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Mesenchymal Stem Cells: Biological Characteristics and Potential Clinical Applications for Haematopoietic Stem Cell Transplantation

Teresa de Souza Fernandez and
Cecília de Souza Fernandez

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells that can be expanded and manipulated *ex vivo*. These cells demonstrated three biological characteristics that qualify them for the use in cellular therapy: (1) potential of differentiation, (2) secretion of trophic factors and (3) immunoregulatory properties. The bone marrow (BM) has been considered as the traditional source of MSCs and much knowledge for potential clinical applications has been obtained from studies using MSCs derived from adult bone marrow. MSCs need to be expanded *in vitro* for the purpose of cell therapy. However, sometimes, the culture expansion could generate cytogenetic and molecular alterations. Accumulation of these alterations during many passages of culture could lead to malignant cell transformation. So, it is important to perform a rigorous control using different methods to test the safety and efficacy of MSCs for cell therapies. BM-MSCs have potential clinical applications in haematopoietic stem cell transplantation (HSCT) as an adjuvant cellular therapy. This chapter reviews the advances in the study of MSCs and the potential clinical applications of MSCs in haematopoietic stem cell transplantation (HSCT). We also describe the importance of statistical methods to aid the analysis of the efficacy and safety for the clinical use of MSCs for HSCT.

Keywords: mesenchymal stem cells, biological characteristics, cell therapy, haematopoietic stem cell transplantation, statistical methods.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate *in vitro* into mesenchymal cell lineages such as adipocytes, osteocytes and chondrocytes. MSCs can be

expanded and manipulated *ex vivo*. According to the minimal criteria of the International Society for Cellular Therapy, MSCs are defined by their growth pattern *in vitro* (the cultured plastic-adherent cells), the specific surface antigen expression (CD73, CD90 and CD105, in the absence of lineage commitment markers such as CD14, CD19, CD34, CD45 and HLA-DR) and multilineage potential (these cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*) [1]. MSCs can be derived from adult bone marrow (BM), adipose tissue and several fetal tissues as umbilical cord. The bone marrow has been considered as the conventional source of MSCs and most knowledge for potential clinical applications has been obtained from studies using MSCs derived from adult bone marrow [2].

MSCs can be expanded and manipulated *ex vivo* and can demonstrate immunomodulatory functions *in vitro* and *in vivo*. Thus, they represent promising tools to be used in immunoregulatory and regenerative cell therapies. Recently, many studies have revealed the clinical use of MSCs as an emerging field for treating cardiovascular disorders, neurodegenerative diseases, bone defects and fractures, inflammatory arthritis and in the field of haematopoietic stem cell transplantation (HSCT) [3]. Bone marrow MSCs constitute approximately 0.01% of mononuclear cells in the bone marrow [4]. Hence, MSCs have to be expanded *in vitro* on tissue culture plastic for the purpose of cell therapy. An extensive amplification *in vitro* is necessary without affecting the cells' genomic characteristics and differentiation properties. But, sometimes, the culture expansion could generate cytogenetic and molecular alterations. Accumulation of these alterations during many passages of culture could lead to malignant cell transformation. Hence, it is important to perform a quality control using different methods to test the safety and efficacy of MSCs for cell therapies.

MSCs generate most of the stromal cells present in the bone marrow (BM). They form part of the haematopoietic stem cell (HSC) niche and produce various factors regulating haematopoiesis. It has been proposed that BM-MSCs are useful as an adjuvant cellular therapy for promoting rapid haematopoietic recovery in the HSCT patients. This chapter reviews the advances in the study of MSCs and the potential clinical applications of MSCs in haematopoietic stem cell transplantation (HSCT). We will also describe the importance of statistical methods to aid the analysis of the efficacy and safety for the clinical use of MSC for HSCT.

2. Definition and biological characteristics of mesenchymal stem cells (MSCs)

MSCs were first described in 1966 by Friedenstein and colleagues. They reported the presence of fibroblastoid cells that could be obtained from bone marrow of adult mice and when transplanted subcutaneously, they could differentiate toward osteogenesis [5]. After this discovery, several studies have been done using human mesenchymal stem cells. These studies confirmed that it is possible to culture and do sub-passages of the whole bone marrow into plastic culture dishes and after discarding the non-adherent cells a few hours later, the cells adhered to the plastic were capable of forming colonies (colony-forming unit: fibroblastic, CFU-F). It was observed that the MSCs have two important properties. First, they can

differentiate into distinctive end-stage cell types, including bone, cartilage, muscle, bone marrow stroma, tendon/ligament, fat, dermis and other connective tissues. Second, MSCs, themselves, secrete a broad spectrum of bioactive macromolecules that are immunoregulatory and serve to structure regenerative microenvironments in an injured tissue [6].

MSCs are not only found in bone marrow. MSCs have been isolated from multiple tissues such as skeletal muscle, adipose tissue, synovial membranes, dental pulp, periodontal ligaments, cervical tissue, umbilical cord, amniotic fluid and placenta. However, much knowledge regarding the biological characteristics and clinical experiences has been obtained from studies of MSCs derived from adult bone marrow [2, 7–9]. MSCs, also known as multipotent cells, are found in adult tissues of different sources. They are self-renewable, multipotent, easily accessible, and culturally expandable *in vitro* [10].

When cultivated *in vitro*, MSCs have three biological characteristics that qualify them for use in cellular therapy: (1) potential of differentiation, (2) secretion of trophic factors that help tissue remodelling and (3) immunoregulatory properties [2]. Therapeutic benefits of MSCs are dependent on their capacity to act as a trophic factor pool. After MSCs home to damaged tissue sites for repair, they interact with local stimuli, such as inflammatory cytokines, ligands of Toll-like receptors (TLRs) and hypoxia, which can stimulate MSCs to produce a large amount of growth factors that act with multiple functions for tissue regeneration. Many of these factors are critical mediators in angiogenesis and prevention of cell apoptosis such as vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1) and IL-6 [11].

Many studies have demonstrated the immunoregulatory properties of MSCs. These cells affect the immune response through their interactions with the cellular components of the immune system: T lymphocytes, B lymphocytes, natural killer (NK) cells and dendritic cells (DCs). MSC immunoregulation can occur through cellular contact and/or by secretion of diverse factors. Because of these properties, MSCs can prevent the inappropriate activation of T lymphocytes and generate a tolerogenic environment during repair or stop an immune response during healing, thus contributing to the maintenance of immune homeostasis [12,13]. Immunomodulatory properties of MSCs can be grouped into three categories: being hypoinmunogenic, modulating T cell phenotype and immunosuppressing the local environment [14, 15].

MSCs have decreased the expression of surface molecules including low levels of MHC class I and costimulatory CD40, CD80, and CD86 and no MHC class II molecules. This distribution of surface markers allows MSCs to evade detection from certain immune cells and contributes to their hypoinmunogenicity. MSCs also have the ability to immunosuppress the local environment and this can be attributed to their effect on cytokine secretion profiles. Specifically, in co-cultures with immune cells, MSCs had an indirect effect on T cell maturation and proliferation by up regulating the secretion of suppressive cytokines (IL-4 and IL-10) to decrease the secretion of proinflammatory cytokines (TNF- α and IFN- γ) from dendritic cells, T helper cells and macrophages. MSCs have the ability to induce regulatory T cells, which ultimately inhibit the proliferation and function of T cells, B cells and natural killer cells. Several soluble mediators, such as transforming growth factor β 1, prostaglandin E2 (PGE2), human leukocyte antigen G5, haemoxygenase I, nitric oxide, IL-6 and indoleamine 2,3-dioxygenase

(IDO), are important for this process [13]. Indoleamine 2,3-dioxygenase, which is induced by IFN- γ , catalyzes the conversion from tryptophan to kynurenine and inhibits T-cell responses [16,17].

As we can observe, the immunomodulatory characteristics of MSCs are important for cell therapy. But, approximately 2×10^6 cells/kg are required for clinical application of MSCs [3]. Therefore, for cell therapy, it is necessary to expand the MSCs using culture methods.

2.1. Isolation and culture expansion of MSCs for cell therapy

Clinical protocols employ cell culture technologies that use a small fraction of primary MSCs isolated from a selected tissue source and expanded by multiple passages in order to generate a clinically relevant number of cells. Consequently, once the tissue source of MSCs is determined for a specific clinical application, the safety and efficacy may be significantly influenced by cell bioprocessing protocols [18, 19].

