T cells (CTL) and PD-1 memory Treg cells, and they differ from aGvHD on selected tissue factors [4, 36–39].

Currently, there is no targeted treatment to dampen tissue pathology, and the presence of steroid resistance can often be an omnious sign of poor outcomes. MSCs can downregulate both innate and specific immune systems, and they are likely to downregulate key factors involved in the perpetuation of inflammation, including cells (such as NK and dendritic cells) and cytokines (such as IL-2, IL-15, and IFN-ϒ). This is in tandem with increased regulatory mechanisms including increased T regulatory (Treg) cells and polarization of macrophages from proinflammatory (M1) to antiinflammatory (M2) phenotype. This results in direct and indirect inhibition of specific immunity and activities of CTL and B cells [40–42]. MSCs are known to promote angiogenesis and tissue tropism toward homeostasis, at large, by releasing paracrine factors [43]. Our results are novel in that they provide head-to-head comparisons of treatment responses between the subtypes of GvHD that bears a direct impact on clinical applications. The results also emphasize the importance of MSCs as an investigational tool. Furthermore, our results support the view that the pathogenesis of aGvHD and late aGvHD must have unique properties for each, although they share similar clinical and histopathological findings.

Recently, new approaches have been encountered in the treatment of MSC in steroid-resistant GvHD. Kuçi et al. developed a novel approach by generating MSCs from pooled bone marrow mononuclear cells of eight healthy "3rd-party" donors, and they reported that those MSC products increased the overall response rates in GvHD by up to 77% [44]. On the other hand, case series demonstrating that decidua stromal cells derived from the placenta are effective in this group have also been reported [45, 46].

In conclusion, MSCs are safe and effective in the treatment of multi-therapy-resistant late aGvHD. So far, there has been no consensus or published guidelines on applications of MSCs for the timing or numbers of infusions. Further studies are warrented to confirm our findings and develop treatment protocols using cell-based treatment. Furthermore, based on our long-term outcomes, it was evident that the sustainability of homeostasis induced by MSCs was more stable for late aGVHD than aGVHD. Thus, incoorporating omics in future MSC trials can help gain knowledge on the mechanisms of improvement and the discovery of predictive biomarkers.

Data Availability The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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