Allogeneic mesenchymal stem cell therapy for refractory cytopenias after hematopoietic stem cell transplantation

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BACKGROUND: Posttransplant cytopenias are a severe complication after allogeneic stem cell transplantation (allo-SCT) and their origin is often multifactorial or unknown. They are frequently refractory to standard therapy, which may include steroids and/or immunoglobulins. Mesenchymal stem cells (MSCs) are an attractive therapeutic tool in the allo-SCT setting for the ability to enhance engraftment as well as acting as immunosuppressants for graft-versus-host disease. There is no prior experience in the literature of the use of MSCs to treat cytopenias after allo-SCT.

CASE REPORTS: In this work we report for the first time four cases of refractory posttransplant cytopenias (three patients with thrombocytopenia and one with neutropenia) that were treated with MSCs from a third-party donor. MSCs were expanded from 100 mL of marrow obtained under standard good manufacturing practice conditions. Most patients received more than one cell dose, and median dose of MSCs administered was 1×10^6/kg.

RESULTS: All patients recovered normal blood counts, with a mean follow-up of 12.5 months. There were no adverse events related to MSC administration.

CONCLUSION: MSC therapy may contribute to the recovery of refractory posttransplant peripheral cytopenias in patients undergoing allo-SCT.

Peripheral blood (PB) cytopenias after allogeneic hematopoietic stem cell (HSC) transplantation have been attributed to several factors, including graft failure, relapse of underlying disease, stromal damage, cytomegalovirus (CMV) infection or therapy, hemolysis, anemia of chronic disease, or autoimmune diseases, including graft-versus-host disease (GVHD).[1,2] Autoimmune cytopenias after HSC transplantation are generally expressed by monolineage or bilineage cytopenias rather than a generalized pancytopenia,[3] and the ultimate mechanism of action is not well established, although cellular immunity may play a role. In addition, marrow assessment may not show a reactive hypercellular response as occurs in the nontransplant setting, since the hematopoietic niche could be also a target of GVHD.[4] Some authors have recently shown that autoimmune cytopenias are more frequent in young patients undergoing umbilical cord blood transplantation, although it can be observed in adult patients receiving either marrow or PB HSCs.[5] Regarding treatment, steroids are generally the first choice.[1] Nevertheless, for

ABBREVIATIONS: allo-SCT = allogeneic stem cell transplantation; AML = acute myeloid leukemia; CR = complete remission; HSC(s) = hematopoietic stem cell(s); MSC(s) = mesenchymal stem cell(s); PB = peripheral blood.

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refractory patients, several options have been assessed, including immunoglobulins[6,7] and rituximab.[8,9] However, there is a number of cases where responses to the above-mentioned therapies are not maintained, or even not achieved at all, and in this situation further therapeutic tools are needed.[3]

Mesenchymal stem cells (MSCs) are nonhematopoietic precursors that are present in a small proportion of marrow mononuclear cells (MNCs; between 0.01 and 0.001%), although they have a high expansion ability "in vitro." In addition, they have multidifferentiation capacity into cells of the stromal lineage (mainly osteoblasts, adipocytes, and chondrocytes) under appropriate culture conditions.[10] On top of this, and maybe most importantly, they have immunomodulatory properties and are able to inhibit lymphocyte activation and are themselves immune privileged.[11] For all these reasons they are an attractive therapeutic tool not only in regenerative medicine but also in immune-based diseases. Thus, in the transplant setting, they have a potential therapeutic role in graft failure, GVHD, and other complications (e.g., hemorrhagic cystitis).[12] Nevertheless, their therapeutic use for posttransplant cytopenias has not been reported yet. In this article we report for the first time the outcome of four patients who developed PB cytopenias after allo-SCT and were successfully treated with marrow-derived MSC.