There are no standard culture protocols for isolation and expansion of MSCs. Hence, the way in which these cells are cultured *in vitro* varies considerably between research groups. Consequently, it is difficult to compare results from different studies [19–21]. But, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define MSCs *in vitro*: (1) MSCs must be adherent to plastic under standard tissue culture conditions; (2) MSCs must express certain cell surface markers such as CD73, CD90 and CD105 and lack expression of other markers including CD45, CD34, CD14, or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; (3) MSCs must have the capacity to differentiate into osteoblasts, adipocytes and chondroblasts under *in vitro* conditions [1].

The procedures used to isolate MSCs, for example, from bone marrow, usually use density centrifugation (with Ficoll™, Lymphoprep™ or Percoll™ density mediums) to separate the mononuclear cell fraction from the other marrow constituents such as red blood cells, plasma and lipids. This mononuclear cell fraction contains an enriched population of T cells, B cells, monocytes, hematopoietic stem cells (HSCs), endothelial progenitor cells and MSCs. Following plating onto tissue culture flasks, MSCs, which represent the adherent cell population, form colonies. The adherent cells remain in culture and the other non-adherent cells are discarded while changing the medium [19, 21]. The MSCs are anchorage-dependent cells that expand when maintained in culture conditions such as medium DMEN supplemented with 10% of FBS. Initial growth of MSCs in primary BM cell culture on a plastic surface is characterized by the formation of single cell-derived colonies. The efficiency with which they form colonies still remains an important assay for the quality of cell preparations. In general, MSCs have a great propensity for expansion in culture, although their proliferation potential is highly variable, mainly between young and older donors who retain reduced proliferative potential [19,22].

MSCs seeding densities range between 2.000 and 5.000/cm²; however, there is evidence that lower seeding densities enhance proliferation, which is thought to be attributed to a reduction in contact inhibition. Some studies demonstrated that MSCs proliferate more rapidly when passaged by plating the cells at low densities as 10–100 cells/cm² [19, 23, 24]. MSCs are most

commonly expanded in a basal media such as Dulbecco's modified Eagle's medium (DMEM)/DMEM F-12 or alpha-MEM with 10% fetal bovine serum (FBS) [21]. All current protocols for *in vitro* culture of MSCs include FBS as a nutritional supplement [25]. However, some problems are associated with the use of FBS, for example, risk of contamination associated with harmful pathogens such as viruses, mycoplasma, prions or unidentified zoonotic agents. The chance of contamination or immunological reaction towards xenogeneic compounds must be also taken into consideration [19, 26]. Hence, for using FBS, tests are necessary for providing optimal growth conditions [21].

Successful expansion techniques aim to facilitate significant increases in cell number without affecting the MSC therapeutic potential. MSCs can be cultured *in vitro* for 8–15 passages, corresponding to approximately 25–40 population doublings and 80–120 days. MSCs demonstrate a marked decrease in proliferation as a function of duration in culture and passage number, thereby becoming senescent and ceasing to proliferate [4, 7, 27]. MSCs may lose differentiation capacity during the time in culture to assess multilineage potential. The potency of MSCs rapidly decline as a function of 2D expansion and this shows a demand for alternative expansion techniques [4].

Bone is a 3D substrate composed of water, organic collagen and inorganic hydroxyapatite. MSCs reside within crevices of the blood-submerged bone and among several cell types with which they engage in a complex orchestra of crosstalk. Numerous aspects of the bone marrow niche, which regulate MSC behavior, are absent in 2D culture. Hence, it is necessary to develop new techniques to recreate characteristics of the elaborate niche to preserve MSC progenitor potency through 3D expansion [4, 28–30]. Some studies have been demonstrated that MSCs can be expanded using scaffolds or scaffoldless approaches, usually in combination with a bioreactor. 3D MSC expansion has been performed on hydroxyapatite (HA), chitosan gelatin and HA/chitosan gelatin and gelatin microcarriers [4, 28, 31].

Bioreactors are devices that facilitate the development of biological and/or biochemical processes through operating parameters such as pH, temperature, nutrient supply and waste removal. Bioreactor systems are essential tools to achieve the goals in the clinical-scale expansion and tissue engineering applications [4]. They maintain the minimal criteria to define MSCs, which include plastic adhesion, expression of a set of specific surface markers and the ability of differentiation along osteogenic, adipogenic and chondrogenic lineages [1]. MSCs also bear broad regenerative and trophic activities, including the secretion of extracellular matrix (ECM), pro-mitotic and pro-angiogenic factors, anti-inflammatory and immune-regulatory factors, and other bioactive molecules that stimulate tissue regeneration by reconstructing a pro-regeneration microenvironment and by modulating immune and inflammatory responses. Thus, these unique properties play a central role to the success of MSC-based therapeutic applications [32–34].

2.1.1. Multilineage potential

MSCs have the potential for multilineage differentiation. This property has been studied for the development of MSC transplantation as a regenerative therapy. Multilineage potential is a criterion to define the MSCs *in vitro*. The multilineage potential may be observed under

culture conditions that induce cell differentiation into three lineages: osteogenic, adipogenic and chondrogenic [35]. A number of *in vitro* assays can be used to assess the multipotentiality of these cell preparations. Osteogenic differentiation of MSCs can be induced using dexamethasone, ascorbate-2-phosphate and beta glycerolphosphate. Osteoblasts may be identified using alizarine red S staining. Adipogenic differentiation can be induced with the medium containing dexamethasone, indomethacin, isobutylmethylxanthine and insulin. Oil red O staining may be used to detect lipid accumulation. Chondrogenic differentiation can be induced in a defined medium containing dexamethasone, ascorbate-2-phosphate, insulin, selenious acid, transferrin, sodium pyruvate and transforming growth factor-beta [3, 36, 37]. The ability of MSCs to differentiate along these lineages is strongly associated with their multipotency and stem cell nature. However, MSCs do not maintain these characteristics indefinitely and MSCs senesce with extensive subcultivation *in vitro* whereby they lose their proliferation and differentiation potential [37]. Such culture expansion could also generate genetic and epigenetic instability, including chromosome alterations. The accumulation of genetic changes during cell culturing and subsequent risk of cell transformation are other important points of stem cell therapy [3, 38].

2.1.2. Cytogenetic and molecular characteristics of MSCs

The utilization of MSCs for cell therapy requires large-scale *in vitro* expansion, thus increasing the probability of cytogenetic and molecular instabilities [38]. The expansion of MSCs in culture could generate chromosomal abnormalities such as aneuploidy (the presence of an abnormal number of chromosomes in a cell) or structural chromosomal alterations, reflecting the chromosomal instability. But, it is not clear how many passages can be performed before these cells acquire chromosome instability or lose their multipotency [3, 39, 40]. Some studies have been shown that *in vitro* culture of MSCs from bone marrow and adipose tissue retained normal karyotypes between passages 1 and 5 [3, 39, 41]. At later passage cultures, MSCs began to show chromosomal abnormalities such as aneuploidy. However, other studies observed that MSC cultures derived from bone marrow and adipose tissue had normal karyotypes up to passage 20 [42, 43]. Although these results are debatable, they show the necessity of cytogenetic analyses for safety before therapeutic application of mesenchymal stromal cells.

Molecular studies also have an important role to determine appropriate MSCs to be used for cell therapy. All primary human cells, including MSCs, undergo only a limited number of cell divisions under standard culture conditions, in a process called cellular senescence. Senescence is considered to be a stress response triggered by activation of some mechanisms as telomere erosion and accumulation of DNA damage [44, 45]. *In vitro* cultures cause significant telomere shortening. Telomeres are the termini of eukaryotic chromosomes and their principal function is to protect chromosomes from illegitimate fusion and recombination, thereby preserving genome integrity [45, 46]. Since MSCs have only a finite ability for self-renewal like most somatic cells, assaying for telomere length in hMSCs provides critical information on the replicative capacity of the cells, an important criterion in the selection of MSCs for therapy. Telomere length is generally quantified by Southern blotting and fluores-

cence *in situ* hybridization and more recently by polymerase chain reaction (PCR)-based methods [47].

