

Evaluation of a cell-based osteogenic formulation compliant with good manufacturing practice for use in tissue engineering

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Research

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Abstract

Background: Proper bony tissue regeneration requires mechanical stabilization, an osteogenic biological activity and appropriate scaffolds. The latter two elements can be combined in a hydrogel format for effective delivery, so it can readily adapt to the architecture of the defect.

Results: We evaluated a Good Manufacturing Practice-compliant formulation composed of bone marrow-derived mesenchymal stromal cells in combination with bone particles ($\emptyset = 0.25$ to $1 \mu\text{m}$) and fibrin, which can be readily translated into the clinical setting for the treatment of bone defects, as an alternative to bone tissue autografts. Remarkably, cells survived with unaltered phenotype (CD73+, CD90+, CD105+, CD31-, CD45-) and retained their osteogenic capacity up to 48 h after being combined with hydrogel and bone particles, thus demonstrating the stability of their identity and potency. Moreover, in a subchronic toxicity *in vivo* study, no toxicity was observed upon subcutaneous administration in athymic mice and signs of osteogenesis and vascularization were detected two months after administration.

Conclusions: The preclinical data gathered in the present work, in compliance with current quality and regulatory requirements, demonstrated the feasibility of formulating an osteogenic cell-based tissue engineering product with a defined profile including identity, purity and potency (*in vitro* and *in vivo*), and the stability of these attributes, which complements the preclinical package required prior to move towards its use of prior to its clinical use.

Background

Failure of the physiologic reaction to acute or chronic bone disorders (e.g. fractures, non-unions, large defects after trauma or tumors) typically requires surgical intervention and implantation of bone grafts. Autografts and heterologous transplants of bony tissue, as well as the use of implants made of biomaterials, are the most common approaches in today's orthopedics field (1). Among them, the bony autograft sourced from the iliac crest is the preferred treatment option for a wide range of orthopedic conditions, including the management of complex fractures or in non-union defects (1, 2). The use of autologous tissue as a vector for bony regeneration fulfils three key requirements: 1) introduces cells with osteogenic potential, 2) offers structural support (osteoconduction), and 3) contributes with growth factors that promote vascularization and osteoinduction (1). However, the complete substitution of the damaged bony tissue is not always achieved through the use of autografts, which can lead to the failure of the autograft at long term (3). On the other hand, the surgical collection of autologous bone is highly associated with morbidity (4), which in some cases can be overcome by using allogeneic, decellularized human bone from tissue banks. However, such strategy lacks the benefits associated to the regenerating activity displayed by osteogenic cells, which have been demonstrated to be a key factor in our hands, after its successful use in animal models and clinical cases (5, 6), in accordance with the "diamond concept" referring to the need of osteoinductive mediators, osteogenic cells, an osteoconductive scaffold and mechanical stability (1). In fact, autografts are preferred for treating large bony defects even when bony tissue from tissue bank is available. Unfortunately, this option is not valid for all patients and it is

not exempt of risks of the procedure required for tissue extraction, as discussed previously. Therefore, when this approach is not feasible (i.e. re-interventions, donor site morbidity, infections), the use of osteogenic cells isolated from bone marrow (BM), either as bulk concentrates or enriched in multipotent mesenchymal stromal cells by *ex vivo* expansion, is an alternative that has already been explored in large animal models, and also in early Phase I/II clinical trials with encouraging results (5–8). It is particularly interesting the development of products that combine culture-expanded multipotent Mesenchymal Stromal Cells (MSC) and scaffolds, namely “Tissue Engineering Products” (TEP), resulting in a new medicinal entity with osteogenic potential that is specifically regulated as advanced therapy and needs to comply with pharmaceutical regulations (9).

In the present study, we formulated TEP based on the use of cells with osteogenic potential (namely, *ex vivo* expanded MSC that were compared to BM concentrates) combined with bony particles from tissue bank embedded in a hydrogel that, altogether, can induce the generation of new tissue while adapting to the diverse architecture of the simulated cylindrical bony defects. In agreement with current quality and regulatory requirements, TEP’s stability, osteogenic potential and *in vivo* safety were assessed comprehensively under Good Laboratory and Manufacturing Practices.