MSC ISOLATION AND EXPANSION

MSCs were obtained from marrow of healthy donors, after informed consent was obtained. Only in Case 1 was the donor related (patient's father). In the remaining three cases MSCs were from an unrelated third-party subject different from the HSC donor. MSCs were isolated and expanded in the good manufacturing practice facility of the Hospital Universitario de Salamanca as previously described.[13] Briefly, 100 mL of marrow from the donors was obtained under propofol sedation in the surgical room under strict sterile conditions. Then, in our good manufacturing practice facility, MNCs from marrow were isolated by a density-gradient centrifugation (Ficoll-Paque, GE Healthcare Bio-Sciences, AB, Uppsala, Sweden) and were resuspended and plated in noncoated 175-cm^2 polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) in modified Eagle's medium-α with 1% penicillin-streptomycin (Gibco, Paisley, UK) at a concentration of 160,000 cells/cm^2. The medium was supplemented with 5% of PLT lysate.[14] The latter was obtained from pooled PLTs frozen at –80°C and then thawed at 37°C. They were further centrifuged and the supernatant (PLT lysate) was used for MSC expansion. Cells were incubated at 37°C in an atmosphere with 90% humidity and 5% CO_2. The medium was completely replaced twice a week. The first passage was performed when cells reached a confluence of 80% (Days 10-15). MSCs were then replated at a concentration of 1000 to 5000 cells/cm^2 and passaged when 80% confluence was reached.[15]

Cells were characterized by flow cytometry and differentiation assays. For flow cytometric analysis, 200,000 MSCs were incubated with the following antibodies: CD34-allophycocyanin, CD44-fluorescein isothiocyanate (FITC), CD45-peridinin chlorophyll protein, CD73-phycoerythrin, and CD90-FITC (Becton Dickinson, San Diego, CA). A flow cytometer was used (FACSCalibur, BD), and data were analyzed using a computer program (Paint-A-Gate, BD), as previously reported.[16] In addition, osteogenic, adipogenic, and chondrogenic differentiation was demonstrated following standard procedures described elsewhere.[17]

PATIENTS' CHARACTERISTICS AND OUTCOME AFTER MSC THERAPY

A summary of patients' characteristics is shown in Table 1, whereas results are outlined in Table 2. In addition, responses to prior therapies as well as to MSCs are depicted in Fig. 1. A synopsis of each case is indicated below. In all four cases MSCs were administered as compassionate use after informed consent was obtained.

Case 1

The first patient was a 34-year-old male who received a PB allo-SCT from a matched unrelated donor after myeloablative conditioning with cyclophosphamide and total body irradiation in October 2007 as treatment for a refractory acute myeloid leukemia (AML) in second complete remission (CR). Tacrolimus and methotrexate were used for GVHD prophylaxis. The patient was admitted on Day +146 for posttransplant peripheral thrombocytopenia (marrow was megakaryocytic, and PLT antibodies were not detected) with a PLT count of 16×10^9/L, that responded transiently to endovenous immunoglobulins (IVIG). Ten days later (Day +156) PLTs again had dropped from more than 120×10^9/L to 18×10^9/L, and prednisone (1 mg/kg) was started, again with a good initial response (>150×10^9 PLTs/L), that lasted to Day +221 posttransplant, when a new episode occurred. He was subsequently treated with rituximab (375 mg/m^2) weekly for 4 weeks, without response. He then received MSC therapy on Day +271, with progressive full recovery of PLT counts from Day +290 to normal values, maintained after 34 months of follow-up.

Case 2

The second patient was a 23-year-old male who received a marrow allo-SCT from a matched unrelated donor after cyclophosphamide plus total body irradiation conditioning in January 2009 as treatment for refractory AML in first CR. GVHD prophylaxis was performed with tacrolimus,

TABLE 1. Patients and transplant characteristics

Patient	Age/sex	Disease and status at transplantation	Conditioning and GVHD prophylaxis	Donor and cell source	GVHD (yes/no)	CMV reactivation (yes/no)	Day of neutrophil engraftment (>0.5/>1 × 10^9 per L)	Day of PLT engraftment (>20/>50 × 10^9 per L)
1	34/male	AML in second CR	Cy + TBI; tacrolimus and Mtx	MUD/PB	No	No	+14/+16	+10/+12
2	23/male	AML in first CR	Cy + TBI; tacrolimus; Mtx, ATG	MUD/marrow	Yes	No	+33/+44	+29/+49
3	31/male	HL in partial response	Fluda + Mel; cyclosporine + Mtx	MRD/PB	Not confirmed	No	+16/+19	+12/+14
4	61/male	CML in first CP	Fluda + Bu; tacrolimus + rapamycin	MUD/marrow	Yes	Yes	+14/+21	+19/+27

Bu = busulfan; CP = chronic phase; Cy = cyclophosphamide; Fluda = fludarabine; HL = Hodgkin's lymphoma; Mel = melphalan; MRD = matched related donor; Mtx = methotrexate; MUD = matched unrelated donor; TBI = total body irradiation.

