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Role of mesenchymal stromal cells derivatives in diabetic foot ulcers: a controlled randomized phase 1/2 clinical trial

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ABSTRACT

Background: Diabetes-related foot complications have been identified as the most common isolated cause of morbidity among patients with diabetes and the leading cause of amputation. Therefore, new strategies to stimulate skin regeneration may provide a novel therapeutic approach to reduce non-healing ulcer disease. Recently, we demonstrated in proof-of-concept in humans that administration of allogeneic bone marrow mesenchymal stromal cellss derivatives (allo-hBM-MSCDs) is effective in a similar way to the use of allogeneic bone marrow mesenchymal stromal cellss (allo-hBM-MSCs) in grade 2 diabetic foot ulcers (DFUs).
Aim: To assess the safety and efficacy profile of the allo-hBM-MSCDs relative to the conventional approach (PolyMen® dressing) in 1/2 clinical trial phases in patients with grade 1 and 2 DFUs.
Methods: In the present study, we used 2 doses of allo-hBM-MSCDs (1 mL) or 1 dose of allo-hBM-MSCs (1×106 cells) intradermally injected around wounds and assessed their safety and effectiveness, relative to the conventional approach (PolyMem dressing). Allo-hBM-MSCDs and allo-hBM-MSCs were produced in a certified Good Manufacturing Practice-type Laboratory. Patients with grade 1 and 2 DFUs were randomized to receive allo-hBM-MSCDs (n=12), allo-hBM-MSCs (n=6) or conventional treatment (PolyMem dressing) (n=10). The wound-healing process was macroscopically evaluated until the complete closure of the ulcers.
Results: No adverse events were reported. Patients with grade 1 and 2 DFUs treated with either allo-hBM-MSCDs or allo-hBM-MSCs, achieved greater percentages of wound closure, enhanced skin regeneration in shorter times and a greater ulcer-free survival relative to the patients who received conventional treatment. Finally, through proteomic analysis, we elucidated the proteins and growth factors that are secreted by allo-hBM-MSCs and relevant to the wound-healing process. In addition, by combining proteomics with Gene Ontology analysis, we comprehensively classified secreted proteins on both biological process and molecular function.
Conclusions: In this phase 1/2 trial, our cumulative results suggest that 2 doses of allo-hBM-MSCDs combined with a wound dressing are a safe and effective treatment for grade 1 and 2 DFUs.

Introduction

Diabetes mellitus is an important cause of morbidity, mortality and economic burden [1]. It is accompanied by significant health complications, which decrease quality of life of the affected individuals. Diabetic foot ulcers (DFUs) are among the most common complications; they are usually the result of poor glycemic control, underlying neuropathy, peripheral vascular disease or poor foot care [2]. In addition, these ulcers are usually in foot areas with repetitive trauma and pressure sensations [3]. DFUs are responsible for more clinical admissions than any other diabetic complication. Today, DFUs are the leading cause of non-traumatic amputations in the USA [2]. Overall, the

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lifetime risk for patients with diabetes to develop a DFU is 25%, with a high risk of amputation. It has been reported that more than 50% of non-traumatic lower-extremity amputations are related to DFU infections, and DFUs precede 85% of all lower-extremity amputations in patients with diabetes; up to 70% of patients with diabetes with a DFU-related amputation die within 5 years of their amputation; mortality increases according with the level of amputation. Inevitably, DFUs have a high financial cost; this cost has been estimated at >$1 billion annually in the United States, approximately £650 million annually in the United Kingdom and >€10 billion annually in Europe [4].

Despite these high healthcare costs, about 20% of patients have unhealed DFUs after 1 year. Even after wound resolution, subsequent DFUs are common, with a recurrence rate of roughly 40% of patients within 1 year [5]. Although there are well-established principles for managing DFUs, their treatment remains challenging. Currently, available treatments for DFUs involve debridement, traditional wound dressings and antibiotics. Nevertheless, approximately 50% of DFUs are refractory to these therapies, even when using promising techniques such as chemicals, active wound dressings and skin grafts [6]. Therefore, a broad spectrum of novel interventions to improve wound healing is being studied, among which regenerative medicine offers the greatest hope for these patients. Recent clinical trials reports suggest that direct application of stem cells may accelerate the healing of non-healing chronic wounds [7,8]. Particularly, mesenchymal stromal cellss (MSCs) are currently explored as an attractive and harmless therapeutic agent to treat skin lesions [9,10]. Nevertheless, recent studies have suggested that the secretome of MSCs, including cytokines, growth factors, chemokines and extracellular vesicles containing mRNA, proteins and microRNAs, is responsible for orchestrating different cellular processes that lead to its regenerative effect [11,12].

In this context, we have previously demonstrated, using a diabetic mouse model, that the administration of bone marrow mesenchymal stromal cellss derivatives from mice (mBM-MSCDs) is more effective than using mBM-MSCs alone. mBM-MSCDs favored wound closure progression and reduced severe leukocyte infiltration [13]. Also, their use increased the formation of granulation tissue and remodeled the orientation of deposited collagen [13,14]. This therapeutic effect is attributed to the presence of pleiotropic bioactive molecules in the acellular derivatives produced by MSCs, which appeared to initiate and improve the wound-healing process as well as facilitate the host response to tissue repair [13,14]. Thus, BM-MSCDs could be potentially used as an effective therapeutic tool. In addition, we recently performed a proof-of-concept in patients with grade 2 DFUs, where we suggest that combining intradermal administration of allogeneic bone marrow mesenchymal stromal cellss derivatives (allo-hBM-MSCDs) with a wound dressing in patients with grade 2 DFUs enhances the wound-healing process, similarly to that observed for patients treated with allogeneic bone marrow mesenchymal stromal cellss (allo-hBM-MSCs) and a wound dressing [15], resulting in improved healing, relative to conventional treatment (wound dressing). Although allo-hBM-MSCDs appears to be a promising option to treat DFUs, their safety and efficacy profile needs to be demonstrated in a larger trial.

In our present work, we conducted a phase 1/2 controlled, randomized, double-blind trial to assess the safety and efficacy of allo-hBM-MSCDs in the treatment of grade 1 and 2 DFUs. Here, we compare 2 doses of allo-hBM-MSCD, 1 dose of allo-hBM-MSCs or 1 dose of vehicle (saline solution with 5% of human albumin), which were intradermally injected around wounds. The wound-healing process and changes on re-epithelialization were macroscopically evaluated until the complete closure of the ulcers. All ulcers were simultaneously treated with conventional treatment (PolyMem dressing; Ferris, Fort Worth, TX, USA). In contrast, proteomic analysis was performed to evaluate the proteins and growth factors relevant to wound healing contained in the allo-hBM-MSCDs.

Methods

Study design

A phase 1/2 randomized, prospective, double-blind, controlled and parallel-group clinical trial was performed at the Fundación Oftalmológica de Santander (FOSCAL) (Bucaramanga, Colombia), assessing the safety and efficacy of 2 doses of allo-hBM-MSCDs (1×) or 1 dose of allo-hBM-MSCs (1×10^6 cells) intradermally injected around wounds compared with conventional treatment (PolyMem dressing) in patients with grade 1 and 2 DFUs (classification system for research purposes described by the International Working Group of the Diabetic Foot) [16]. The study was conducted following Good Clinical Practice guidelines and the Declaration of Helsinki. All protocols were approved by the Research Ethics Committee at FOSCAL, Colombia (Act. No. 46/May 20, 2016), and the study was registered at ClinicalTrials.gov (No. NCT124174455522). Before MSC isolation and wound treatment, informed consent was obtained from both bone marrow donors and study participants, respectively.

Participants

The target population included patients 40–80 years old with grade 1 and 2 DFUs who were recruited between August 2018 and November 2020 at FOSCAL. All participants met the inclusion criteria described in Table 1. They did not have appropriate metabolic control of diabetes (7.52–9.48% glycosylated hemoglobin values before and during the study). In addition, demographic characteristics of patient, co-morbidities, and concomitant medications are described in Table 2, and wound baseline characteristics are presented in Table 3.

Forty-one subjects with DFU were screened, and 13 were excluded before the study because of the unmet eligibility criteria such as ulcer size >5.5 cm^2 (n=6), ulcer type (n=5) and non-diabetic participant (n=2). Of the remaining 28 subjects, 11 had grade 1 DFUs and 17 had grade 2 DFUs, which were randomly assigned as follows: grade 1 DFUs conventional treatment (n=3), allo-hBM-MSCs (n=3) and allo-hBM-MSCDs (n=5); grade 2 DFUs conventional treatment (n=7), allo-hBM-MSCs (n=3) and allo-hBM-MSCDs (n=7) (Figure 1).