The multipotency of MSCs has allowed a significant progress in our understanding about differentiation pathways of various lineages for tissue engineering and therapeutic purposes [48]. Runt-related transcription factor 2 (Runx2) has been considered as a master regulatory gene responsible for early osteogenic differentiation [49]. While Runx2 acts to promote osteoblastic differentiation, another important osteogenic inducer, osterix, suppresses chondrogenesis and promotes osteoblastic differentiation at a later stage. Low levels of osterix are sufficient to inhibit chondrogenesis, while a high expression level is necessary for osteogenic differentiation [48, 50]. Furthermore, *ex vivo* MSCs have successfully differentiated into osteoblasts in osteogenic media supplemented by dexamethasone and ascorbate. The selective capability to promote osteogenic differentiation has potential clinical implications in bone repair and regeneration [48, 51].

In-vitro differentiation of MSCs into a chondrogenic lineage has been studied through exposure to growth factors, co-culture with cartilage and overexpression of specific genes such as SRY-box 9 (Sox9) to promote chondrocytic differentiation. Sox9 cooperates with its downstream proteins Sox5 and Sox6 to promote chondrocyte proliferation and maturation and matrix formation [48, 52]. MSCs also have the capacity to differentiate into an adipogenic lineage. PPAR- γ plays a critical role in this process by regulating the function of many adipocyte-specific genes. In addition, PPAR γ interacts with members of the CCAAT/enhancer-binding protein (C/EBP) family to regulate adipogenesis. Cells can also be induced to undergo adipogenesis through exposure to exogenous factors or by culturing them in adipogenic media containing insulin and dexamethasone [48, 53]. As the multilineage potential is one of the three criteria to define the MSCs *in vitro* according to the International Society of Cellular Therapy [1], the use of molecular tests to analyze the expression of the genes involved in the differentiation of the osteogenic, chondrogenic and adipogenic are important to associate the biological function of the MSCs for their clinical use.

The expansion of MSCs *in vitro* is associated with genetic instability. Hence, molecular studies comparing the molecular profile during the culture passages are important to acquire knowledge about molecular modifications and potential risks for cell therapy. In this sense, proteomic and transcriptomic approaches have been used to verify molecular modifications of MSCs from different culture passages [3, 54].

We need to be careful before using BM-MSCs for clinical applications. Some changes may be analyzed such as enlarged morphology, decreased number of cell divisions, random loss of genomic regions and telomere shortening. These modifications process could lead to a reduction in the multipotent state of MSCs and might lead to tumour formation under specific conditions. It is very important to characterize the cytogenetic and molecular profiles during expansion *in vitro* of BM-MSCs; thus, appropriate tests should be applied to ensure the integrity of the genome and epigenome [54].

2.1.3. Quality control for cell therapy

There are many challenges associated with characterizing and quantifying cells for use in cell- and tissue-based therapies. From a regulatory perspective, these advanced treatments must not only be safe and effective, but also must be made by high-quality manufacturing processes [55]. Prolonged exposure to stressful conditions during the cell enrichment and differentiation processes has raised concerns about the safety of stem cell therapy. The International Society for Stem Cell Research has created “Guidelines for the Clinical Translation of Stem Cells” [56]. Some cytogenetic tests that may be performed to ensure the safety of the stem cells include: G-banding, fluorescence in situ hybridization (FISH) and array comparative genomic hybridization (array CGH) [38, 57]. Molecular genetic tests may be performed as the analysis of the telomere length, the expression of genes involved in the osteogenic, adipogenic and chondrogenic differentiation. Some characteristics and tests, which may be considered as a quality control for the use of MSCs for cell therapy, are shown in **Figure 1**. The practical application of these recommended tests can be standardized for the sensitivity and specificity between laboratories.

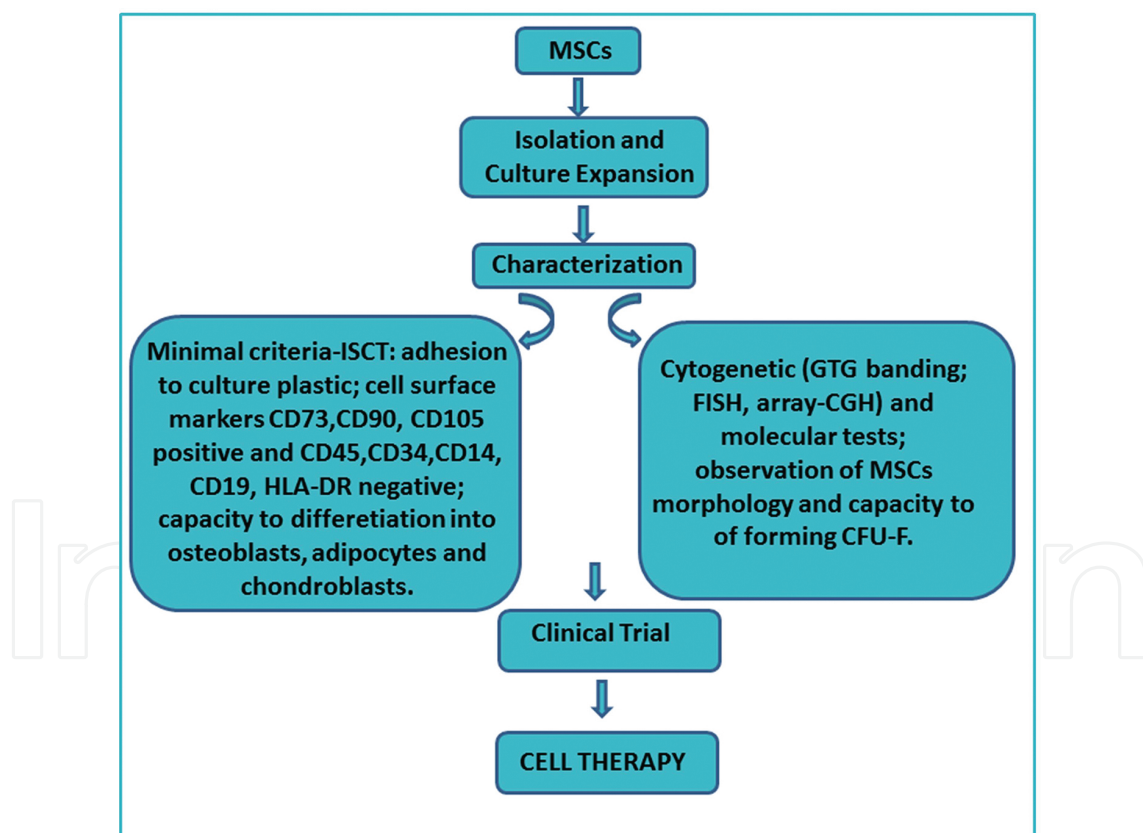


Figure 1. Some characteristics and tests that may be considered as a quality control for the use of MSCs for cell therapy.

The first clinical trial using culture-expanded MSCs was performed in 1995 and bone marrow samples were obtained from 23 patients with haematologic malignancies in complete

remission. In this study, as no adverse reactions were observed with the infusion of the MSCs, Lazarus and colleagues concluded that MSCs obtained from cancer patients can be collected, expanded *in vitro* and infused intravenously without toxicity [58]. Many completed clinical trials have demonstrated the efficacy of MSC infusion for diseases including acute myocardial ischaemia, liver cirrhosis, amyotrophic lateral sclerosis and graft versus host disease (GVHD) [12, 59]. The statistical methods are important tools to evaluate the quality, safety and efficiency of MSCs for cellular therapy as we will observe in the last section.

3. Potential clinical applications of MSCs in haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is the first field wherein human stem cell therapy was successful. Allogeneic HSCT has been an important modality to cure various diseases, including haematologic malignancies, various non-malignant haematologic diseases and primary immunodeficiency diseases. However, autologous HSCT is generally performed to rescue bone marrow aplasia following high-dose chemotherapy for solid tumour or multiple myeloma [60].

The first successful HSCT using bone marrow from a relative donor was performed in 1968 in a boy with X-linked severe combined immunodeficiency disease (SCID). Since the first successful achievement in human, numerous trials and errors were repeated until 1980, when allogeneic HSCT began to be actively performed under a better recognition of transplant immunology. Dr. Donnall Thomas received Nobel Prize for his pioneering work in bone marrow transplantation to cure leukaemia and other haematologic malignancies [60].