TABLE 2. Outcome after MSC therapy

Patient	Type of cytopenia	Day of onset	Prior treatments for cytopenia	MSC donor	Number of doses (median cell number per dose)	Outcome	Duration of response or time to last follow-up (months)
1	Thrombocytopenia	+146	Steroids, IVIG, rituximab	Related (father)	1 (0.85 × 10^9/kg)	Full recovery	34
2	Neutropenia	+398	IVIG	Unrelated	4 (1.45 × 10^9/kg)	Full recovery	8
3	Thrombocytopenia	+649	Steroids, IVIG	Unrelated	4 (0.97 × 10^9/kg)	Full recovery	5
4	Thrombocytopenia	+51	Steroids	Unrelated	3 (0.87 × 10^9/kg)	Full recovery	3

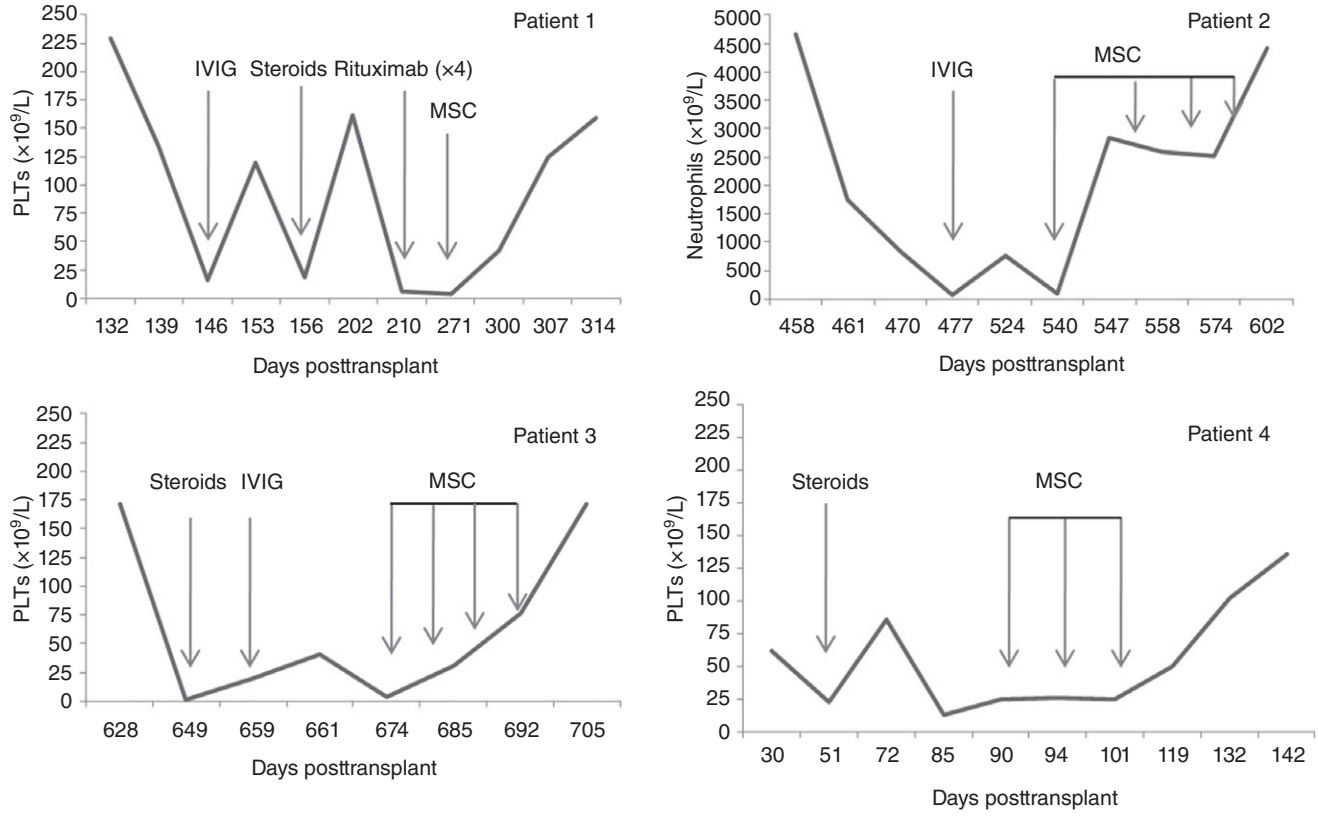

Fig. 1. Summary of patients' sequential therapies and responses.

methotrexate, and thymoglobulin. PLT engraftment was slightly delayed (see Table 1), but recovered normal PB counts after transplantation. He developed Grade 2 acute GVHD with skin involvement on Day +65 and extensive chronic GVHD on Day +163, with gastrointestinal, mucosal, and skin involvement, treated with steroids and budesonide achieving CR of the disease by 1 year posttransplant.