Treatments

Patients were randomly assigned to receive 1 of the following treatments:

(i) Conventional treatment (n=10), which consisted of intradermally applying 1 mL of vehicle (saline solution with 5% human albumin) at 4 equidistant peripheral sites from the ulcer (0.25 mL of the vehicle on each side of the lesion) with a distance from wound

Table 1
Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• Adult male or female, 40 y of age or older (to 80 y old)	• Cancer
• Diagnosis of diabetes	• Presence of osteomyelitis
• Presence of grade 1 or 2 DFUs	• Diagnosis of brain or hematologic disorders
• Surface area between 0.5 and 5.5 cm^2	• Use of immunosuppressive or cytotoxic drugs
	• Any acute systemic infectious disease process

DFU, diabetic foot ulcer.

Table 2
Patient characteristics.

	Grade 1 DFUs			Grade 2 DFUs		
	Conventional treatment (PolyMem dressing)	Allo-hBM-MSCs	Allo-hBM-MSCDs	Conventional treatment (PolyMem dressing)	Allo-hBM-MSCs	Allo-hBM-MSCDs
Sex						
Male, *n* (%)	2 (67)	2 (67)	3 (60)	5 (71)	1 (33)	4 (57)
Female, *n* (%)	1 (33)	1 (33)	2 (40)	2 (29)	2 (66)	3 (43)
Age, y						
18-50 y, *n* (%)	0 (0)	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)
> 50 y, *n* (%)	3 (100)	2 (67)	5 (100)	7 (100)	3 (100)	7 (100)
Mean ± SD	59.67 ± 6.65	55.50 ± 13.44	65.80 ± 6.83	62.86 ± 8.45	63.33 ± 3.78	62 ± 8.12
Median	63	55.50	65	59	65	58
Min/max	52/64	46/65	57/74	55/77	59/66	54/74
Glycated hemoglobin A1c (%) at first and second month of study						
	Grade 1 DFUs		Grade 2 DFUs			
	First month	Second month	First month	Second month		
Mean ± SD	8.58 ± 2.48	9.48 ± 3.57	7.93 ± 1.13	7.52 ± 0.91		
Median	7.8	9.4	7.8	7.4		
Min/max	6.1 / 12.20	6.1 / 14	5.3 / 9.8	6.4 / 9.5		

Allo-hBM-MSCDs, allogeneic human bone marrow mesenchymal stromal cells derivatives; allo-hBM-MSCs, allogeneic human bone marrow mesenchymal stromal cells; DFUs, diabetic foot ulcer; SD, standard deviation.

Table 3
Wound baseline characteristics.

	Grade 1 DFUs			Grade 2 DFUs		
	Conventional treatment (PolyMem dressing)	Allo-hBM-MSCs	Allo-hBM-MSCDs	Conventional treatment (PolyMem dressing)	Allo-hBM-MSCs	Allo-hBM-MSCDs
Duration of ulcer, mo						
1 mo, *n*	0	0	0	3	0	2
2 mo, *n*	1	0	2	1	2	1
>2 mo, *n*	2	3	3	3	1	4
Mean ± SD	24.67 ± 23.01	21.50 ± 20.51	5.08 ± 4.26	2.14 ± 1.21	3.66 ± 2.88	6.28 ± 8.26
Median	24	21.5	5	2	2	2
Min/max	2/48	7/36	2/2	1/4	2/7	1/24
Initial wound area, cm^2						
Mean ± SD	0.80 ± 0.19	0.66 ± 0.31	0.53 ± 0.23	3.06 ± 1.38	3.59 ± 2.12	2.92 ± 1.26
Median	0.72	0.50	0.48	2.77	4.42	2.83
Min/max	0.66 / 1.03	0.46/1.02	0.35/0.92	1.65/5.60	1.17/5.17	1.25/5.30
Ulcer location, *n* (%)						
Foot right (plantar)	3 (100)	2 (66)	4 (80)	5 (71.4)	1 (34)	3 (42.8)
Foot left (plantar)	0 (0)	1 (34)	1 (20)	2 (28.6)	2 (66)	4 (57.2)

Allo-hBM-MSCDs, allogeneic human bone marrow mesenchymal stromal cells derivatives; allo-hBM-MSCs, allogeneic human bone marrow mesenchymal stromal cells; DFUs, diabetic foot ulcer; SD, standard deviation.

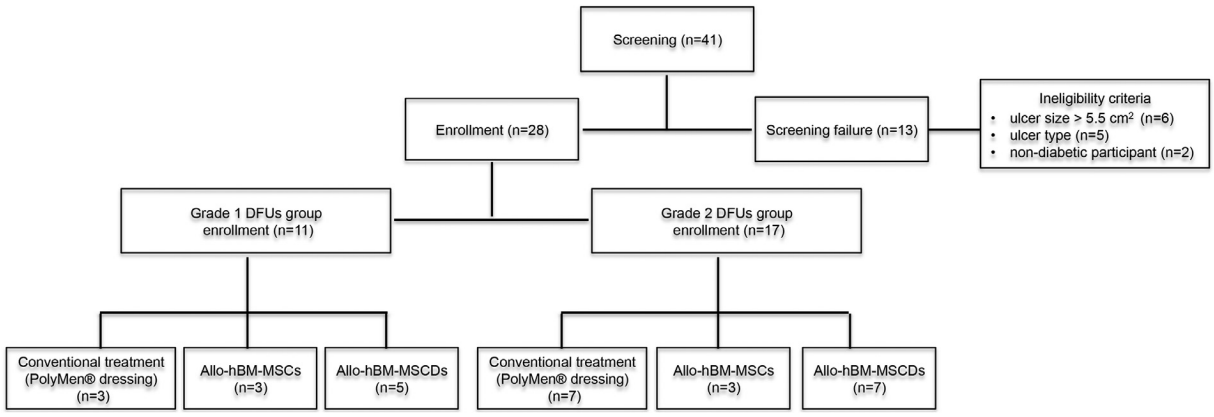

Figure 1. Participant flowchart. Reasons of screening failure: ulcer size >5.5 cm^2 (6 participants), ulcer type (5 participants) and participant without diabetes (2 participants).

edges of 1 to 2 mm approximately in 1 dose at day 0. Therefore, these patients were only treated with the wound dressing based on PolyMem Ferris, Fort Worth, TX, USA.

(ii) Allo-hBM-MSCs (n=6) were obtained from a healthy 27-year-old female donor unrelated to the patient. Surface markers of allo-hBM-MSCs were evaluated and results were positive for $CD73^+$, $CD90^+$ and $CD105^+$ and negative for $CD45^-$, $CD34^-$, $CD11b^-$ and human leukocyte antigen-DR isotype$^-$ (Supplementary Figure 1). One million of allo-hBM-MSCs were intradermally administered around the wound area at 4 equidistant sites (2.5×10^5 cells on each side of the lesion) with a distance from wound edges of 1 to 2 mm approximately in 1 dose at day 0.

(iii) Allo-hBM-MSCDs (n=12) were obtained from culturing the allo-hBM-MSCs. Like the other 2 treatments, 1 mL of allo-hBM-MSCDs was intradermally administered at 4 equidistant peripheral points of the lesion (0.25 mL of allo-hBM-MSCDs on each side of the lesion) with a distance from wound edges of 1 to 2 mm approximately in 1 dose at day 0. A second dose was repeated on day 7.

Outcomes measures

Safety profile

The trial's primary end point was the safety of allo-hBM-MSCD and allo-hBM-MSC administration, according to the number of treatment-related adverse events (AEs) reported for each study group as coded by the Common Terminology Criteria for AE classification. AEs were defined as (i) local toxicity, including signs of local inflammation (swelling, warmth, impairment of function), worsening of ulcer, new ulcer or hematomas after allo-hBM-MSCDs or allo-hBM-MSCs administration; (ii) systemic toxicity as fever, allergies; and (iii) other AEs, graded according to the Common Terminology Criteria for AEs, expressed as maximum grade toxicity for skin and subcutaneous tissue disorders.

Secondary safety outcomes were the incidence of any serious AEs post-treatment, defined as events leading to hospitalization, malignancy, death, persistent or significant disability. AEs were documented at each visit and described in terms of incidence, severity and relatedness with macroscopic changes in skin wounds.

Efficacy profile

The secondary end point of the trial was efficacy assessed by (i) wound closure, (ii) wound healing rate, (iii) changes in other ulcer dimensions, (iv) ulcer-free survival, (iv) hazard ratios (HRs) and closure rate person day, (v) quality of life by the validated scale Short-form 36 (SF-36) questionnaire [17] and (vi) pain evaluation by the validated scale McGill Pain Questionnaire [18].

Follow-up

The follow-up visits were at days 1, 3, 7 and after this, every week until wound closure to evaluate the efficacy of treatments and support the healing process, which consisted of treating the ulcers with a wound dressing (PolyMem). After the wound closure, all participants were contacted by the study center at 3, 6 and 12 months to collect information about the recurrence of DFUs.

During each visit, wound size (area, perimeter, volume, mean depth, and max depth) was accurately measured using 3D laser technology (SilhouetteStar camera).