Material And Methods

Study design

As part of a larger preclinical package required by Regulatory Authorities that grant permission to move forward clinical testing of a TEP composed of an active ingredient (BM- MSC) and scaffold (fibrin and bone particles), the present regulatory study complements previous work on the active ingredient (10), and a proof-of-concept of using allogenic BM- MSC for bone formation in a translational animal model (11, 12).

Cell cultures

Clinical grade *ex vivo* expanded MSC derived from the mononuclear cell (MNC) fraction of BM aspirates were produced in the context of a clinical trial (EudraCT No. 2010- 024041–78) with appropriate donor informed consent following Good Manufacturing Practice (GMP)-compliant methods reported elsewhere (10). Cells were further expanded *in vitro* up to sufficient numbers (always under passage 4) by using Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco) containing 2 mM glutamine supplemented with 10% human serum (hSer) B/AB (13, 14). All cultures were maintained at 37°C and 5% CO₂ in humidified incubators. Cell number and viability were determined by either the hemocytometer-based Trypan blue dye exclusion method or by using ViaCount Reagent and analysis in a Guava EasyCyte Mini device with Guava® Suite Software v3.3 following the manufacturer’s instructions (EMD; Millipore, Billerica, MA, USA). Each sample was analyzed twice and the average value was calculated. *Differentiation assays*

Specific differentiation media (StemPro; Gibco) were used for the adipogenic, chondrogenic and osteogenic induction of undifferentiated MSC cultures *in vitro*. Oil Red O (Sigma), Safranin O (Sigma) and Alkaline Phosphatase (Takara Bio Inc.) stainings were performed for the determination of the outcome of the differentiation assays in triplicates, as described elsewhere (15).

Stability assessment

The effect of fibrinogen on the stability of BM-MSC (from 4 independent donors) was assessed by determining cell viability, phenotype, capacity to adhere to plastic surfaces and osteogenic differentiation capacity at different times (up to 48h) with freshly prepared cellular suspensions (16).

Cell Proliferation Assay

Cell proliferation was monitored by using the ATP-based CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison WI, USA) following the vendor's instructions. Briefly, cells were cultured in multiwell format plates and, at the time of analysis, luminescent reagent was added to each well at 1:1 (v/v) ratio with respect to culture medium and shook for 2 min using an orbital shaker. After 15 min incubation in the dark, 100 µL of supernatant from each well were transferred into opaque-walled 96 well-plates and their luminescence was measured in triplicates on a Triad Multimode detector plate reader with Concert Triad Series software v2.1 (Dynex Technologies, Chantilly, VI).

Flow cytometry

Fluorescence-Activated Cell Sorting (FACS) analysis was performed to evaluate expression of surface markers using 1:20 dilution of antibodies described next: CD31 (WM59, BD Pharmingen ref. 555445), CD45 (HI30, BD Pharmingen ref. 555482), CD73 (AD2, BD Pharmingen ref. 550257), CD90 (F15-42-1-5, Beckman Coulter ref. IM1839U), CD105 (43A4E1, Miltenyi Biotec ref. 130-094-941) and HLA-DR (L243, BD Biosciences ref. 347400) in a FACSCalibur device with CellQuest Pro software v5.1 (Becton Dickinson). Each sample was analyzed twice and the average value was calculated. Validation of the identification methods was reported elsewhere (14).

Preparation of osteogenic tissue engineering product

Osteogenic hydrogels were prepared following a Good Manufacturing Practice compliant procedure. Briefly, a cellular pellet was obtained and then resuspended in a volume of saline solution supplemented with 2% (w/v) of human serum albumin (HSA; Albutein®, Grifols). Then the same volume of fibrinogen (Tissucol Duo, Baxter) was added. This mixture was combined with cadaveric, particulated, decellularized and deantigenized bone from tissue bank approved for human use (diameter of particles comprised

between 0.25–1 mm) and commercial thrombin (Tissucol Duo, Baxter) diluted 1:100 in saline solution (final concentration 50 UI/mL).