Thirteen months posttransplant he developed the first episode of neutropenia (with less than 0.1×10^9 neutrophils/L) that was treated with filgrastim (5 μg/kg/day) until normalization of neutrophil counts. He had subsequent episodes of neutropenia ($<0.5 \times 10^9$/L) for the following 3 months, treated again with granulocyte–colony-stimulating factor (G-CSF). Anti-neutrophil antibodies were then demonstrated. Marrow examination showed normal cellularity and granulocyte maturation. Thus, IVIG therapy was started, without sustained response, so MSC therapy was initiated (18 months after transplant), with a first dose of 1.45×10^6 MSCs/kg, with normalization of neutrophil counts after 1 week. Three additional weekly doses of MSC were administered for the following 3 weeks. The patient has maintained normal neutrophil counts in the past 8 months, with the exception of a single episode of neutropenia ($<0.5 \times 10^9$/L), 2 months after the last dose of MSCs, that responded to a single dose of G-CSF.

Case 3

The third patient was a 31-year-old male who received a PB allo-SCT from a matched related donor in January 2009 to treat a Hodgkin's lymphoma in partial response after auto-SCT. He received a reduced intensity conditioning regimen with fludarabine and melphalan and GVHD prophylaxis with cyclosporine and methotrexate. He had no confirmation of acute or chronic GVHD, although he experienced mild liver function test abnormalities during cyclosporine tapering, which was corrected by increasing cyclosporine dose to therapeutic trough levels. One year 9 months after transplantation, he was admitted with severe peripheral thrombocytopenia (1×10^9/L). Marrow assessment showed a megakaryocytic marrow with complete donor chimerism. The patient had no evidence of PLT antibodies and was treated with steroids without response in a week, thus followed by IVIG therapy, with a transient response (PLTs over 40×10^9/L). One week later the PLT count again dropped, and he received 40 mg/day dexamethasone for 4 days, followed by MSC treatment (1×10^6 cells/kg), without response to the first dose. He subsequently received another course of IVIG and three additional weekly doses of MSCs, and he progressively reached normal PLT counts, that remain to date (5 months of follow-up).

Case 4

The last patient was a 61-year-old male who received a marrow allo-SCT in October 2010 from a matched unrelated donor after a reduced intensity conditioning regimen (fludarabine plus busulfan) for a chronic myeloid leukemia in chronic phase, refractory to imatinib, dasatinib, and nilotinib therapy. For GVHD prophylaxis, tacrolimus and rapamycin were used. In the first marrow assessment (Day +28), he had mixed chimerism with 76% cells from the recipient and 44% Ph+ chromosomes. Thus, immunosuppression was promptly diminished and then discontinued. On Day +45, the patient developed acute gastrointestinal GVHD that responded initially to topical treatment with oral beclomethasone, and rapamycin was also added. In addition, the PLT count dropped to 22×10^9/L on Day +51. Marrow was hypocellular but with megakaryocytes. PLT antibodies were negative. Thus, systemic steroids were initiated. With the onset of GVHD, BCR-ABL ratio and marrow chimerism notably improved until negative and 100% donor, respectively. Besides an improvement of PLT count up to 85×10^9/L (Day +72), on Day +85 PLT counts dropped again to 13×10^9/L. Thus, in the context of GVHD and with the same features in marrow, we decided to treat the patient with three doses of MSCs, with full recovery of PLT counts to date (3 months).