Time elapsed to complete wound closure was defined as the time in which the wound bed became completely re-epithelialized and filled with new tissue.

The percentage of wound closure was calculated using the equation:

$$[(\text{original wound area} - \text{actual wound area}) / (\text{original wound area})] \times 100.$$

The percentage of perimeter reduction was calculated using the same equation (by using perimeter measurements instead of area measurements).

The wound healing rates were calculated using the following equations:

$$\text{Surface area change } \Delta A = Aa - Ab$$
$$\text{Surface area change per day } \Delta Ad = \Delta A/\text{days}$$

Where Δ refers to change, A to the area, a is initial area, and b is final area.

The clinical outcome scales (McGill Pain Questionnaire and SF-36 questionnaire) were evaluated at the beginning of the study and 1-month post-treatment.

Human allo-hBM-MSCs characterization

Isolation and *ex vivo* expansion of allo-hBM-MSCs

The allo-hBM-MSCs for this trial were processed and manufactured in a Good Manufacturing Practice type Laboratory (Centro de Terapias Avanzadas FOSCAL, Colombia), under Good Manufacturing Practice conditions according to the Food and Drug Administration Guidance for industry (current good tissue practice and additional requirements for manufacturers of human cells, tissues and cellular and tissue-based products). Bone marrow was obtained after informed consent from healthy donors, and it was aseptically stored in sterile Hank's Balanced Salt Solution (Gibco, Grand Island, NY, USA) supplemented with heparin (Fresenius Kabi, Santiago, Chile). To summarize in brief, mononuclear cells were separated by centrifugation in a Ficoll-Hypaque gradient (density 1.077 g/cm^3; Sigma, St Louis, MO, USA) following the manufacturer's instructions. The mononuclear cells were suspended in Minimum Essential Medium Eagle Alpha Modifications (Alpha-MEM) high glucose (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, USA), 1% gentamicin, and 2 mM L-glutamine (Gibco), and seeded at a concentration of 1×10^6 cells/cm^2. After 72 hours, non-adherent cells were removed, and fresh medium was added to the cells. Cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide in air. One week later, when the monolayer of adherent cells reached confluence, cells were trypsinized (0.25% trypsin and 2.65 mM ethylenediaminetetraacetic acid [EDTA]; Gibco), washed, resuspended in Alpha-MEM containing 10% FBS, and sub-cultured at a concentration of 7000 cells/cm^2. When the cell culture reached 70–80% confluence, cells were detached by treatment with (0.25% trypsin and 2.65 mM EDTA; Gibco), harvested and cryopreserved.

Preparation and immunophenotypic analysis of allo-hBM-MSCs

For the trial, cells were thawed and expanded until passage 4 using Alpha-MEM supplemented with 10% plasma obtained from donors with type AB blood. When cell cultures reached 80% confluence, adherent cells were detached by treatment with (0.25% trypsin and 2.65 mM EDTA; Gibco). Live cells were counted using trypan blue staining and a hemocytometer. The release criteria for clinical use of allo-hBM-MSCs comprised the absence of macroscopic clumps, contaminating pathogenic micro-organisms (bacteria, mycoplasma, syphilis, hepatitis B virus, hepatitis C virus, HIV, cytomegalovirus and fungi) or endotoxin (≤0.5 EU/mL) [19]. At passage 4, allo-hBM-MSCs had a viability >95%, and they were characterized according to the International Society for Cellular Therapy Guidelines [20], with an identity and purity pattern characterized by ≥95% positivity for CD73

clone AD2 (PerCP-Cy5.5-conjugated; BD Biosciences, San Jose, CA, USA), CD90 clone 5E10 (FITC-conjugated; eBioscience, San Diego, CA, USA) and CD105 clone MJ7/18 (eFluor450-conjugated; eBioscience), and negativity (≤2%) for the expression of CD45 clone HI30 (PE-Cyanine 7-conjugated; eBioscience), CD34 clone 4H11 (PE-conjugated; eBioscience), CD11b clone ICRF44 (APC-conjugated; eBioscience), CD14, and Human Leukocyte Antigen-DR isotype clone G46-6 (APC-H7-conjugated; BD Biosciences). Thereafter, the cell pellet was rinsed once with staining buffer, resuspended in loading buffer and a total of 10,000 events were analyzed per condition. Flow cytometry analysis was performed using an Amnis CellStream benchtop system (Luminex; MilliporeSigma, Burlington, MA, USA).

The cells were washed twice with PBS before final suspension and packaging. Cells (1×10^6) were suspended in a final volume of 1 mL (0.9% saline solution, 5% human serum albumin) and dispensed in masked 1-mL syringes to treat individual patients accordingly with the study design.

Chromosome stability assessment

The genomic stability of allo-hBM-MSCs at passage 4 cultured in Alpha-MEM supplemented with 10% plasma obtained from donors with type AB blood was evaluated by karyotyping analysis. Cells were seeded at 2000 cells/cm^2 and cultured until 80% confluence. Then, allo-hBM-MSCs were treated with colcemid for 1 h at 37°C and 5% CO_2, harvested and collected by centrifugation at 300*g* for 10 min. The cell pellet was resuspended in a hypotonic solution and incubated for 10 min at 37°C. Then, the solution was centrifuged at 100*g* for 10 minutes, and Allo-hBM-MSCs were resuspended in a cold fixative solution. Two to 3 drops were added to slides and dried at RT. Chromosomes were observed under a microscope (Zeiss, White Plains, NY, USA), and analyzed using GenASIs software (Applied Spectral Imaging, Carlsbad, CA, USA) (Supplementary Figure 2).

Cell differentiation assays

Differentiation potential of allo-hBM-MSCs cultured in Alpha-MEM supplemented with 10% plasma obtained from donors with type AB blood was assessed via exposure to established differentiation media. Allo-hBM-MSCs were seeded at 2.5×10^4 cells/cm^2 and allowed to spread for 24 h. Then, medium was replaced with adipogenic or osteogenic differentiation media. For the adipogenic condition, we used adipogenic medium that contained 1 μmol/L dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 100 μg/mL 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 100 μmol/L indomethacin (Sigma-Aldrich), and 0.2 U/mL insulin (Sigma-Aldrich). For osteogenic differentiation, allo-hBM-MSCs were exposed to medium that contained 0.1 μmol/L dexamethasone (Sigma-Aldrich), 10 mmol/L β-glycerophosphate (Sigma-Aldrich) and 50 μg/mL ascorbic acid (Sigma-Aldrich) [21]. For both conditions, cells were maintained at 37°C and 5% CO_2, with media changes every 2 days during 2 and 3 weeks, for adipogenic and osteogenic differentiation experiments, respectively.

Adipogenic differentiation analyses

Adipogenic differentiation was further analyzed using standard staining for lipid deposition. In brief, after 2 weeks of culture, cells were stained with 60% (w/v) Oil Red O (Sigma-Aldrich) in isopropanol for 1 h at room temperature. Stained cells were imaged with an inverted microscope (Primovert; Zeiss) using the phase contrast option.

Osteogenic differentiation analyses

Calcium phosphate deposition was evaluated via Alizarin Red S staining. For each condition, cells were stained with 40 mmol/L Alizarin Red S (Sigma-Aldrich) for 10 minutes at room temperature, washed and imaged using an inverted microscope (Primovert; Zeiss).

Preparation and characterization of allo-hBM-MSCDs

The allo-hBM-MSCDs were produced using allo-hBM-MSC cultures at 80% confluence (passage 1) in a 75-cm^2 tissue culture flask (1.5×10^6 cells approximately). Allo-hBM-MSCs were prewashed twice with serum-free Alpha-MEM medium, maintained using 6 mL/flask of this medium and incubated for 24 h under normoxic conditions (37°C in a humidified atmosphere containing 95% air and 5% CO_2). The medium was collected and centrifuged at 1500 rpm = 300 g for 5 min. The supernatant was re-centrifuged at 3000 rpm = 700 g for 3 min, followed by collecting the second supernatant, named allo-hBM-MSCDs, to remove all cell debris. The pH was confirmed using an electric pH meter. Total allo-hBM-MSCDs were collected, filtered, mixed and aliquoted in 500 μL for storage at −80°C.

The release criteria for clinical use of allo-hBM-MSCDs and allo-hBM-MSCs involved the absence, contaminating pathogenic microorganisms (aerobic and anaerobic bacteria, fungi, mycoplasma, syphilis, hepatitis B virus, hepatitis C virus, human immunodeficiency virus [HIV 1-2], human T-lymphotropic virus type 1 and 2 cytomegalovirus) and endotoxin (≤0.5 EU/mL).