Results from basic and clinical research have allowed the improvement of HSCT. Some of these improvements were the use of haematopoietic stem cells from peripheral blood or cord blood, which promoted a change in the terminology from bone marrow transplantation to haematopoietic stem cell transplantation [61]; a better understanding of the complexities of the human leukocyte (HLA) system, which has allowed selecting compatible sibling donors and the establishment of larger registries of HLA-typed volunteers; advances in the immunogenetics of HLA, especially typing of molecular techniques; the development of the preparative regimen of cyclophosphamide and busulfan, which avoids the use of irradiation for some diseases [62]; the development of non-myeloblastic conditioning regimens for allogeneic HSCT, avoiding regimen-related toxicity and death and that opened the way to include elderly patients [61, 63].

These advances make HSCT one curative treatment modality for many patients mainly with malignant haematological diseases. But, remaining challenges include further advances in the prevention and treatment of both infections and graft-versus-host disease (GVHD). Despite significant progress in HSCT, GVHD remains a significant cause of morbidity and mortality after allogeneic HSCT [63–67].

Homeostasis of the haematopoietic system, which is a balance between self-renewal and differentiation, is thought to be tightly regulated by interactions among haematopoietic stem

cells (HSCs) and the specialized microenvironment where they reside, the haematopoietic niche. The haematopoietic niche consists of a heterogeneous cellular population of non-haematopoietic and haematopoietic origin as well as of extracellular matrix, which collectively provide the structural scaffold, the spatial framework and the appropriate physiological and trophic cues to control HSC maintenance and function [68]. The key component of the haematopoietic microenvironment is bone marrow mesenchymal stromal cells (BM-MSCs).

Many studies have reported that MSCs can promote HSC expansion *in vitro*. Koc and colleagues (2000) first reported rapid haematopoietic recovery after co-infusion of autologous BM-MSCs at the time of HSCT without significant side effects [69]. Lazarus and colleagues (2005) showed, in a multicenter trial with 46 patients receiving allogeneic HSCT and MSCs from HLA-identical siblings, a rapid haematopoietic recovery in most patients [58]. These studies showed the beneficial effects of MSC on engraftment after HSCT [2].

BM-MSCs have potential clinical applications in HSCT as an adjuvant cellular therapy for promoting the rapid reconstitution of haematopoiesis after HSCT, to prevent and treat of graft failure, in graft versus tumour effect and in GVHD [2, 70].

3.1. Pathophysiology of graft-versus-host disease (GVHD) and clinical use of MSC for GVHD

Allogeneic HSCT is an effective treatment for many haematologic and genetic diseases. However, donor-derived cells may also recognize recipient organs as foreign and mount an immune attack against the patient's own tissues, known as graft-versus-host disease (GVHD) [71].

Graft-versus-host disease (GVHD) is the major cause of morbidity and mortality after an allogeneic HSCT. GVHD has traditionally been classified by the time of its clinical manifestations. Acute GVHD occurs within the first 100 days after haematopoietic stem cell transplantation, whereas chronic GVHD occurs after day 100. This simple classification is increasingly unsatisfactory particularly as reduced-intensity condition regimens gain wider acceptance. The clinical manifestations of acute GVHD after such conditioning often occur much later, sometimes coinciding with the day 100, the demarcation of chronic GVHD [72]. New recommendations that emphasize the importance of qualitative differences, as opposed to the time of onset after HSCT, are being used to standardize the diagnosis and clinical assessment of chronic GVHD [73].

The pathophysiology of acute GVHD after HSCT can be considered as a three-step process where the innate and adaptive immune system interacts. These three-step processes are as follows: (1) initiation of tissue damage; (2) activation and proliferation of donor T cells; and (3) the effector phase involving cellular and inflammatory factors. The pathophysiology of GVHD is a complex process. Chemotherapy and radiation cause tissue damage, producing pro-inflammatory cytokines, resulting in donor T-cell activation through the host antigen-presenting cell (APC) interaction via MHC-T cell receptor binding and co-stimulatory signals. This leads to T cell expansion and differentiation into various subtypes which traffic through blood vessels to target organs, where they cause tissue destruction and recruitment of other

inflammatory cells through pathways such as perforin/granzyme and cytokine release. Moreover, these inflammatory cells and cytokines can propagate the cycle of GVHD [71, 72, 74]. The main step of GVHD reaction is step 2, where donor T cells are activated by the host antigen-presenting cells (APCs). The GVHD reaction is amplified by the intensity of the recipient-conditioning regimen, which can result in an intensive tissue damage with the release of various cytokines and augment of inflammatory response [75].

Acute GVHD affects mainly skin, liver and gastrointestinal tract. Approximately 50% of the patients treated with HSCT subsequently developed acute GVHD and required systematic treatment. Chronic GVHD occurs in 40% of patients treated with allogeneic HSCT from HLA-identical sibling and more than 50% of patients treated with HSCT from an HLA non-identical-related donor [67, 75].

Chronic GVHD is one of the most significant complications of long-term survivors after allogeneic haematologic stem cell transplantation. Experimental studies and clinical observations have elucidated the mechanisms of acute GVHD, but the biology of chronic GVHD is not well understood. Experimental studies generated at least four theories to explain the pathophysiology of chronic GVHD: (1) thymic damage and the defective negative selection of T cells; (2) regulatory T cells deficiencies; (3) auto-antibody production by aberrant B cells; and (4) the formation of profibrotic lesions [73]. The immunopathology of chronic GVHD is mediated in part by helper T lymphocyte 2 (Th2) cells, with a syndrome of immunodeficiency and an autoimmune disorder [76].

GVHD remains associated with significant morbidity and mortality in allogeneic HSCT. Improving outcomes in HSCT will require additional therapeutic modalities such as the use of MSCs. MSCs may be used to modulate the immune system, as prophylaxis to prevent GVHD and as treatment for established GVHD. Some studies have been performed demonstrating that the MSCs can act in GVHD [76–81]. In a multicenter clinical trial, HSCs and MSCs derived from HLA-identical sibling donors were infused and promoted haematopoietic engraftment and limited GVHD. In this study, 31 patients received myeloblastic conditioning and HLA-identical sibling bone marrow or peripheral blood stem cells. Escalating doses of MSCs from 1 to $5 \times 10^6/\text{kg}$ were given. Toxicity related to MSC infusion was not observed. The incidence of acute GVHD was 15% in the co-transplanted group compared with 40% in a matched control group [17, 77]. Le Blanc and colleagues (2004) reported a child with acute lymphoblastic leukaemia receiving haploidentical MSC infusion for severe acute GVHD with a satisfactory clinical outcome [78]. The immunomodulatory ability of BM-MSCs shows promise in treating GVHD, especially acute GVHD. Zhou and colleagues (2010) also showed the potential clinical application of MSCs for chronic GVHD. In this study, four patients with sclerodermatous chronic GVHD were reported, who received MSCs expanded *ex vivo* from unrelated donors by intra BM injection. After MSC infusion, the symptoms gradually improved in all four patients. During the course of MSC treatment, the patient's vital signs and laboratory studies remained normal. None of the four patients had recurrence of leukaemia. This study, despite its limited number of patients, suggests a benefit of MSC infusion therapy in treating sclerodermatous chronic GVHD [76]. MSCs are capable of escaping recognition by the alloreactive immune system and can exert immunomodulatory and anti-

inflammatory effects. Hence, these cells represent a promising tool in the prevention and treatment of GVHD [2].

3.2. Prevention and treatment of graft failure using MSC

Graft failure or graft rejection after HSCT may occur as either a lack of initial engraftment of donor cells or loss of donor cells after initial engraftment. In the later case, autologous recovery may appear or, alternatively, marrow aplasia may be developed. Rejection is a major cause of graft failure and it is due to recipient immune response against donor Immuno-haematopoietic cells. Graft failure may also occur to other causes, such as viral infections, specifically, cytomegalovirus (CMV) [82]. In patients with leukaemia receiving myeloblastic conditioning, the rejection rate was 0.1% in patients given HLA-identical sibling transplants compared to 5% in those given HLA-mismatched grafts. Another risk factor for graft failure is the reduced intensity conditioning that is used in the elderly patients, with lower doses of chemoradiation therapy, the host immune system may persist, resulting in an increased risk of allograft rejection. Hence, the main risk factors associated with graft failure include HLA disparity in the donor/recipient pair, viral infections and the type of conditioning regimen [82, 83].