DISCUSSION

To the best of our knowledge, this report is the first one describing the outcome of patients with peripheral cytopenias after allo-SCT treated with MSCs. Two of them were in the context of GVHD, a setting where MSCs have demonstrated their efficacy based on their immunomodulatory effects.[13,18-20] In Patient 3 hepatic chronic GVHD was probably present, since it occurred during cyclosporin tapering and responded to dose increase to therapeutic trough levels. Nevertheless, thrombocytopenia developed 8 months later, with normal liver function tests and cyclosporine levels in the therapeutic range. The exact mechanism for the development of posttransplant peripheral cytopenias is not well known, but an immune-mediated component could be involved.[3] In addition, poorly understood tolerance mechanisms impairment after SCT could also be important in their development.[3] Most patients with posttransplant peripheral cytopenias respond to standard therapy (steroids and or IVIG).[3,21] Nevertheless, when those treatments fail the outcome is poor, so alternative therapeutic approaches are needed.[8] MSC administration is currently being explored in a number of immune-based diseases, including Crohn's disease or multiple sclerosis.[22,23] In addition, we have shown that MSC function is abnormal in immune thrombocytopenic purpura,[18,19,24] a clinical entity that shares many aspect with posttransplant peripheral cytopenias. Thus, it can be hypothesized that allogeneic MSCs may improve the situation. It should be emphasized that patients had been refractory to several treatment lines such as steroids, IVIG, or even rituximab, so a delayed response to the former treatments cannot be excluded and may have contributed to the beneficial effects observed after MSC administration. Unfortunately, a group of control patients with refractory cytopenias that did not receive MSCs is not available and would have been of great interest to compare their outcome. It is important to note that in some cases several doses of MSCs were needed to achieve a response and failure to MSC therapy cannot be assumed until several doses have been administered.

In summary, with the cautions indicated above, we can conclude that MSC therapy may contribute to the recovery of refractory posttransplant peripheral cytopenias in patients undergoing allo-SCT. This warrants a prospective trial to confirm these preliminary results.

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CONFLICT OF INTEREST

None.

REFERENCES

1. Couriel D, Carpenter PA, Cutler C, Bolanos-Meade J, Treister NS, Gea-Banacloche J, Shaughnessy P, Hymes S, Kim S, Wayne AS, Chien JW, Neumann J, Mitchell S, Syrjala K, Moravec CK, Abramovitz L, Liebermann J, Berger A, Gerber L, Schubert M, Filipovich AH, Weisdorf D, Schubert MM, Shulman H, Schultz K, Mittelman B, Pavletic S, Vogelsang GB, Martin PJ, Lee SJ, Flowers ME. Ancillary therapy and supportive care of chronic graft-versus-host disease: National Institutes of Health consensus development project on criteria for clinical trials in chronic Graft-versus-host disease: V. Ancillary therapy and supportive care working group report. Biol Blood Marrow Transplant 2006; 12:375-96.
2. Filipovich AH, Weisdorf D, Pavletic S, Socie G, Wingard JR, Lee SJ, Martin P, Chien J, Przepiorka D, Couriel D, Cowen EW, Dinndorf P, Farrell A, Hartzman R, Henslee-Downey J, Jacobsohn D, McDonald G, Mittleman B, Rizzo JD, Robinson M, Schubert M, Schultz K, Shulman H, Turner M, Vogelsang G, Flowers ME. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. Biol Blood Marrow Transplant 2005;11:945-56.
3. Tsirigotis PD, Resnick IB, Or R, Elad S, Zilberman I, Yoffe L, Levovic A, Miron S, Gesundheit B, Slavin S, Shapira MY.