Proteomic assays

A pool of 2 batches of allo-hBM-MSCDs was evaluated for its content of specific growth factors and proteins relevant to wound healing via proteomic assays. Each fraction corresponding to the specific molecular weight cut-off (30, 50 and 100 kDa) was individually processed according to Liu et al [22,23]. The digested peptides were separated using Easy nLC UHPLC 1200 in nanoflow configuration (Thermo Scientific, Bremen, Germany) coupled to QExactive Plus (Thermo Scientific) Quadrupole Orbitrap through a nano-electrospray ion source using Full MS followed by ddMS2 (DDA) mode during 110 min [23]. Mascot Distiller v2.6.2.0 in-house licensed (www. matrixscience. com) and Proteome Discoverer v2.1 (Thermo Scientific) were used to generate the peak list at the mascot generic format (mgf) to identify +1 or multiple charged precursor ions from the mass spectrometry data file. Parent mass (MS) and fragment mass (MS/MS) peak ranges were 250–1800 Da (resolution 70,000) and 65–2000 Da (resolution 17,500), respectively. Mascot server v2.7.0.1 (www.matrix-science.com; Matrix Science Ltd., London, UK) in MS/MS ion search mode (local licenses) was applied to conduct peptide matches (peptide masses and sequence tags) and protein searches against SwissProt 2021_04 (565,928 sequences; 204,173,280 residues) using taxonomy filter for *Homo sapiens* (human) (20,376 sequences). The following parameters were set for the search: carbamidomethyl (C) on cysteine was fixed, and variable modifications included asparagine and glutamine deamidation and methionine oxidation. Only 2 missed cleavages were allowed; monoisotopic masses were counted; the precursor peptide mass tolerance was set at 15 ppm; fragment mass tolerance was 0.02 Da, and the ion score or expected cut-off was set at 5. The MS/MS spectra were searched with MASCOT using a 95% confidence interval threshold ($P < 0.05$), with which a minimum score of 27 was used for peptide identification, indicating identity or extensive homology, and at least 2 peptides for protein identification. In addition, the error-tolerant mode was set up at Mascot search to corroborate potential peptides unidentified at the first search. For more details, see supplemental information. The Gene Ontology (GO) analysis for biological process and molecular function was performed

using the UniProt (www.uniprot.org) proteins accession numbers and exported into David Bioinformatics tools [24,25].

Statistical Analysis

Data are reported as mean ± standard deviation. Once statistical normality was checked, comparison of experimental groups was performed using a Kruskal–Wallis one-way analysis followed by Dunn's multiple comparison test to test significant differences ($P < 0.05$) in quantitative variables among treatment groups (conventional treatment, allo-hBM-MSCs and allo-hBM-MSCDs) at baseline and during follow-up. Association or independence of categorical variables was compared using Pearson's χ^2 test; $P < 0.05$ values were accepted as statistically significant.

Ulcer-free survival analysis was calculated according to Kaplan–Meier function. P values < 0.05 were considered statistically significant. We estimated HRs in a bivariate Cox-proportional hazards model with 95% confidence intervals and a level of statistical significance. Stat Graph Prism 5.0 (GraphPad, San Diego, CA, USA) and STATA 15 software (StataCorp LLC, College Station, TX, USA) were used for statistical analysis.

Results

Patient demographic characteristics

Forty-one individuals were screened: 28 were found to be eligible and were randomized to receive the different treatments (Figure 1). Generally, all the patients enrolled in each group had similar baseline characteristics of age and glycated hemoglobin A1c (Table 2). There were no significant differences in baseline characteristics between groups. The lower limb most affected in the participants was the right one (61%) and the most affected area was the foot sole (76%).

Safety profile

No patient had AEs or serious AEs post-treatment. In addition, no AEs occurred during bone marrow donation. Moreover, separation of the aspirate by Ficoll and subsequent cell culture went smoothly as well.

Efficacy profile: clinical outcomes

Wound closure

Wound closure started to be noticed after 1 day of treatment with either allo-hBM-MSCDs or allo-hBM-MSCs in both grade 1 and 2 DFUs, as compared with the conventional treatment, for which wound closure was first observed at day 7 in both types DFUs (Figures 2A,B and 3A,B). The percentage of wound closure in patients treated with allo-hBM-MSCD and allo-hBM-MSC was higher than those treated with the conventional approach (Figure 2B and 3B). Specifically, after 7 days of treatment, the allo-hBM-MSCD and allo-hBM-MSC patients with grade 1 DFUs achieved 41.13% and 78.27%, respectively, in wound closure, and patients with grade 2 DFUs achieved 30.03% and 22.34% decrease in wound surface area, respectively; instead, the patients treated with the conventional approach only achieved a 10.14% grade 1 DFUs and 3.66% grade 2 DFUs reduction in wound surface area. At days 7, 14 and 21, significant differences were found for grade 1 DFUs between allo-hBM-MSCs versus the conventional approach ($P = 0.0208$), allo-hBM-MSCs versus hBM-MSCDs ($P = 0.0360$), allo-hBM-MSCs versus conventional approach ($P = 0.0104$), hBM-MSCDs versus conventional

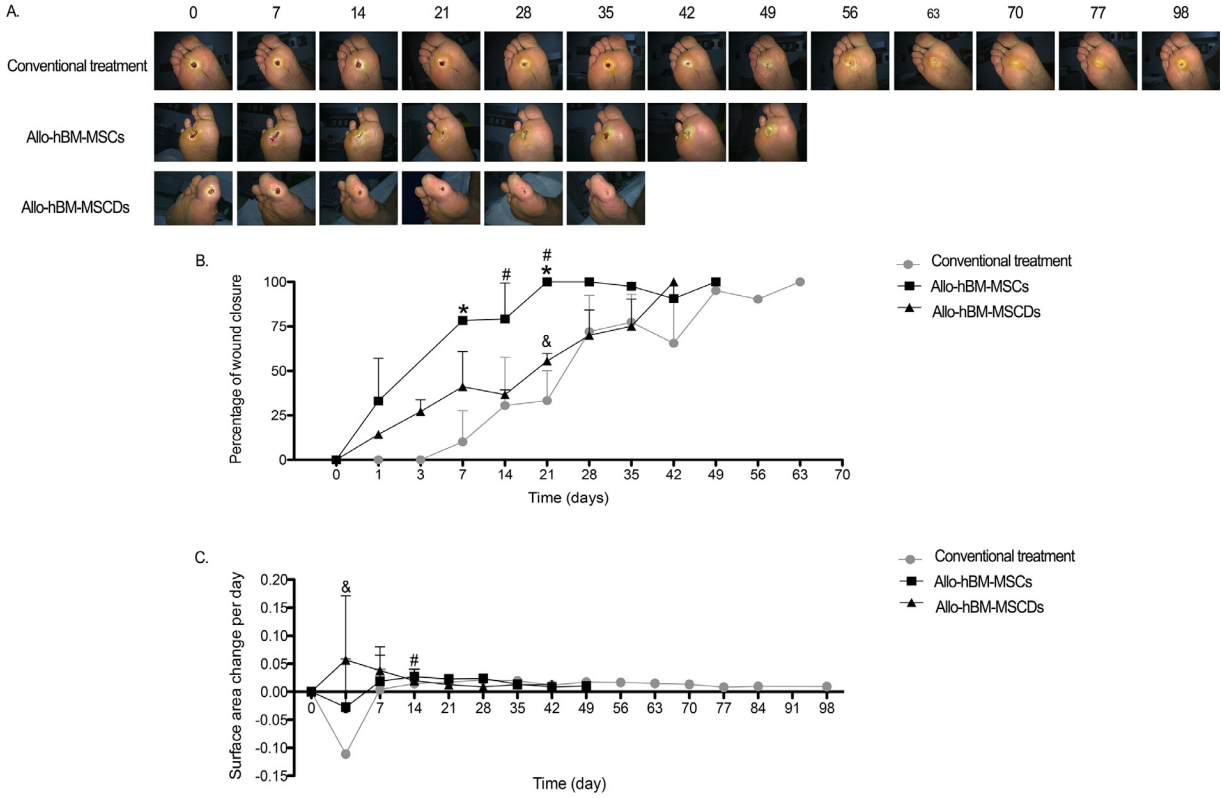

Figure 2. Evolution of wound-healing progression after intradermal administration of allo-hBM-MSCDs until wound closure in patients with grade 1 DFUs. (A) Macroscopic analysis of the chronic wound-healing progress before and after intradermal administration of 1 mL of vehicle, 1×10^6 allo-hBM-MSCs or 1 mL of allo-hBM-MSCDs. (B) Percentage of wound closure and (C) wound-healing rate. Significant differences between: allo-hBM-MSCs versus conventional approach (*), allo-hBM-MSCDs versus conventional approach (&), allo-hBM-MSCs versus hBM-MSCDs (#).