Some studies have demonstrated that the use of MSCs is efficient in prevention and treatment of graft failure [2, 9, 70]. Ball and colleagues carried out a pilot study of co-transplantation of BM-derived, *ex vivo*-expanded MSCs of donor origin in 14 children undergoing transplantation of granulocytic colony stimulating factor (G-CSF) mobilized, CD34-selected progenitor cells from HLA disparate relative. In this study, a graft failure rate of 15% was observed in 47 controls and all patients, who received MSCs, showed sustained haematopoietic engraftment without any adverse reaction. These results suggested that MSC co-transplantation may modulate host alloreactivity and/or promote better engraftment of donor haematopoiesis, thus reducing the risk of graft failure [9].

3.3. Graft-versus-tumour effect and MSCs

Two mechanisms are involved in the cure of a malignant disease by stem cell transplantation. The first is the conditioning regimen, which confers a powerful anti-tumour effect from myeloblastic doses of chemotherapy or radiotherapy. The second is the graft-versus-leukaemia (GVL) effect or the graft-versus-tumor (GVT) effect exerted by transplanted donor T cells and NK cells against malignant tissue. The T lymphocytes recognize antigens presented by HLA molecules on malignant cells. They destroy tumour cells by direct cytotoxicity inducing death by lysis through the perforin-granzyme pathway and by apoptosis through activation of Fas on the cell surface. The graft-versus leukaemia effect requires donor immune competence that often accompanies graft-versus-host disease (GVHD) [84].

Despite HLA identity between a patient and donor, approximately 40% of patients receiving HLA-identical grafts develop acute GVHD due to genetic differences that lie outside the HLA loci, or minor histocompatibility antigens (HA). Some of such antigens, such as HY and HA-3, are expressed on all tissues and are targets for both GVHD and GVL. Others, such as HA-1

and HA-2, are expressed most abundantly on haematopoietic cells (including leukaemic cells) and may therefore induce a greater GVL effect with less GVHD [75].

Bearing in mind two fundamental aspects to the success of allogeneic HSCT, the GVL effect and the GVHD, the co-infusion of MSCs in transplantation should aim at reducing the severity of GVHD while preserving the GVL. In this sense, the study of Baron and colleagues (2010) demonstrated that the MSC co-infusion appeared to be safe; furthermore, MSC co-infusion might have prevented death from GVHD without abrogating GVT effects. In this study, 20 patients with haematologic malignancies received MSCs from HLA-mismatched donors after conditioning with TBI (total body irradiation) and fludarabine. The HLA-mismatched non-myeloblastic HSCT with MSC co-infusion had a therapeutic effect on the haematologic malignancies. MSCs may have important beneficial characteristics in terms of promoting GVT effects due to their immunomodulatory properties after HSCT and their tropism towards the microenvironment [85, 86]. **Figure 2** shows the potential clinical applications of MSCs for haematopoietic stem cell transplantation.

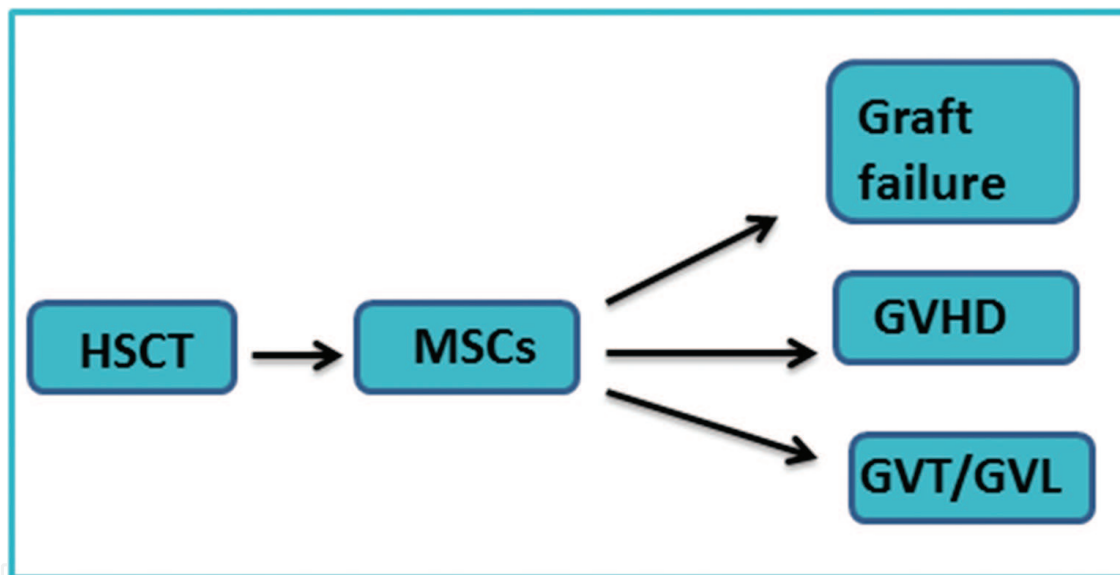


Figure 2. Potential clinical applications of MSCs for HSCT.

4. Using probability techniques to study clinical applications of mesenchymal stem cells

Nowadays, people recognize the importance of Mathematics in Medicine. Many statements in this area can be better understood using mathematical concepts and results. In this section, we will present mathematical techniques based on the concept and results of probability, as we are interested in making decisions in the face of uncertainty. In fact, in Medicine, clinical outcomes, such as the occurrence of disease, death, symptoms or functional impairment, can be counted and expressed as numbers, but in most clinical situations, the diagnosis, progno-

sis and treatment outcomes are uncertain for an individual patient. A person will experience a clinical outcome or not: the prediction is rarely exact. Therefore, the prediction must be expressed as a *probability*.

Although earlier work on probability was done by the Italian mathematician Giralamo Cardano (1501–1576), the investigation of probability as a branch of Mathematics sprang about 1654 with two great French mathematicians: Blaise Pascal (1623–1662) and Pierre Fermat (1601–1665) [87]. Of course, we shall not do a discourse on probability. But, we need to say that the theory of probability underlies the procedures in testing hypotheses, which are very useful to Medicine and other disciplines in the health field.

In this section, we will avoid mathematical formulas and theorems.

4.1. Descriptive statistics and inferential statistics

Statistics is a branch of Mathematics. The word “statistics” derives from the Latin word *status*, meaning “manner of standing” or “position.” Statistics were first used by tax assessors to collect information for determining assets and assessing taxes. Statistics applied to Medicine and other health disciplines is called biostatistics or biometrics. For those who would like to study this subject, we recommend the book of J. H. Zar [88].

Statistics is divided into two branches: descriptive and inferential. *Descriptive statistics* is used to organize and summarize data. *Inferential statistics* is used to draw inferences and reach conclusions about a population, when only a sample from that population has been studied. A population is a complete set of observations, patients, measurements and so forth. A sample is a subset of a certain population.

The mathematical techniques used on descriptive statistics are graphic (*tables and graphs*) and numerical (*quantitative indices*). Tables are often used to present qualitative and quantitative data. Graphs are used widely to provide a visual display data. The bar diagram, histogram and frequency polygon are three graphic formats that are commonly used to present medical data. Quantitative indices are numbers that describe the centre and the variation of a distribution, which are called *parameters* if they are referred to a population and called *statistics* if they are referred to a sample. Quantitative indices that describe the centre of a distribution are referred to as *measures of central tendency*. The mean, known also as the arithmetic mean, median and mode are three common measures of central tendency. Quantitative indices that describe the variation or dispersion of a distribution are referred to as *measures of dispersion*. The range, variance and standard deviation are three common measures of dispersion. Other quantitative indices such as risk difference, relative risk and odds ratio are also used in Medicine.

One mathematical technique used on inferential statistics is hypothesis tests. They are much more sophisticated than the techniques used in descriptive analysis, because hypothesis tests are based on probability models.

A probability model for a particular experiment is a probability distribution that *predicts* the relative frequency of each outcome if the experiment is performed a large number of times. A

probability distribution is a table, a graph or a formula that describes what probably will happen instead of describing what really happened.

A probability distribution can be discrete or continuous. An important example of a discrete probability distribution is the *binomial distribution* and an important example of a continuous distribution is the *normal distribution*.

4.2. Hypothesis tests

A hypothesis test is a method used to determine if there is enough evidence in a sample to infer that a certain property holds for the entire population. It works with two hypothesis: the null hypothesis (designated H_0) and the alternative hypothesis (designated H_A).