Animals

Eighteen healthy 7 to 8 weeks old Crl:NIH-Foxn1^{rnu} female mice (Charles River Laboratories, France) were randomly distributed at a maximum of 3 animals per cage according to the assigned experimental group (namely, acellular control, MNC-loaded TEP, and MSC-loaded TEP), after a 7-day acclimatization period in autonomous pressurized units. Animals were housed together in Specific Pathogen Free (SPF) facilities, fed a standard diet and allowed access to water *ad libitum*. All animal care and experimental procedures adhered to the recommendations of local, national, and European laws (Decret 214 de 1997, Real Decreto 53 de 2013, European directive 86/609/CEE of 1986, respectively) and were approved by the *Universitat Autònoma de Barcelona's* Ethical Committee on Human and Animal Experimentation (Ref. No. CEAAH 1972), and registered by *Generalitat de Catalunya* (Reg. No. DMAiH 6967). This study was performed under Good Laboratory Practices (GLP) and inspected by our institutional Quality Assurance Unit (QAU) and verified by the competent regulatory authority. The athymic mouse strain Crl:NIH-Foxn1^{rnu} (17, 18) was chosen in this study for carrying a mutation in the *mu* locus in chromosome 10 resulting in a deficiency of functional T lymphocytes, a characteristic required for the heterologous transplantation of human biologic material (18–20).

Experimental surgery

Animals were induced with inhalatory anesthesia consisting of 4% isoflurane mixed with 100% oxygen in an anesthetic chamber. Anaesthetized animals were placed on a heating blanket and maintained on isoflurane 2% mixed with 100% oxygen administered through an anesthetic mask. Two mg/kg meloxicam were administered subcutaneously for analgesia, and ophthalmic lubricant was placed on the eyes in order to prevent eyes dryness. The surgical zone was sterilized and a 1 cm long incision was made in the epidermis of the dorsal zone, caudal to the shoulder blade and perpendicular to the spinal column. In order to perform the incision, the skin was pinched and pulled using blunt-ended surgical forceps and incised with straight-bladed Mayo scissors. Then, the subcutaneous area was dissected cranially to the incision forming a pouch where the test and reference items were placed using curved Adson forceps. After implantation, the surgical wound was closed with two surgical staples and the animals were maintained on the heating blanket until total recovery before returning them to the original cage (*Supplemental Figure 1*).

Necropsy and histology

A systematic, ordered and full necropsy was performed in all animals to assess any potential toxicity of the treatments. To this end, animals were euthanized by an overdose of sodium pentobarbital (200

mg/kg, 60 mg/mL) administered intraperitoneally after the two-month follow-up period. The macroscopic analysis included the assessment of the musculature, fur, skin and natural orifices. Additionally, the brain and cranium, thoracic cavity and mediastinum, trachea, esophagus, glands and lymph nodes, lungs, heart, abdominal cavity including stomach, small and large intestine, liver, spleen, kidneys and genitourinary system were examined. All these organs were then extracted, minced, fixed and embedded in paraffin for further histological studies. Two μm thick microtome sections of the specimens were cut in the sagittal plane and examined by routine hematoxylin and eosin (H&E) staining. Osteogenic potential of the treatments was evaluated by blinding histopathology following a grading score for specific bone formation parameters (*Supplemental Table 1*).

Data analysis

Descriptive data was expressed as mean \pm standard deviation (number of replicates) or mean (range of values). One-way ANOVA test and Bonferroni's multiple comparison tests were performed to evaluate differences in water and food consumption, body weight gain. Paired t-tests were performed to evaluate differences in size and histological scores. Statistical significance was set at * $p < 0.05$ and determined using GraphPad Prism program v5.03 (GraphPad software Inc., La Jolla, CA).

Results

Sourcing of cells, characterization and formulation of an osteogenic hydrogel

First, cells isolated from BM aspirates were successfully expanded in culture up to sufficient numbers for use in the series of *in vitro* and *in vivo* experiments described next. Phenotypic profiles of BM-derived MSC (BM-MSC) were consistent with their mesenchymal identity, being positive for the expression of CD90, CD73, CD105; negative for CD31 and CD45; and slightly positive for HLA-DR (*Figure 1A*). *In vitro* differentiation assays confirmed the multipotentiality of culture expanded BM-MSC into the adipogenic, chondrogenic and osteogenic lineages (*Figure 1B*).