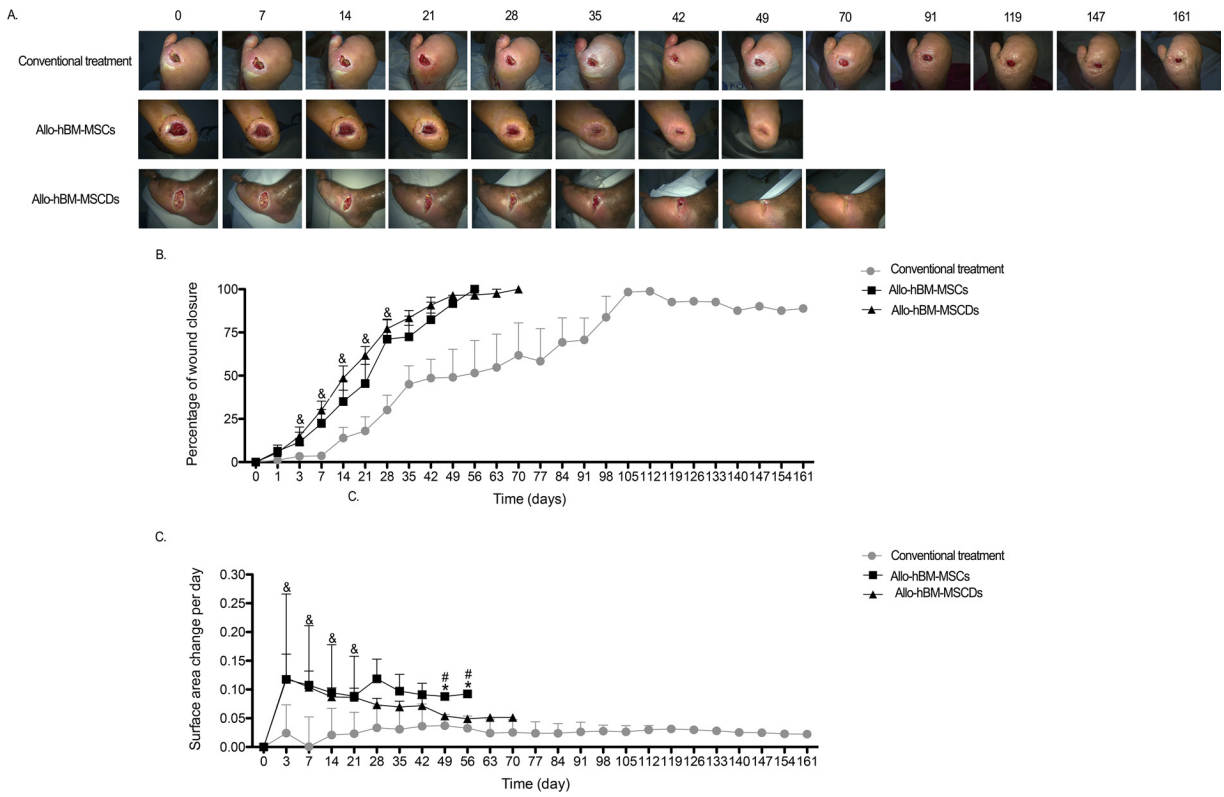

Figure 3. Evolution of wound-healing progression after intradermal administration of allo-hBM-MSCDs until wound closure in patients with grade 2 DFU. (A) Macroscopic analysis of the chronic wound-healing progress before and after intradermal administration of 1 mL vehicle, 1×10^6 allo-hBM-MSCs or 1 mL allo-hBM-MSCDs, (B) percentage of wound closure and (C) wound healing rate. Significant differences between: allo-hBM-MSCs versus conventional approach (*), allo-hBM-MSCDs versus conventional approach (&), allo-hBM-MSCs versus hBM-MSCDs (#).

approach (P = 0.0493) and allo-hBM-MSCs versus hBM-MSCDs (P = 0.0090), respectively. At days 3, 7, 14, 21 and 28, differences were found for grade 2 DFUs between hBM-MSCDs versus conventional approach (P = 0.0332, P = 0.0086, P = 0.0119, P = 0.0012 and P = 0.0052, respectively).

Furthermore, the data suggested that patients with grade 1 DFUs treated with allo-hBM-MSCs and allo-hBM-MSCDs reached 50% of wound closure after approximately 3 and 14 days respectively; in contrast, the patients treated with conventional therapy achieved the same extent of wound closure after approximately 21 days (Figure 2B), whereas those patients with grade 2 DFUs treated with allo-hBM-MSCs and allo-hBM-MSCDs reached 50% of wound closure after approximately 21 and 14 days, respectively; in contrast, the patients treated with conventional therapy achieved the same extent of wound closure after approximately 42 days (Figure 3B).

Wound healing rate

Similarly, changes in wound healing rate per day in both grade 1 and 2 DFUs were noticed earlier in patients treated with allo-hBM-MSCDs and allo-hBM-MSCs compared with the conventional treatment. In particular, after 3 days of treatment, in patients with grade 1 DFUs, allo-hBM-MSCDs–treated wounds showed significant changes in surface area compared with the patients treated with allo-hBM-MSCs or conventional therapy (0.057 cm^2/day, −0.02765 cm^2/day and −0.1111 cm^2/day, respectively) (Figure 2C), whereas at 3 days of treatment in grade 2 DFUs either allo-hBM-MSCDs or allo-hBM-MSCs treated wounds showed greater changes in surface area compared with the patients treated with conventional therapy (0.12015 cm^2/day, 0.11773 cm^2/day and 0.02416 cm^2/day, respectively) (Figure 3C).

After 7 days of treatment in grade 1 DFUs using either allo-hBM-MSCDs or allo-hBM-MSCs, significant surface area changes were obtained, unlike patients treated with conventional therapy (0.0378 cm^2/day, 0.0186 cm^2/day and 0 0004 cm^2/day, respectively) (Figure 2C). In contrast, the results obtained for grade 2 DFUs after 7 days of treatment using both allo-hBM-MSCDs and allo-hBM-MSCs showed greater surface area changes as opposed to patients treated with conventional therapy (0.1039 cm^2/day, 0.1076 cm^2/day and 0.0005 cm^2/day, respectively) (Figure 3C).

At day 14 post-treatment, significant differences were found for grade 1 DFUs comparing allo-hBM-MSCs versus the conventional approach (P = 0.0051), whereas at 3, 7, 14 and 21 days post-treatment, grade 2 DFUs significant differences were found between allo-hBM-MSCDs versus the conventional approach (P = 0.0304, P = 0.0019, P = 0.0071 and P = 0.0176, respectively) and at 49 and 56 days post-treatment, significant differences were found between allo-hBM-MSCs versus the conventional approach (P = 0.0351 and P = 0.0295, respectively) and allo-hBM-MSCs versus allo-hBM-MSCDs (P = 0.0097 and P = 0.0290, respectively) (Figure 3C).

Changes in other ulcer dimensions

The time course of wound healing progress was also monitored by measuring the % reduction in wound perimeter in both grade 1 and 2 DFUs. As shown in Table 4, since week 2, patients treated with allo-hBM-MSCDs and allo-hBM-MSCs presented higher decreases in wound perimeter values compared to patients treated with the conventional approach.

Ulcer-free survival

Ulcer-free survival analysis in DFUs (grade 1 and 2) between allo-hBM-MSCDs versus allo-hBM-MSCs versus conventional approach

Table 4
Percentage reduction in wound perimeter.

Percentage perimeter reduction Time, wk	Grade 1 DFUs			Grade 2 DFUs		
	Conventional treatment (PolyMem dressing)	Allo-hBM-MSCs	Allo-hBM-MSCDs	Conventional treatment (PolyMem dressing)	Allo-hBM-MSCs	Allo-hBM-MSCDs
1	5.61 ± 9.72	29.23 ± 25.95	21.02 ± 26.28	2.95 ± 4.07	28.72 ± 23.15	15.96 ± 8.74
2	18.11 ± 16.44	43.67 ±11.49	36.91 ± 36.69	5.55 ± 8.67	35.27 ± 19.38	28.58 ± 13.82
3	27.44 ± 13.33	81.79 ± 18.21	46.28 ± 36.94	7.79 ± 11.43	52.01 ± 44.30	39.16 ± 11.88
4	50.83 ± 20.96	85.84 ± 14.16	64 ± 36.37	10.13 ± 13.76	80.63 ± 19.38	57.45 ± 20.70
5	52.55 ± 19.19	87.67 ± 12.33	72.46 ± 38.13	23.84 ± 20.29	84.80 ±15.20	64.06 ±18.65
6	75.97 ± 23.84	78.96 ± 21.04	93.86 ± 13.73	29.47 ± 22.81	100 ± 0	83.15 ± 15.68
7	82.45 ± 17.55	100 ± 0	100 ± 0	32.41 ± 28.87	100 ± 0	86.84 ± 14.48