In the null hypothesis we use the words *no difference* or *equal to* and in the alternative hypothesis we use the words *different from*, *less than* or *greater than*. But let us mention that, in fact, we should say *no statistical difference*, *statistically equal to*, *statistically different from*, *statistically less than* or *statistically greater than*, because we are dealing with the probabilities of an event happens or not. When we retain H_A (equivalently reject H_0), we say the results are significant and when we retain H_0 (equivalently reject H_A), we say the results are not significant. Because we are dealing with probabilities, this implies in making two possible errors from four possible relations between the conclusions obtained using a hypothesis test and real situations, as shown in **Table 1**.

		Real difference	
		Presence	Absence
Conclusion of the statistical test	Results are significant	True	Type I error
	Results are not significant	Type II error	True

Table 1. Relations between statistical conclusions and real situations.

The two errors mentioned in the previous paragraph are known as Type I error and Type II error. A Type I error leads to a *false positive* conclusion. The probability of such an error occurs is noted by α . Mathematically, α is a conditional probability: α is the probability of reject H_0 when there is no real difference. A Type II error leads to a *false negative* conclusion. The probability of such an error occurs is noted by β . Mathematically, β is a conditional probability: β is the probability of retain H_0 when there is a real difference.

Hypothesis tests are used to estimate the *probability* of a Type I error. So, they are based on probability models. In the literature, we usually use $\alpha < 0.05$. This means we are assuming a probability less than 0.05 of rejecting H_0 when there is no real difference between treatments, drugs or procedures. In other words, if the study were repeated one-hundred times, we *probably* would find five outcomes showing H_0 should be accepted.

The hypothesis tests commonly used in the medical literature are presented in **Table 2**. They can be parametric or nonparametric. The choice in using one of them depends on the pur-

pose of the study, the size of the sample and the type of the variables involved at the study, for instance. If we can guarantee that the sampling distribution is normal or approximately normal, we can use a parametric test. Because a normal distribution has good mathematical properties (bell-shaped, symmetric, and so on), using a parametric test leads to better results compared with a nonparametric test. In other words, we say that nonparametric tests are less powerful, in the sense that, they lead to a small probability to reject H_0 , when H_0 is false.

To test the statistical significance of the difference between ...		
Two or more proportions	Chi-square	Nonparametric
Two proportions	Fisher's exact	Parametric
Two medians	Mann-Whitney	Nonparametric
Two means	Student's t	Parametric
More than two means	Kruskal-Wallis (one-factor)	Nonparametric
More than two means	ANOVA (one-factor)	Parametric
More than two means	ANOVA (more factors)	Parametric

Table 2. Statistical tests usually used in the medical literature.

When we use a hypothesis test, we compute a p -value. The p -value is the probability of obtaining a result as extreme or more extreme than the sample value, assuming that the null hypothesis is true. The sample value is calculated. Depending on the test we use, there is a specific formula to calculate the sample value. An appropriate computer software can do such a calculation.

We finish this subsection noting that many methods described above are univariate methods, because they are only concerned with analyzing only one variable. The point in using a multivariate analysis is to consider several variables simultaneously in the study. With multivariate methods, we can reduce data information, we can classify objects or variables, we can investigate the dependence among variables and, finally, we can make predictions. Principal component analysis, factor analysis and clustering methods can be used in order to reduce data and investigate the dependence among variables. To make predictions, we can use Hotelling's T^2 test, which allows inference about one mean vector, and we can use MANOVA, which allows inference about a finite number of mean vectors. It is important to say that both Hotelling's T^2 test and MANOVA are generalizations of Student t -test and ANOVA, respectively. So, they are based on the multivariate normal distribution. For those who would like to study this subject, we recommend the book of R.A. Johnson and D.W. Wichern [89].

4.3. Probability techniques on the study of mesenchymal stem cells

In cellular therapy, safety remains one of the main characteristic and refers to validation tests. The culture process should be reproducible, robust and efficient. The increasing use of MSCs

has led to a production of processes which needs to be in accordance with current Good Manufacturing Practice (cGMP) [90, 91]. For the validation of the tests, it is important to use probability techniques comparing the results of the tests and the results between different laboratories, with the main to standardize the procedures to characterize the MSCs at the stage of production for cell therapy. The evidence of clinical efficacy is also required. In this case, the application of probability methods is also an important tool in clinical trials and clinical outcomes revealing the impact on the use of MSCS in cellular therapy.

5. Conclusion

Mesenchymal stem cells (MSCs) represent promising tools to be used in immunoregulatory and regenerative cell therapies. For this purpose, an extensive amplification *in vitro* of MSCs is necessary without affecting the cells' genomic characteristics and differentiation properties. However, sometimes, the culture expansion could generate cytogenetic and molecular alterations. Accumulation of these alterations during many passages of culture could lead to malignant cell transformation. Hence, it is important to do a quality control using different methods to test the safe and efficacy of MSCs for cell therapies. Many studies have revealed the clinical use of MSCs as an emerging field for treating cardiovascular disorders, neurodegenerative diseases, bone defects and fractures, inflammatory arthritis and in the field of haematopoietic stem cell transplantation. The BM-MSCs have potential clinical applications in HSCT as an adjuvant cellular therapy for promoting the rapid reconstitution of haematopoiesis after HSCT, to prevent and treat of graft failure, in graft-versus tumor effect and in GVHD. Although these studies showed positive results, it is necessary to continue the scientific and clinical research to clarify some points as: the characterization of the appropriate cell passage during the culture of MSCs to ensure the genomic stability; the definition of the tests for quality control to ensure the safety of the MSCS for clinical practice; the practical application of these recommended tests can be standardized for the sensitivity and specificity between the laboratories; it is necessary to define the optimum cell dose and the number of infusions of MSCs during the treatment; a long follow-up to characterize the positive clinical effects and also the adverse clinical effects that may occur with the use of MSCs. With the advancement of basic and clinical research, we hope that the use of MSCs in cell therapy brings excellent results especially for patients treated with HSCT.

Author details

Teresa de Souza Fernandez^{1*} and Cecília de Souza Fernandez²

*Address all correspondence to: teresafernandez@inca.gov.br

1 National Cancer Institute (INCA), Bone Marrow Transplantation Center (CEMO), Laboratory Division, Rio de Janeiro, RJ, Brazil

2 Mathematics and Statistics Institute, Federal Fluminense University (UFF), Niterói, RJ, Brazil

References

- [1] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8:315–317.
- [2] Wu KH, Wu HP, Chan CK, Hwang SM, Peng CT, Chao YH: The role of mesenchymal stem cells in hematopoietic stem cell transplantation: from bench to bedside. *Cell Transpl* 2013; 22:723–729. DOI: 10.3727/096368912X655217.
- [3] Binato R, de Souza Fernandez T, Lazzarotto-Silva C, Du Rocher B, Mencalha A, Pizzatti L: Stability of human mesenchymal stem cells during in vitro culture: considerations for cell therapy. *Cell Prolif*. 2013; 46:10–22. DOI: 10.1111/cpr.12002.
- [4] Hoch AI, Leach JK: Optimizing expansion of bone marrow mesenchymal stem/stromal cells for clinical applications. *Stem Cells Transl Med* 2014; 3:643–652. DOI: 10.5966/sctm.2013-0196.
- [5] Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV: Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966; 16:381–390.
- [6] Caplan AI: Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 2007; 213:341–347.
- [7] Banfi A, Muraglia A, Dozin B, Mastrogiacomo M, Cancedda R, Quarto R: Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. *Exp Hematol* 2000; 28:707–715.
- [8] Deans RJ, Moseley AB: Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* 2000; 28:875–884. DOI: 10.1016/S0301-472X(00)00160-0.
- [9] Ball LM, Bernardo M E, Roelofs H, Lankester A, Cometa A, Egeler RM: Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. *Blood* 2007; 110:2764–2767. DOI:10.1182/blood-2007-04-087056.
- [10] Ullah I, Subbarao RB, Rho GJ: Human mesenchymal stem cells – current trends and future prospective. *Biosci Rep* 2015; 35: e00191. DOI: 10.1042/BSR20150025.
- [11] Wei X, Yang X, Han ZP, Qu FF, Shao L, Shi YF: Mesenchymal stem cells: a new trend for cell therapy. *Acta Pharmacol Sin* 2013; 34:747–754. DOI: 10.1038/aps.2013.50.
- [12] Wang S, Qu X, Zhao RC: Clinical applications of mesenchymal stem cells. *J Hematol Oncol* 2012; 5:19.