The osteogenic formulation studied here was composed of A) a cellular component made of cells resuspended in saline solution and fibrinogen at 1:1 (v/v); and B) an acellular component made of bony particles and 1% (v/v) diluted thrombin to avoid immediate polymerization of fibrin. Provided that current clinical practice employs bone marrow concentrates as source of osteogenic cells in several indications, we decided to include an additional formulation with MNC as comparator for BM-MSC in animal studies.

MSC embedded in fibrinogen survived and maintained their phenotype and osteogenic potential

The effect of fibrinogen on cellular stability was investigated by assessing cell viability, which remained stable up to 48 hours at 2–8 °C (*Figure 2A*). Phenotype at 24 h was consistent with initial MSC identity determined at the start of the experiment (*Figure 2B*). In order to understand whether cells retained their capacity to adhere to plastic surfaces along the course of the study, as a surrogate marker of cell viability, we evaluated this parameter at 0, 6, 18, 24 and 48 h to complement cytometric data. Interestingly, cells were viable along the study time and no gross differences were observed regarding the morphology of adherent cells during the first 24 hours (*Figure 2C*). However, at 48 h, most of the cells stayed in the supernatant and did not display the capacity to adhere to cell-culture treated plastic surfaces. Despite the presence of fibrinogen for 24 hours, incubation of BM-MSC in osteogenic medium for 8 days resulted in readily differentiation into osteoblasts as revealed by positive ALP staining (*Figure 2D*).

Preparation of constructs

First, a pellet of cells was obtained and then resuspended in a volume of saline solution supplemented with 2% (w/v) of human albumin and fibrinogen at 1:1 (v/v) with a final concentration of fibrinogen in the range of 35–55 mg/mL. This mixture was combined with cadaveric, particulated, decellularized and deantigenized bone from tissue bank (diameter of particles comprised between 0.25–1 mm) and thrombin diluted 1:100 (v/v) in saline solution resulting in a final concentration of 5 UI/mL. We tested this formulation in 1 mL syringes whose edges were previously cut, with the aim of simulating a cylindrical bone defect (*Supplemental Figure 1C*). Under such conditions, the jelly mixture clotted within minutes while adapting perfectly its shape to the cylindrical shape.

Next we generated cylindrical constructs as a model experimental situation of cylindrical defect, using three formulations: acellular (control group), MNC-loaded and MSC-loaded TEPs. The cellular doses were 48×10^7 MNC/cm³ of bone and 60×10^6 MSC/cm³ of bone (*Figure 3A*). Those cylinders were cut in a way that 0.2 cm³ constructs were obtained for the following *in vivo* verification of their safety and osteogenic and angiogenic capacity.

In vivo experiments demonstrating vascularization and osteogenesis

In addition to *in vitro* analyses, *in vivo* studies were performed to demonstrate safety and efficacy of the osteogenic formulation reported here. Constructs of 0.2 cm³ were implanted subcutaneously between the scapulae of athymic mice and they were followed up for 2 months. All animals survived the surgery without any adverse reactions to the procedure. Several clinical parameters were monitored throughout the study (including weight, general condition, wound appearance, food and water intake). The surgical wound healed normally in all animals, which presented a healthy general condition throughout the experimental phase. No spontaneous mortality occurred in the course of the study, nor was any pathological condition observed. All animals increased in body weight throughout the study, only a slight

decrease in the body weight gain immediately after administration of the constructs was observed, which can be attributed to animal handling and surgery (*Figure 3B*). A full necropsy at termination was performed to all animals in order to rule out any potential toxicity of the TEP, but only unspecific findings were observed in all three experimental groups, irrespective of treatment, which were not considered to have pathologic relevance thus confirming the lack of toxicity (*Supplemental Figure 2*). In addition, no signs of rejection of the implants were observed macroscopically.