P value wound perimeter Time, wk	Grade 1 DFUs			Grade 2 DFUs		
	Conventional treatment (PolyMem dressing) versus Allo-hBM-MSCs	Conventional treatment (PolyMem dressing) versus Allo-hBM-MSCDs	Allo-hBM-MSCs versus Allo-hBM-MSCDs	Conventional treatment (PolyMem dressing) versus Allo-hBM-MSCs	Conventional treatment (PolyMem dressing) versus Allo-hBM-MSCDs	Allo-hBM-MSCs versus Allo-hBM-MSCDs
1	0.3600 (NS)	0.5204 (NS)	0.1139 (NS)	0.0357 (*)	0.0087 (**)	0.2619 (NS)
2	0.1907 (NS)	0.4547 (NS)	0.9819 (NS)	0.1954 (NS)	0.0476 (*)	0.7528 (NS)
3	0.0233 (*)	0.5311 (NS)	0.3403 (NS)	0.1084 (NS)	0.0139 (*)	0.6359 (NS)
4	0.0451 (*)	0.3267 (NS)	0.7294 (NS)	0.0358 (*)	0.0057 (**)	0.0667 (NS)
5	0.0121 (*)	0.5162 (NS)	0.6668 (NS)	0.0357 (*)	0.0025 (*)	0.1167 (NS)
6	0.2074 (NS)	0.2229 (NS)	0.2254 (NS)	0.0509 (NS)	0.0043 (**)	0.2911 (NS)
7	0.2254 (NS)	0.2254 (NS)	0.2254 (NS)	0.0819 (NS)	0.0095 (**)	0.0932 (NS)

Allo-hBM-MSCDs, allogeneic human bone marrow mesenchymal stromal cells derivatives; allo-hBM-MSCs, allogeneic human bone marrow mesenchymal stromal cells; DFUs, diabetic foot ulcer; NS, not significant.
(*) (**) statistical significance

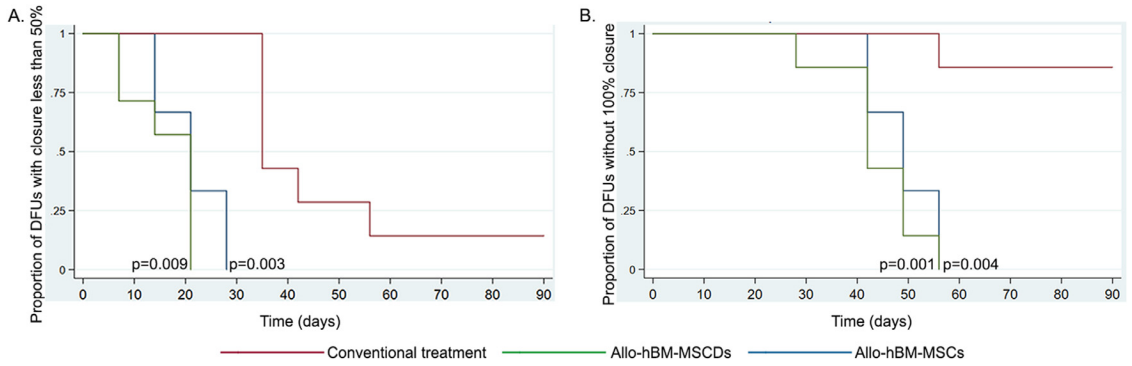

Figure 4. Ulcer-free survival analysis in patients with grade 1 and 2 DFUs. (A) Proportion of DFUs with closure less than 50% between conventional treatment versus allo-hBM-MSCDs versus allo-hBM-MSCs, (B) proportion of DFUs without 100% closure comparing conventional treatment versus allo-hBM-MSCDs versus allo-hBM-MSCs.

showed that DFUs treated with allo-hBM-MSCDs reached 50% of wound closure at 21 days post-treatment, whereas allo-hBM-MSCs reached 50% of wound closure at 28 days post-treatment compared with the conventional approach, where 90% of DFUs had 50% wound closure at 90 days post-treatment with a significant difference (P = 0.009 and P = 0.03, respectively) (Figure 4A).

In addition, DFUs treated with allo-hBM-MSCDs and with allo-hBM-MSCs achieved 100% of wound closure at 56 days post-treatment, compared with the conventional approach, where 60% of DFUs had a complete wound closure at 90 days post-treatment with significant differences (P = 0.001 and P = 0.004, respectively) (Figure 4B). No significant differences were found between the allo-hBM-MSCDs and allo-hBM-MSCs groups (Figure 4). Ulcer-free survival analysis was adjusted by age and baseline glycated hemoglobin A1c; however, these variables did not interfere with the final outcome.

HRs and healing rate (person-day)

Age, female sex and baseline glycated hemoglobin A1c variables were not significantly related to HRs for DFU closure at 50% and 100% (Table 5).

Grade 1 DFU closure rate at 50% was 2.46 times greater than grade 2 DFUs. Furthermore, DFUs treated with allo-hBM-MSCs and allo-hBM-MSCDs had a ≈10-fold greater closure rate than DFUs treated with conventional approach, with a statistically significant difference (P = 0.002 and P = 0.003, respectively). The DFUs closure rate person/day for the closure at 50% was 0.064 person/day for grade 1 DFUs, and 0.031 person/day for grade 2 DFUs. In addition, the closure rate person/day for the 50% closure of the DFU treated with the conventional approach was 0.022 compared with the allo-hBM-MSC group (0.078) and the allo-hBM-MSCDs group (0.062) (Table 5).

In addition, the closure rate of the grade 1 DFUs at 100% was 2.8 times greater than grade 2 DFUs. Furthermore, DFUs treated with

Table 5
Hazard ratios and healing rate (person/day).

Variable	Closure rate at 50%				Closure rate at 100%			
	HRs	CIs	*P* value	Healing rate (person/day)	HRs	CIs	*P* value	Healing rate (person/day)
Age	0.989	0.937–1.043	0.688	NA	0.995	0.941–1.052	0.868	NA
Female	1.206	0.532–2.734	0.653	NA	0.850	0.362–1.990	0.708	NA
HbA1c	1.166	0.884–1.539	0.276	NA	1.038	0.796–1.354	0.779	NA
Closing rate according to DFUs type								
Grade 1				0.0647				0.025
Grade 2	2.468	1.040–5.853	0.04	0.0318	2.844	1.218–6.638	0.016	0.010
Treatment								
Conventional treatment (PolyMen dressing)				0.022				0.005
Allo-hBM-MSCs	9.921	2.390–41.182	0.002 *	0.078	7.795	1.891–32.135	0.004 *	0.025
Allo-hBM-MSCDs	9.132	2.175–38.335	0.003 *	0.062	10.085	2.668–38.112	0.001 *	0.025

Allo-hBM-MSCDs, allogeneic human bone marrow mesenchymal stromal cells derivatives; allo-hBM-MSCs, allogeneic human bone marrow mesenchymal stromal cells; CI, confidence interval; DFUs, diabetic foot ulcer; HRs, hazard ratios; NA, not applicable.
(*) statistical significance

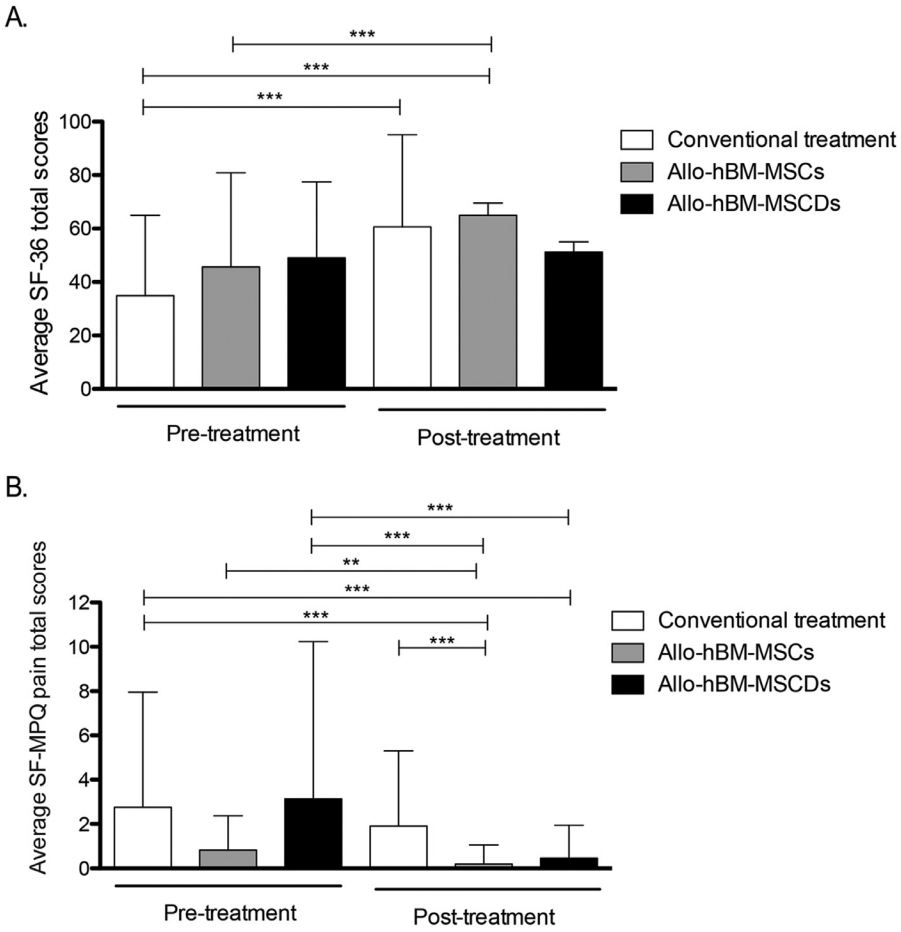

Figure 5. Evaluation of quality of life and pain in patients with grade 1 and 2 DFUs. (A) Average SF-36 total scores in patients with DFUs treated with conventional, allo-hBM-MSCDs and allo-hBM-MSCs and (B) average SF-MPQ pain total scores in patients with DFUs treated with conventional, allo-hBM-MSCDs and allo-hBM-MSCs. Significant differences (*** <0.0001) (** <0.0072). SF-MPQ, short-form McGill Pain Questionnaire.