- [13] Castro-Manrreza ME, Montesinos JJ: Immunoregulation by mesenchymal stem cells: biological aspects and clinical applications. *J Immunol Res* 2015; 2015:394917. DOI: 10.1155/2015/394917.
- [14] Atoui R, Chiu RCJ: Concise review: immunomodulatory properties of mesenchymal stem cells in cellular transplantation: update, controversies, and unknowns. *Stem Cells Transl Med* 2012; 1:200–205. DOI: 10.5966/sctm.2011-0012.
- [15] Faiella W, Atoui R: Immunotolerant properties of mesenchymal stem cells: updated review. *Stem Cells Int* 2016; 2016:1859567. DOI: 10.1155/2016/1859567.
- [16] Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B: Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science*. 1998; 281:1191–1193. DOI: 10.1126/science.281.5380.1191.
- [17] Le Blanc K, Ringdén O: Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; 11:321–334. DOI: 10.1016/j.bbmt.2005.01.005.
- [18] Horwitz EM, Maziarz RT, Kebriae P: MSCs in hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2011; 17: S21-S29. DOI: 10.1016/j.bbmt.2010.11.026.
- [19] Jung S, Panchalingam KM, Rosenberg L, Behie LA: Ex vivo expansion of human mesenchymal stem cells in defined serum-free media. *Stem Cells Int* 2012; 2012:123030. DOI:10.1155/2012/123030.
- [20] Penfornis P, Pochampally R: Isolation and expansion of mesenchymal stem cells/multipotential stromal cells from human bone marrow. *Methods Mol Biol* 2011; 698:11–21. DOI: 10.1007/978-1-60761-999-4_2.
- [21] Bara JJ, Richards RG, Alini M, Stoddart MJ: Concise review: bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic. *Stem Cells* 2014; 32:1713–1723. DOI: 10.1002/stem.1649.
- [22] Kassem M: Stem cells: potential therapy for age-related disease. *Ann N Y Acad Sci* 2006; 1067:436–442.
- [23] Sekiya I, Larson BL, Smith JR, Pochampally R, Cui J-G, Prockop D: Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 2002; 20:530–54. DOI: 10.1634/stemcells.20-6-530.
- [24] Both SK, van der Muijsenberg AJ, van Blitterswijk CA, de Boer J, de Bruijn JD: A rapid and efficient method for expansion of human mesenchymal stem cells. *Tissue Eng* 2007; 13:3–9. DOI:10.1089/ten.2005.0513.
- [25] Shahdadfar A, Katrine Frønsdal K, Haug T, Reinholt FP, Brinchmanna JE: In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell

- proliferation, differentiation, gene expression, and transcriptome stability. *Stem Cells* 2005; 23:1357–1366. DOI: 10.1634/stemcells.2005-0094.
- [26] Tonti GA, Mannello F: From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera? *Int J Dev Biol* 2008; 52:1023–1032. DOI: 10.1387/ijdb.082725gt.
- [27] Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B: Aging of mesenchymal stem cell in vitro. *BMC Cell Biol* 2006; 7:14. DOI: 10.1186/1471-2121-7-14.
- [28] Zhao F, Chella R, Ma T: Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: experiments and hydrodynamic modeling. *Biotechnol Bioeng* 2007; 96:584–595.
- [29] Keung AJ, Kumar S, Schaffer DV: Presentation counts: microenvironmental regulation of stem cells by biophysical and material cues. *Annu Rev Cell Dev Biol* 2010; 26:533–556. DOI: 10.1146/annurev-cellbio-100109-104042.
- [30] Frith JE, Thomson B, Genever PG: Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods* 2010; 16:735–749. DOI: 10.1089/ten.TEC.2009.0432.
- [31] Braccini A, Wendt D, Farhadi J, Schaeren S, Heberer M, Martin I: The osteogenicity of implanted engineered bone constructs is related to the density of clonogenic bone marrow stromal cells. *J Tissue Eng Regen Med* 2007; 1:60–65. DOI: 10.1002/term.11.
- [32] Bartosh TJ, Ylostalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K: Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their anti-inflammatory properties. *Proc Natl Acad Sci U S A* 2010; 107:13724–13729. DOI: 10.1073/pnas.1008117107.
- [33] Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ: The meaning, the sense, the significance: Translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013; 19:35–42. DOI:10.1038/nm.3028.
- [34] Sart S, Agathos SN, Li Y, Ma T: Regulation of mesenchymal stem cell 3D microenvironment: From macro to microfluidic bioreactors. *Biotechnol J* 2016; 11:43–57. DOI: 10.1002/biot.201500191.
- [35] Shi C. Recent progress toward understanding the physiological function of bone marrow mesenchymal stem cells. *Immunology* 2012; 136:133–138. DOI: 10.1111/j.1365-2567.2012.03567.x.
- [36] Bobis S, Jarocha D, Majka M. Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem Cytobiol* 2006; 44:215–230.

- [37] Solchaga LA, Penick KJ, Welter JF: Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks. *Methods Mol Biol* 2011; 698:253–278. DOI: 10.1007/978-1-60761-999-4_20.
- [38] Kim JA, Im KO, Park SN, Kwon JS, Kim SY, Oh K: Cytogenetic heterogeneity and their serial dynamic changes during acquisition of cytogenetic aberrations in cultured mesenchymal stem cells. *Mutat Res* 2015; 777:60–68. DOI: 10.1016/j.mrfmmm.2015.04.003.
- [39] Zhang ZX, Guan LX, Zhang K, Wang S, Cao PC, Wang YH: Cytogenetic analysis of human bone marrow-derived mesenchymal stem cells passaged in vitro. *Cell Biol Int* 2007; 31:645–648. DOI: 10.1016/j.cellbi.2006.11.025.
- [40] Nikitina VA, Osipova EY, Katosova LD, Rumyantsev SA, Skorobogatova EV, Shamanskaya TV: Study of genetic stability of human bone marrow multipotent mesenchymal stromal cells. *Bull Exp Biol Med* 2011; 150:627–631.
- [41] Bochkov NP, Voronina ES, Kosyakova NV, Liehr T, Rzhabinina AA, Katosova LD: Chromosome variability of human multipotent mesenchymal stromal cells. *Bull Exp Biol Med* 2007; 143:122–126.
- [42] Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A: Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 2007; 67:9142–9149. DOI: 10.1158/0008-5472.CAN-06-4690.
- [43] Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J: Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. *Cancer Res* 2008; 68:4229–4238. DOI: 10.1158/0008-5472.CAN-07-5272.
- [44] Collado M, Blasco MA, Serrano M: Cellular senescence in cancer and aging. *Cell* 2007; 130:223–233.
- [45] Estrada JC, Torres Y, Benguría A, Dopazo A, Roche E, Carrera-Quintanar L: Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy. *Cell Death Dis* 2013; 4: e691. DOI: 10.1038/cddis.2013.211.
- [46] Zimmermann S, Voss M, Kaiser S, Kapp U, Waller CF, Martens UM: Lack of telomerase activity in human mesenchymal stem cells. *Leukemia* 2003; 17:1146–1149. DOI: 10.1038/sj.leu.2402962.
- [47] Samsonraj RM, Raghunath M, Hui JH, Ling L, Nurcombe V, Cool SM: Telomere length analysis of human mesenchymal stem cells by quantitative PCR. *Gene*. 2013; 519:348–55. DOI:10.1016/j.gene.2013.01.039.
- [48] Rastegar F, Shenaq D, Huang J, Zhang W, Zhang BQ, He BC: Mesenchymal stem cells: molecular characteristics and clinical applications. *World J Stem Cells* 2010; 2:67–80. DOI:10.4252/wjsc.v2.i4.67.