In the control group, the constructs were absorbed gradually until becoming unnoticeable macroscopically (with the exception of one animal, as shown in *Supplemental Figure 3* at week 5). In the groups treated with cell-based TEPs, a reduction of the initial size of the constructs was observed although they increased gradually thereafter reaching a peak of growth at five weeks after surgery and further shrunk until the euthanasia at week 8. At termination, the dimensions of the constructs varied between experimental groups ($\emptyset = 1 \pm 2.4$, 6.8 ± 6.1 and 12 ± 10.0 mm² for control, MNC and BM-MSC, respectively), being MSC-loaded TEP the one showing a significantly higher size increase compared to the control group ($p = 0.0191$) (*Figure 3C*). Interestingly, the presence of MSC prevented the fragmentation of the test item that remained in one single piece along the course of the study, whilst multifocal fragmented particles were observed in some animals from the group treated with MNC (*Supplemental Figure 2*). Despite the evident increased size of new tissue in the BM-MSC treated group, differences in the overall histological scores were non-significant in all cases although highest values were observed in the animals treated with BM-MSC (6.1 ± 1.7 , 5.9 ± 1.8 , and 7.7 ± 1.3 , for control, MNC- and BM-MSC-loaded TEPs, respectively) (*Table 1*). Remarkably a statistically significant increment of vascularization was observed in the BM- MSC-treated group compared to both control (2.6 ± 0.5 vs 0.75 ± 1.0 ; $p = 0.0650$) and MNC- treated animals (2.6 ± 0.5 vs 1.2 ± 0.8 ; $p = 0.0285$). Although no evident osteoblastic lines were found, which may have indicated the formation of secondary bone substance associated to the bone particles, eosinophilic islands of osteoid were observed in all experimental groups, as early sign of osteogenesis (*Figure 3D*).

Discussion

Treatment of bone defects using osteogenic cell-laden hydrogels that can adapt to the architecture of the lesion might soon become a useful tool for orthopedic surgeons (21). In the present study, we explored whether BM-MSC expanded under a Good Manufacturing Practice (GMP)-compliant production process preserved their viability, identity and osteogenic potential when combined with commercial fibrin and decellularized and deantigenized bone particles sourced from tissue bank as a TEP. We found that indeed cells remained viable and preserved their osteogenic capacity in such osteogenic formulation, so it could be potentially used in the treatment of a wide range of orthopedic conditions or even as bioink in 3D bioprinting strategies (22, 23). Regarding the phenotype, although HLA-DR expression was higher than the criteria established by the International Society for Cell and Gene Therapy (ISCT) (24), it did comply with product specifications that were approved by the competent authority (14). Indeed, varying percentages of HLA-DR expression are often reported in MSC cultures, despite meeting the rest of defining criteria (25–29).

In current clinical practice, the use of BM concentrates are becoming popular in an attempt to provide stem cells to the fracture site. Although BM aspirates are typically processed in order to isolate the MNC fraction, this is not composed only of MSC but a heterogeneous population of B-cells, T-cells and monocytes, as well as rare progenitor cell types such as hematopoietic stem progenitor cells (HPCs) and endothelial progenitor cells (EPCs), it is still unclear which component or combination of components exactly determines its tissue induction activity, which can be exerted either by direct differentiation or by paracrine activity. It has been previously described that the MNC fraction from BM can promote angiogenesis (30), mediate vascular repair, produce cytoprotective growth factors and cytokines (31), and regenerate bone (32). From all the different subpopulations present in the bone marrow, MSC contribute to a very small fraction, estimated in the range from 0.001% to 0.1% of MNC (33, 34). Despite of such low occurrence, BM-MSCs can be efficiently expanded *ex vivo* and induced to differentiate into multiple lineages when subjected to defined culture conditions (32). In the orthopedics field, the use of pure populations of MSC is thought to promote bone formation more efficiently.