allo-hBM-MSCs and allo-hBM-MSCDs had a closure rate ≈7 and ≈10 times greater, respectively, than DFUs treated by the conventional approach, with a statistically significant difference (P = 0.004 and P = 0.001, respectively). The closure rate of DFUs person/day for 100% closure was 0.025 person/day for grade 1 DFUs and 0.010 person/day for grade 2 DFUs. In addition, the closure rate person/day for 100% closure of DFU treated with the conventional approach was 0.005 compared with the allo-hBM-MSC group of 0.025 and the allo-hBM-MSCD group of 0.025 (Table 5).

Quality of life

Data regarding the SF-36 scores of the participants are shown in Figure 5. Participants' quality of life improved after treatments. Significant differences were found between conventional and allo-hBM-

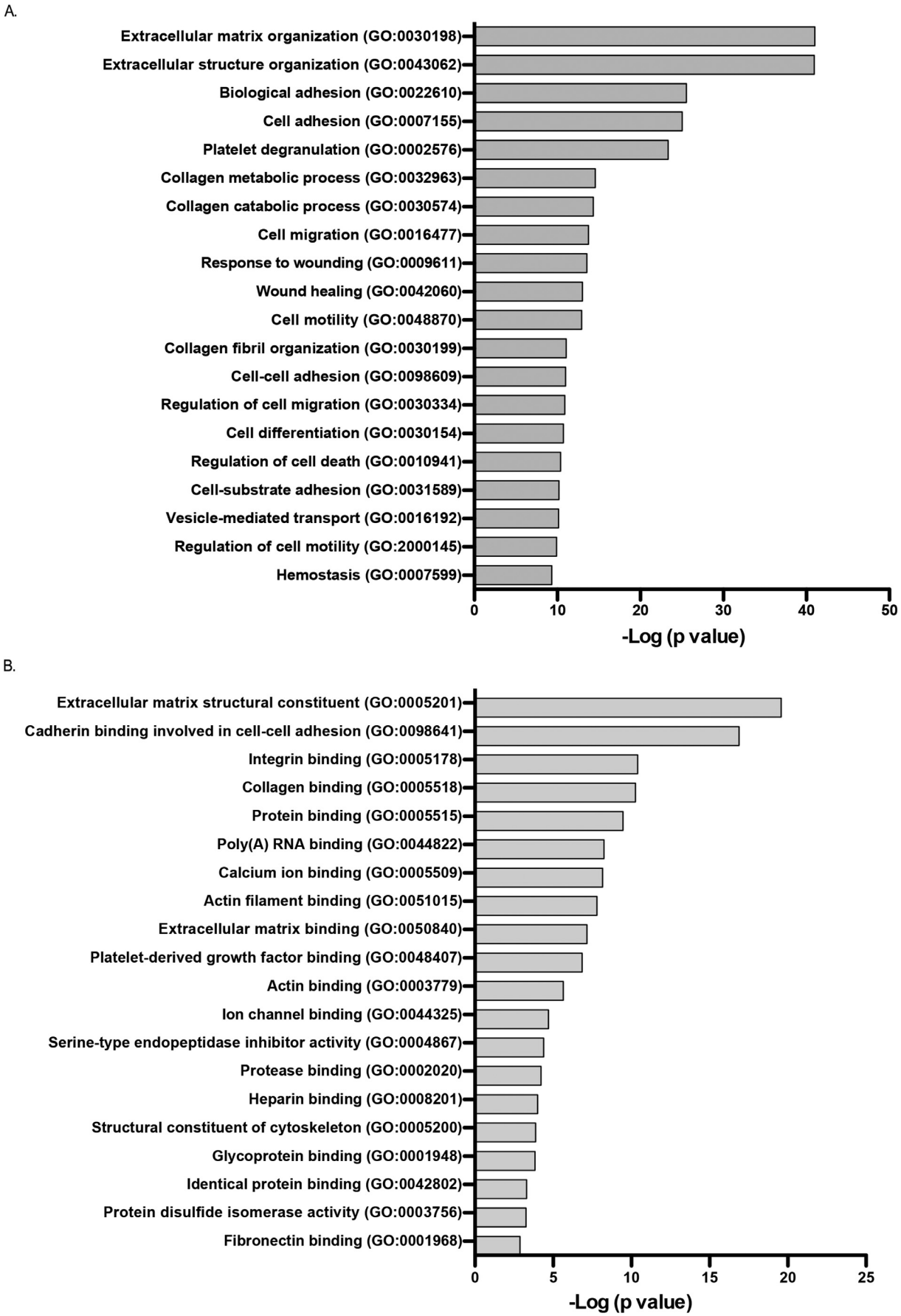

Figure 6. Proteomic analysis of the allo-hBM-MSCDs. Gene ontology (GO) analysis represents the top 20 GO terms and their number of enrichment in (A) biological process and (B) molecular function.

MSC groups before and 1-month post-treatment ($P < 0.0001$) (Figure 5A).

Pain evaluation

Evaluating pain in the participants with DFUs treated with a conventional, allo-hBM-MSCs and allo-hBM-MSCDs approach, we found there were statistically significant differences between the allo-hBM-MSC and allo-hBM-MSCD groups after 1-month post-treatment ($P = 0.0072$ and $P = 0.0003$, respectively) (Figure 5B).

Proteomic analysis of the allo-hBM-MSCDs

Finally, proteomic analysis revealed the biological process highly involved in the wound healing process. A total of 348 different proteins were found in the allo-hBM-MSCDs, in the absence of the plasma proteins (Supplementary Table 1). Among them, many of the representative proteins involved in matrix organization, e.g., fibronectin, collagen families, vimentin and laminin, among others were found. In addition, a variety of growth factors, such as the vascular endothelial growth factor (VEGF) family, transforming growth factor-beta 1 (TGF-β1), epidermal growth factor and insulin-like growth factor 1 (IGF-1) and inflammatory cytokines such as tumor necrosis factor superfamily and interleukins (interleukin-6, interleukin-3) were found. In addition, by combining proteomics with GO analysis, we were able to comprehensively classify the secreted proteins by both biological process and molecular function (Figure 6). Allo-hBM-MSCDs would be highly involved in the wound-healing process, exhibiting GO terms of extracellular matrix organization, angiogenesis, cell migration and wound healing, among others.

Discussion

MSC therapies are emerging as a promising strategy to promote tissue repair and may extend their utility to treat skin lesions. In addition, their secretome has shown promising results in tissue repair (including heart, nerves and skin), indicating that released factors induce regeneration rather than the cells themselves [11]. Based on this, the present work evaluated the effect of allo-hBM-MSCDs in grade 1 and 2 DFUs as a novel therapeutic healing approach. To our knowledge, this is the first phase 1/2 clinical trial to test allo-hBM-MSCD-based therapy for human DFUs healing.

A major concern with MSC therapy is the safety profile. To date, most of the published pre-clinical and clinical trials have reported that MSCs or their derivatives are safe when being used for the treatment of numerous injuries and diseases [21,26-34].

The present study did not detect serious AEs, like malignancy, infection, organ system complications or acute toxicity related to allo-hBM-MSCD and allo-hBM-MSC injections used to treat DFUs. These findings were consistent with the evidence from numerous clinical trials that evaluated the safety of MSC-based therapy in skin wound healing [35–45]. However, several studies about the susceptibility of malignant transformation of previous DFUs becoming verrucous carcinoma have been described [46–48].