Post-hematopoietic stem cell transplantion immune-mediated cytopenias. Immunotherapy 2009;1:39-47.
4. Shono Y, Ueha S, Wang Y, Abe J, Kurachi M, Matsuno Y, Sugiyama T, Nagasawa T, Imamura M, Matsushima K. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. Blood 2010;115:5401-11.
5. Page KM, Mendizabal AM, Prasad VK, Martin PL, Parikh S, Wood S, Sempowski GD, Szabolcs P, Kurtzberg J. Posttransplant autoimmune hemolytic anemia and other autoimmune cytopenias are increased in very young infants undergoing unrelated donor umbilical cord blood transplantation. Biol Blood Marrow Transplant 2008;14:1108-17.
6. Hartert A, Willenbacher W, Gunzelmann S, Roemer E, Basara N, Fauser AA, Kiehl MG. Successful treatment of thrombocytopenia and hemolytic anemia with IvIG in a patient with lupus-like syndrome after mismatched related PBSCT. Bone Marrow Transplant 2001;27:337-40.
7. Khouri IF, Ippoliti C, Gajewski J, Przepiorka D, Champlin RE. Neutropenias following allogeneic bone marrow transplantation: response to therapy with high-dose intravenous immunoglobulin. Am J Hematol 1996;52:313-5.
8. Raj K, Narayanan S, Augustson B, Ho A, Mehta P, Duncan N, Tauro S, Mahendra P, Craddock C, Mufti G. Rituximab is effective in the management of refractory autoimmune cytopenias occurring after allogeneic stem cell transplantation. Bone Marrow Transplant 2005;35:299-301.
9. Ratanatharathorn V, Pavletic S, Uberti JP. Clinical applications of rituximab in allogeneic stem cell transplantation: anti-tumor and immunomodulatory effects. Cancer Treat Rev 2009;35:653-61.
10. Dominici M, Le BK, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.
11. Tolar J, Le BK, Keating A, Blazar BR. Concise review: hitting the right spot with mesenchymal stromal cells. Stem Cells 2010;28:1446-55.
12. Battiwalla M, Hematti P. Mesenchymal stem cells in hematopoietic stem cell transplantation. Cytotherapy 2009; 11:503-15.
13. Perez Simon JA, Lopez-Villar O, Andreu EJ, Rifon J, Muntion S, Diez-Campelo M, Sanchez-Guijo FM, Martinez C, Lopez D, Del Cañizo C. Mesenchymal stem cells expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease: results of a phase I/II clinical trial. Haematologica 2011;96:1072-6.
14. Carrancio S, Lopez-Holgado N, Sanchez-Guijo FM, Villaron E, Barbado V, Tabera S, ez-Campelo M, Blanco J, San Miguel JF, Del Canizo MC. Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. Exp Hematol 2008;36: 1014-21.
15. Perez-Ilzarbe M, Diez-Campelo M, Aranda P, Tabera S, Lopez T, Del CC, Merino J, Moreno C, Andreu EJ, Prosper F, Perez-Simon JA. Comparison of ex vivo expansion culture conditions of mesenchymal stem cells for human cell therapy. Transfusion 2009;49:1901-10.
16. Sanchez-Guijo FM, Sanchez-Abarca LI, Villaron E, Lopez-Holgado N, Alberca M, Vazquez L, Perez-Simon JA, Lopez-Fidalgo J, Orfao A, Caballero MD, Del Canizo MC, San Miguel JF. Posttransplant hematopoiesis in patients undergoing sibling allogeneic stem cell transplantation reflects that of their respective donors although with a lower functional capability. Exp Hematol 2005;33:935-43.
17. Sanchez-Guijo FM, Blanco JF, Cruz G, Muntion S, Gomez M, Carrancio S, Lopez-Villar O, Barbado MV, Sanchez-Abarca LI, Blanco B, Brinon JG, Del Canizo MC. Multiparametric comparison of mesenchymal stromal cells obtained from trabecular bone by using a novel isolation method with those obtained by iliac crest aspiration from the same subjects. Cell Tissue Res 2009;336:501-7.
18. Ringden O, Le BK. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. Best Pract Res Clin Haematol 2011; 24:65-72.
19. Sato K, Ozaki K, Mori M, Muroi K, Ozawa K. Mesenchymal stromal cells for graft-versus-host disease: basic aspects and clinical outcomes. J Clin Exp Hematop 2010;50:79-89.
20. Le BK, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-86.
21. Klumpp TR, Block CC, Caligiuri MA, Rabinowe SN, Soiffer RJ, Ritz J. Immune-mediated cytopenia following bone marrow transplantation. Case reports and review of the literature. Medicine (Baltimore) 1992;71:73-83.
22. Hoogduijn MJ, Popp F, Verbeek R, Masoodi M, Nicolaou A, Baan C, Dahlke MH. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. Int Immunopharmacol 2010;10:1496-500.
23. Siatskas C, Payne NL, Short MA, Bernard CC. A consensus statement addressing mesenchymal stem cell transplantation for multiple sclerosis: it's time! Stem Cell Rev 2010;6: 500-6.
24. Perez-Simon JA, Tabera S, Sarasquete ME, Diez-Campelo M, Canchado J, Sanchez-Abarca LI, Blanco B, Alberca I, Herrero-Sanchez C, Canizo C, San Miguel JF. Mesenchymal stem cells are functionally abnormal in patients with immune thrombocytopenic purpura. Cytotherapy 2009;11:698-705.