In the present study we employed fibrin hydrogel as a clotting agent, which is a commercially available product for clinical use that can also be manufactured *in situ* on demand either as allogeneic or autologous product (35, 36). Typically, fibrin is presented in a formulation of two components: A) concentrate or purified fibrinogen, which is the precursor glycoprotein of fibrin, and B) a mixture of factor XIII, thrombin and calcium, which triggers the polymerization reaction (35). In fact, fibrin glue is a widely known product in the surgery field that is used for rapid hemostasis (37), acceleration of wound healing (38), reduction of blood loss (39), protection against bacterial infections (40) and its capacity for shaping to the architecture of the application zone (6). Since human fibrin is highly biocompatible and resorbable, it can be used as a vehicle for bioactive ingredients in tissue engineering strategies (36). Remarkably, MSC maintained their features and viability, when combined with fibrinogen. Such biocompatibility was key in the outcome observed after administration into animals, in which new tissue formation may result from either direct differentiation to osteogenic cells or paracrine activity, which is known to provide chemotactic factors both locally and systemically, or as a result of the combination of both mechanisms (11, 12, 41, 42). Our results are in the same line as those obtained by Seebach and collaborators, who demonstrated the compatibility of fibrin with MSC and obtained similar results regarding the angiogenic activity in a study inducing bone healing in rats (43).

Both fibrin gels and bony particles used as scaffolds in the present study displayed key features as a support for the repair of damaged bone tissue, providing a jelly texture that allows for the spatial localization of the cellular component with osteogenic potential, therefore adapting itself to the morphology of the fracture site. Particulated decellularized/deantigenized bone matrix from human cadaveric donors can be found commercially on different size formats and are commonly used in the clinics for bone mass augmentation. Small particles, like we employed in the present study, make possible to increase the surface area so, their natural osteoinductive and osteoconductive properties are available to cells embedded in fibrin hydrogels (5). The non-cellular components of the TEPs have the capacity to generate a biological environment that ensures the supply of nutrients to the cells and facilitates their regenerative function. Remarkably, both acellular and cell-laden osteogenic formulations

showed osteogenic properties *in vivo* being BM-MSCLoaded TEP clearly the most potent formulation for generating extensive bone-like tissue and inducing improved vascularization. These findings are in accordance with similar studies published by Yamada and collaborators demonstrating bone regenerative activity when applying MSC-based TEP in a canine preclinical model first, and in the clinical setting afterwards (44–46). Kargozar and collaborators also reported a comparative study in rats with positive results regarding bone regeneration in the case of BM-MSCLoaded TEP (47). Despite of the promising outcomes of the present study, similar TEPs based in cell aggregates or cell sheets are gaining ground among the regenerative medicine field suggesting that different delivery modes of the active ingredient within the TEP may enhance tissue regeneration and therefore much effort is currently being made in order to improve such formulations (48, 49).

Conclusions

The present study provides evidence of the feasibility of TEP preparation with clinical-grade reagents while preserving the identity, osteogenic potency and safety of cells used in its formulation. Moreover, the preclinical data gathered in the present work, in compliance with current quality and regulatory requirements, complementing the required preclinical package, which is fundamental for subsequent translation of the new treatment into the clinical setting.

Table 1

TABLE 1. Summary of histological scores. MNC: Mononuclear cells from bone marrow aspirate; BM-MSCLoaded TEP: Bone Marrow-derived multipotent Mesenchymal Stromal Cells.

Category	Score		
	Acellular control	MNC	BM-MSCLoaded TEP
Bone formation	0.8 ± 1.0	0.8 ± 0.8	1.0
Tissue reaction	1.6 ± 0.5	1.5 ± 1.1	1.3 ± 0.8
Inflammatory reaction	2.0	1.4 ± 0.5	1.8 ± 0.4
Inflammatory cell type	1.0	1.0	1.0
Vascularisation around bone particles	0.8 ± 1.0	2.6 ± 0.5	1.2 ± 0.8

List Of Abbreviations

ALP: Alkaline phosphatase BM: Bone Marrow

DMEM: Dulbecco's Modified Eagle's Medium EPC: endothelial progenitor cells

GMP: Good Manufacturing Practice HLA: Human Leukocyte Antigen

HPC: hematopoietic stem progenitor cells HSA: human serum albumin

MNC: Mononuclear cell

MSC: Multipotent Mesenchymal Stromal Cells P: Passage number

PBMC: Peripheral Blood Mononuclear Cells TEP: Tissue Engineering Product

Declarations

Ethics approval and consent to participate: cells were sourced from Banc de Sang i Teixits' Biobank (Barcelona, Spain) and had appropriate donor informed consent for use in research. Authorization issued by Hospital de la Vall d'Hebron's Ethics Committee (Barcelona, Spain) to JV. All animal care and experimental procedures adhered to the recommendations of local, national, and European laws (Decret 214 de 1997, Real Decreto 53 de 2013, European directive 86/609/CEE of 1986, respectively) and were approved by the *Universitat Autònoma de Barcelona's* Ethical Committee on Human and Animal Experimentation (Ref. No. CEAAH 1972), and registered by *Generalitat de Catalunya* (Reg. No. DMAiH 6967).