In contrast, the transmission of viruses by animal sera represents a considerable risk for humans and animals, particularly when the serum is used for the production of biological products. Procedures applicable for inactivating large numbers of different viruses are therefore mandatory. For this purpose, in our study, the FBS used is subjected to gamma irradiation to reduce viral contamination [49–52]. In addition, the company that provided the FBS guarantees the least risk of bovine spongiform encephalopathy and lower viral risk; also, this product also meets quality specification tests that include virus testing panels according to EMA/CHMP/BWP/457920/2012, EMEA/CVMP/743/00 and CFR Title 9 part 113.53(c)[113.46, 113.47]. Furthermore, several researchers have made significant efforts to develop and validate FBS substitutes, including serum-free media [53], and alternatives derived from human blood components such as autologous serum, umbilical cord blood serum, human plasma and human derivatives from platelet-rich plasma, among others [54,55]. In this context, after thawed, allo-hBM-MSCs were cultured in Alpha-MEM supplemented with 10% plasma obtained from donors with type AB blood for the generation of the allo-hBM-MSCDs and allo-hBM-MSCs to reduce contamination risk.

Regarding the effectiveness profile of the MSC-based treatments, it has been observed that some promising preclinical results often remain far short of expectations in large controlled clinical trials [56]. In particular, our findings in the previous preclinical studies were in accordance with the results of our clinical trial, which showed that the administration of allo-hBM-MSCDs or allo-hBM-MSCs to patients with grades 1 and 2 DFUs led to positive short-term outcomes.

In fact, we demonstrated that allo-hBM-MSCD or allo-hBM-MSC injections increased wound closure rate being evident at day 1 after allo-hBM-MSCD and allo-hBM-MSC administration in grade 1 and 2 DFUs; conversely, this effect was less marked with conventional treatment and only by day 7 post-treatment in grade 1 or day 14 post-treatment in grade 2 DFUs. In addition, the healing rate of wounds treated with allo-hBM-MSCDs and allo-hBM-MSCs was significantly greater than in the control group. In this context, after combined therapy of allo-hBM-MSCDs or allo-hBM-MSCs with PolyMem dressing, patients showed improved wound status, and all wounds were closed entirely within 5- and 7-weeks' post-treatment, in comparison with the control group, where the participants responded poorly to conventional treatment (PolyMem dressing) and some wounds were wholly closed following 12 weeks post-treatment whereas others were still opened.

In contrast, DFUs, even when healed successfully, have a high rate of recurrence in the long term. In our study during 1-year follow-up, we observed the absence of ulcer recurrence in all the patients who received allo-hBM-MSCDs or allo-hBM-MSCs, conversely to the control group, in which there was no long-term complete skin restoration.

These observations suggest that the effect of MSC-based therapy might ensue in response to the following key mechanisms: (i) the ability to differentiate and transdifferentiate into tissue-specific cells, (ii) fuse with the resident cells and (iii) secrete a wide array of paracrine factors to stimulate the survival and functional recovery of the resident cells, or to regulate the local microenvironment and immune response. These mechanisms are probably independent but not mutually exclusive. In many circumstances, a combination of these protective mechanisms might work together to affect cutaneous wound healing [57].

Nevertheless, various investigations using animal models (including ours) [13,14] and our proof-of-concept studies in grade 2 DFUs [15] discern that paracrine factors appear to be the leading MSC-therapeutic element entailed in the repair of skin lesions, as evidenced by the fact that only a small percentage of the engrafted MSCs become incorporated and survive within the damaged tissue. Also, several studies revealed that the implantation time of MSCs is usually too short to have an effective impact, whereas others indicated that transplanted MSCs do not necessarily have to be close to the damaged tissue to promote wound repair and functional recovery.

Wound healing is a highly complex and dynamic process and remains a major challenge in modern medicine. The optimal healing of a cutaneous wound requires a well-orchestrated integration of the complex biological and molecular events of secretion of growth factors, cytokines and chemokines, cell migration and proliferation, as well as extracellular matrix deposition and remodeling during the distinct phases of the process healing of the skin, which includes hemostasis, inflammation, proliferation and remodeling (or maturation phase) [7]. Meanwhile, non-healing, chronic wounds predominantly remain in the early inflammatory stages of wound healing,

lacking the controlled synchronization and succession of events that lead to rapid and complete healing [58]. Particularly, in individuals with diabetes, more than 100 physiological factors have been attributed to wound-healing deficiencies, including decreased or impaired growth factor production, angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, the quantity of granulation tissue, keratinocyte and fibroblast migration and proliferation, number of epidermal nerves, bone healing and the balance between the accumulation of extracellular matrix components and their remodeling by matrix metalloproteinases, among others [59,60]. Due to the strong wound-healing effects observed with the drug substance called allo-hBM-MSCDs, this could contain a large variety of biomolecule classes, including proteins, extracellular vesicles, peptides, nucleotides and lipids. Previously, we evaluated that the biological activity of individual fractions of allo-hBM-MSCDs tested in the full-thickness excisional wound models was significantly inferior compared with that of the entire secretome (data not shown). This suggests that the effect of allo-hBM-MSCDs depends on the synergy of its components.

Proteases are an indispensable element during wound healing and regeneration. They regulate the clearance of damaged proteins and matrix and facilitate cell infiltration [61]. However, in some cases, proteases impair tissue repair through excessive tissue degradation. Especially in chronic wounds such as DFUs, stimuli such as bacteria, foreign material and impaired tissue lead to the elevated and prolonged presence of proteases at the wound site. This aberrant expression of tissue-degrading enzymes results not only in poor healing outcomes but also in the degradation of pro-regenerative growth factors [61–63]. In this context, growth factor–based therapies such as the allo-hBM-MSCDs might present limitations due to the high levels of proteolytic activity in the injured tissues, which leads to poor growth factor stability and rapid enzymatic degradation, and, therefore, a short half-life [64,65]. Thus, multiple administrations and/or supraphysiological doses are often necessary to sustain an effective concentration of growth factors at the delivery site. As a result, we administrated 2 doses of allo-hBM-MSCDs (secretome group). In contrast, some studies have reported that local or intra-tissue delivery of the MSCs has shown a greater delivery retention survival rate, engraftment and maintenance of cellular function as well as efficiency of their full therapeutic potential [66].

Our characterization of the allo-hBM-MSCDs by proteomic analysis showed that it contained a remarkable spectrum of proteins relevant to extracellular matrix organization (e.g., fibronectin, collagen subunits, vimentin, etc.) and growth factors (e.g., TGF-β1, VEGF, IGF-1, epidermal growth factor, collagen, etc.), which are highly involved in the wound-healing process in terms of extracellular matrix remodeling, angiogenesis and cell migration, among others [67]. In this context, several authors agree with our findings that proteins such as VEGF, IGF-1 [68], TGF-β1 [69], connective tissue growth factor [70], collagen alpha-1 chain [71], plasminogen activator inhibitor 1 [72], metalloproteinase inhibitor 1 [73], thrombospondin-1 [74], decorin [75], periostin [76], interleukin-6 [77] and alpha-2-macroglobulin, found in others' proteomic analysis, trigger wound-healing processes [78].

It is highlighted that some of the proteins found in our GO analysis (GO:0042060) have not been involved in the wound-healing process, which glimpses novel factors in this process through the use of allo-hBM-MSCD (Supplementary Table 2).

An apparent reason for obtaining a number of secreted proteins could be that the maturing culture of allo-hBM-MSCs promoted the secretion of the biological molecules as well as maximized the productive efficacy of the allo-hBM-MSCDs, which eventually facilitated the tissue repair process.

Moreover, the use of the allo-hBM-MSCD therapy without cells but with all favorable factors has decisive virtue over cell-containing products, e.g., in the possibility of viral clearance, immune rejection, tumorigenicity, better storage options, longer shelf life, less complex and costly large-scale production, greater reproducibility among them and easier handling in the patient application.

One of the limitations of this study was the relatively small sample size, which restrains the statistical power. Nevertheless, our results provide new knowledge about the safety and efficacy of allo-hBM-MSCDs in the treatment of grade 1 and 2 DFUs and implications for further research. It is recommended to do a multi-centric randomized phase 2 clinical trial, which includes a large number of subjects from different geographic places to gather more information about allo-hBM-MSCDs effectiveness.

Our cumulative results suggest that combining intradermal administration of allo-hBM-MSCDs with a dressing in patients with grade 1 and 2 DFUs enhances the wound-healing process in a similar way that it was observed in patients treated with allo-hBM-MSCs and a dressing. Thus, our phase 1/2 clinical trial is relevant, as it highlights the possible use of allo-hBM-MSCDs, which opens the way to a newly emerged cell-free therapy to treat DFUs, which could be part of the comprehensive management of DFUs.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Conception and design of the study: MLA and LCM. Wound-healing treatment: LCM. Patient follow-up: MLA. Proteomic experiments and analysis of data: SMB, VS, EC and MDP. Bone marrow aspirate: CLS. Analysis of scale Short-form 36 and scale McGill Pain Questionnaire: AKA and LTGV. Writing the manuscript: MLA. Critical feedback to the final version of the manuscript: SMB, VS, EC, CLS, AKT and LCM.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcyt.2022.04.002.

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