- [49] Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M: Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. *J Cell Biol* 2004; 166:85–95. DOI: 10.1083/jcb.200401138.
- [50] Tominaga H, Maeda S, Miyoshi H, Miyazono K, Komiya S, Imamura T: Expression of osterix inhibits bone morphogenetic protein-induced chondrogenic differentiation of mesenchymal progenitor cells. *J Bone Miner Metab* 2009; 27:36–45.
- [51] Griffin M, Iqbal SA, Bayat A: Exploring the application of mesenchymal stem cells in bone repair and regeneration. *Bone Joint Surg Br* 2011; 93:427–34. DOI: 10.1302/0301-620X.93B4.25249.
- [52] Akiyama H: Control of chondrogenesis by the transcription factor Sox9. *Mod Rheumatol* 2008; 18:213–219. DOI: 10.1007/s10165-008-0048-x.
- [53] Farmer SR: Regulation of PPARgamma activity during adipogenesis. *Int J Obes (Lond)* 2005; 29 Suppl 1:S13–S16.
- [54] Redaelli S; Bentivegna A; Foudah D; Miloso M; Redondo J; Riva G: From cytogenomic to epigenomic profiles: monitoring the biologic behavior of in vitro cultured human bone marrow mesenchymal stem cells. *Stem Cell Res Ther* 2012; 3:47. DOI: 10.1186/scrt138.
- [55] Rayment EA, Williams DJ: Concise review: mind the gap: challenges in characterizing and quantifying cell- and tissue-based therapies for clinical translation. *Stem Cells* 2010; 28:996–1004. DOI: 10.1002/stem.416.
- [56] ISSCR. Guidelines for the Clinical Translation of Stem Cells, International Society for Stem Cell Research, 2008; 1–19. Available from: www.isscr.org.
- [57] Kim SY, Im K, Park SN, Kwon J, Kim JA, Choi Q: Asymmetric aneuploidy in mesenchymal stromal cells detected by in situ karyotyping and fluorescence in situ hybridization: suggestions for reference values for stem cells. *Stem Cells Dev* 2015; 24:77–92. DOI: 10.1089/scd.2014.0137.
- [58] Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI: Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitors cells (mesenchymal progenitors cells): implications for therapeutic use. *Bone Marrow Transplant* 1995; 16:557–564.
- [59] Otto WR, Wright NA: Mesenchymal stem cells: from experiment to clinic. *Fibrogenesis Tissue Repair* 2011; 4:20. DOI: 10.1186/1755-1536-4-20.
- [60] Park B, Yoo KH, Kim C: Hematopoietic stem cell expansion and generation: the ways to make a breakthrough. *Blood Res* 2015; 50:194–203. DOI: 10.5045/br.2015.50.4.194.
- [61] Thomas ED, Blume KG: Historical markers in the development of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 1999; 5:341–346. DOI: 10.1016/S1083-8791(99)70010-8.

- [62] Santos GW: Busulfan (Bu) and cyclophosphamide (Cy) for marrow transplantation. *Bone Marrow Transplant* 1989; 4:236.
- [63] Baron F, Storb R: Allogeneic hematopoietic cell transplantation as treatment for hematological malignancies: a review. *Springer Semin Immunopathol* 2004; 26:71–94.
- [64] HoVT, Soiffer RJ: The history and future of T-cell depletion as graft-versus-host-disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood* 2001; 98:3192–3204. DOI:10.1182/blood.V98.12.3192.
- [65] Komanduri KV, Couriel D, Champlin RF: Graft-versus-host disease after allogeneic stem cell transplantation: evolving concepts and novel therapies including photopheresis. *Biol Blood Marrow Transplant* 2006; 12:1–6. DOI:10.1016/j.bbmt.2005.11.003.
- [66] Ball LM, Egeler RM: On behalf of the EBMT Paediatric Working Party. Acute GvHD: pathogenesis and classification. *Bone Marrow Transplant* 2008; 41: S58–S64.
- [67] Skoczen S, Bik-Multanowski M, Pietrzyk JJ, Grabowska A, Fijorek K, Strojny W: Genetic background of immune complications after allogeneic hematopoietic stem cell transplantation in children. *Stem Cells Int* 2016, 2016:2626081. DOI: 10.1155/2016/2626081.
- [68] Pontikoglou C, Deschaseaux F, Sensebé L, Papadaki HA: Bone marrow mesenchymal stem cells: biological properties and their role in hematopoiesis and hematopoietic stem cell transplantation. *Stem Cell Rev.* 2011; 7:569–589. DOI: 10.1007/s12015-011-9228-8.
- [69] Koç ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI: Rapid hematopoietic recovery after coinfusion of autologous blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 2000; 18:307–316.
- [70] Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J: Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia* 2007; 21:1733–1738.
- [71] Sung AD, Chao NJ: Concise Review: acute graft-versus-host disease: immunobiology, prevention and treatment. *Stem Cells Transl Med* 2013; 2:25–32. DOI: 10.5966/sctm.2012-0115.
- [72] Ferrera JMLM, Yanik G, Valdez R. Graft-versus-host-disease. In: Young NS, Gerson SL, High KA, editors. *Clinical Hematology*. Elsevier; Philadelphia, 2006, 1235–1251.
- [73] Min CK. The pathophysiology of chronic graft-versus host disease: the unrevealing of an enigma. *Korean J Hematol* 2011; 46:80–87. DOI: 10.5045/kjh.2011.46.2.80.
- [74] Ichiki Y, Bowlus CL, Shimoda S, Ishibashi H, Vierling JM, Gershwin ME: T cell immunity and graft-versus-host-disease (GVHD). *Autoimmun Rev* 2006; 5:1–9. DOI: 10.1016/j.autrev.2005.02.006.

- [75] Ferrera JLM, Levine JE, Reddy P, Holler E: Graft-versus-host-disease. *Lancet* 2009; 373:1550–1561.
- [76] Zhou H, Guo M, Bian C, Sun Z, Yang Z, Zeng Y: Efficacy of bone marrow derived mesenchymal stem cells in the treatment of Sclerodermatous chronic graft-versus-host disease: clinical report. *Biol Blood Marrow Transplant* 2010; 16:403–412. DOI:10.1016/j.bbmt.2009.11.006.
- [77] Frassoni F, Labopin M, Bacigalupo A: Expanded mesenchymal stem cells (MSC), co-infused with HLA-identical hematopoietic stem cell transplants, reduce acute and chronic graft-vs-host disease: a matched pair analysis. *Bone Marrow Transplant* 2002; 29 (suppl 2):S2.
- [78] Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M: Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; 363:1439–1441.
- [79] Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J: Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. *Biol Blood Marrow Transplant* 2009; 15:804–811 DOI:10.1016/j.bbmt.2008.03.012.
- [80] Lucchini G, Intronà M, Dander E, Rovelli A, Balduzzi A, Bonanomi S: Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. *Biol Blood Marrow Transplant* 2010; 16:1293–1301. DOI: 10.1016/j.bbmt.2010.03.017.
- [81] von Dalowski F, Kramer M, Wermke M, Wehner R, Röllig C, Alakel N: Mesenchymal stromal cells for treatment of acute steroid-refractory graft versus host disease: clinical responses and long-term outcome. *Stem Cells* 2016; 34:357–366. DOI: 10.1002/stem.2224.
- [82] Mattsson J, Ringdén O, Storb R: Graft failure after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2008; 14:165–170. DOI: 10.1016/j.bbmt.2007.10.025.
- [83] Locatelli F, Lucarelli B, Merli P: Current and future approaches to treat graft failure after allogeneic hematopoietic stem cell transplantation. *Exp Opin Pharmacother* 2014; 15:23–36. DOI: 10.1517/14656566.2014.852537.
- [84] Barrett J. Allogeneic Bone Marrow Transplantation. In: Young NS, Gerson SL, High KA. editors. *Clinical Hematology*. Elsevier; Philadelphia, 2006; 1190–1210.
- [85] Baron F, Lechanteur C, Willems E, Bruck F, Baudoux E, Seidel L: Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following non-myeloablative conditioning. *Biol Blood Marrow Transplant* 2010; 16:838–847.

- [86] Kim EJ, Kim N, Cho SG. The potential use of mesenchymal stem cells in hematopoietic stem cell transplantation. *Exp Mol Med* 2013; 45: e2. DOI: 10.1038/emm.2013.2
- [87] Katz VJ. *A History of Mathematics – An Introduction*. HarperCollins College Publishers, New York, 1993.
- [88] Zar JH. *Biostatistical Analysis*. 5th Ed. Pearson Prentice Hall, New Jersey, 2010.
- [89] Johnson RA, Wichern DW. *Applied Multivariate Statistical Analysis*. 6th Ed. Pearson Prentice Hall, New Jersey, 2007.
- [90] Sensebé L, Bourin P, Tarte K: Good manufacturing practice production of mesenchymal stem /stromal cells. *Hum Gen Ther* 2011; 22:19–26.
- [91] Kolkundkar U, Gottipamula S, Majumdar A S: Cell therapy manufacturing and quality control: current process and regulatory challenges. *Stem Cell Res Ther* 2014; 4:1–10.