Consent for publication: Not applicable.

Availability of data and material: All datasets generated for this study are included in the manuscript and the supplementary files.

Competing interests: No competing interests to declare.

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Authors' contributions: DV, MG-V, IOV and JV performed experiments and analysed data; JG-L and JV conceived the study; DV and JV revised data and wrote the manuscript. All authors revised and approved the final version of the manuscript.

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Figures

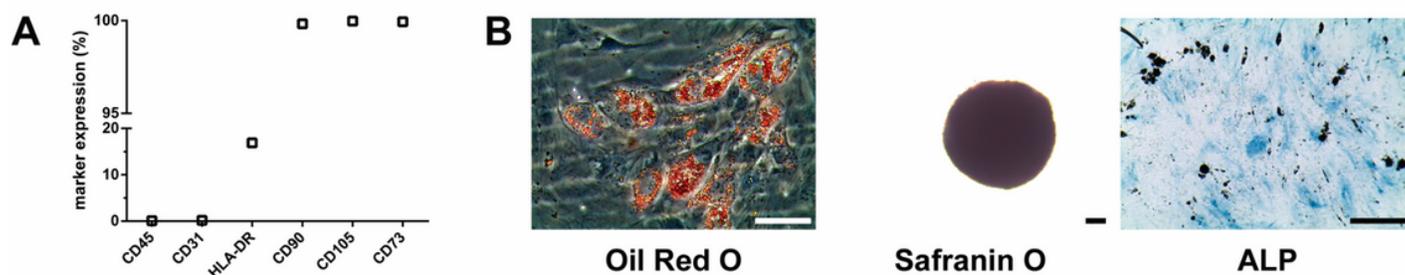


Figure 1

Characteristics of multipotent Mesenchymal Stromal Cells derived from bone marrow. BM-MSC typically lacked the expression of CD31 and CD45 surface markers, whereas they readily expressed CD73, CD90

and CD105. HLA-DR expression varied from batch to batch, but was always lower than 20% in accordance with product specifications (A). The cell line illustrated in this Figure corresponds to the one used in the in vivo study. BM- MSC also displayed differentiation potential into the adipogenic, chondrogenic and osteogenic lineages (B). Specific stainings were performed in order to determine lipidic vacuolae in adipocytes (using Oil Red O stain), presence of proteoglycans in chondrogenic micromasses (using Safranin O stain), and alkaline phosphatase (ALP) activity in osteoblasts. Scale bars = 200 μ m.



Figure 2

Stability study in fibrinogen. Cell viability was maintained above 70% along the study time (up to 48h) (A); Surface marker expression stayed consistent at the 24 h time point (B); Despite of cells remaining viable for 48 hours, they maintained the capacity to attach to plastic and proliferate normally only during the first 24 h (C); MSC taken at the 24 h time point still retained their osteogenic potential when subjected to osteogenic differentiation in vitro, as demonstrated by alkaline phosphatase (ALP) activity at day 8 in osteogenic inducing conditions (at two magnifications) compared to negative control stained at day 0 (top) (D). Scale bars = 200 μ m.



Figure 3

In vivo studies using different formulation of tissue engineering products in athymic mice. Histological assessment of cylindrical constructs evidenced the presence of bone particles, whereas cells were only present in the MNC and BM-MSC groups (A). No adverse effects were observed after treatment and mean body weight gain increased steadily along the course of the study (B). Size of constructs measured at 2 months post-administration were significantly bigger in BM-MSC group compared to acellular control ($p = 0.0191$) (C). Eosinophilic islands of osteoid (arrows) were seen in all three experimental groups (D). * = bone powder particles.

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