Effects of cell culture conditions on Mesenchymal Stem Cells and strategies to improve their therapeutic application

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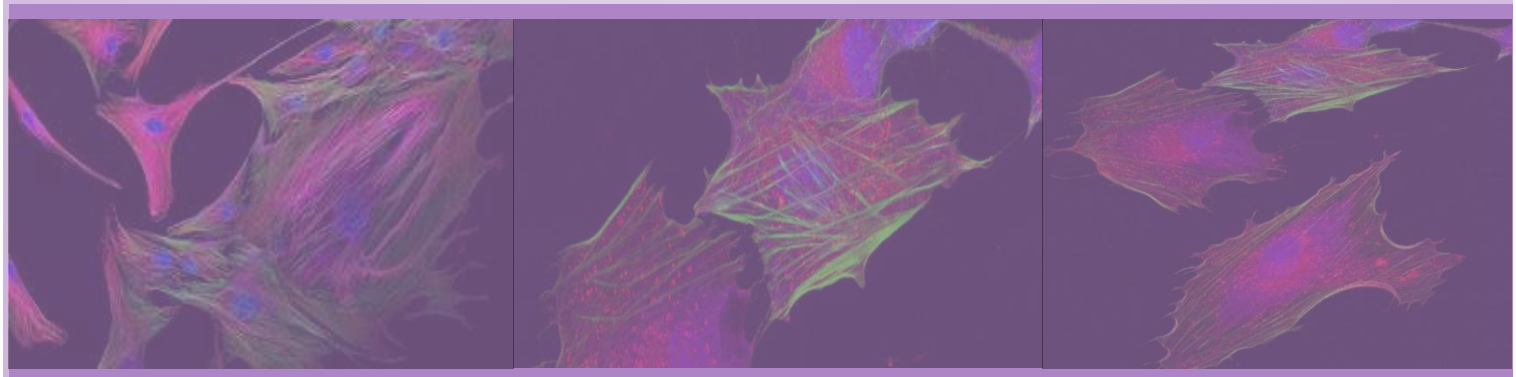

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The beneficial characteristics of Mesenchymal Stem Cells (MSCs) allow us to use them in translational and clinical research. Recent studies have shown beneficial effects of MSCs for the treatment of several pathologies, such as retinal degenerative disorders, neurodegenerative diseases, diabetes, myocardial infarction, skin problems, bone, and liver disorders, among others. These cells are found in various tissues, but they appear in low quantities, which makes necessary to expand MSCs *in vitro* before application. However, *in vitro* manipulation has noticeable consequences on MSCs morphology, physiology and function. The expression profile of molecules and receptors of MSCs undergoes drastic changes during cell culture. These alterations give rise to different results when MSCs are used in cell-based therapies, such as different immune response in the host. In this overview, our main aim will be to analyze the different modifications of MSCs during cell culture, and how these changes alter their therapeutic properties after transplantation. In addition, we will discuss potential strategies to improve the therapeutic effects of MSCs.

1. Introduction.

Mesenchymal Stem Cells (MSCs) were discovered in 1974 by Friedenstein who isolated them from bone marrow and described their morphology *in vitro* as fibroblast-like spindle shaped [1]. MSCs are multipotent cells that can be obtained from various tissues, including placenta [2], umbilical cord [3], amniotic fluid [4], bone marrow [5], muscle [6], compact bone [7] , synovial fluid [8], fat [9], dental pulp [10], hair follicles [11] and blood [12]. MSCs have two principal characteristics: self-renewal and multilineage differentiation [13,14]. Self-renewal concerns to the MSCs ability to generate identical copies of themselves, while multilineage differentiation refers to their capacity to give rise to cells into the mesodermal, ectodermal and endodermal lineages [15]. Given that MSCs show heterogeneous qualities depending on their tissue source, the International Society for Cellular Therapy (ISCT) has established three minimal standards to define MSCs: they adhere to plastic in standard conditions, they express specific markers (positive in antigens like CD73, CD105 and CD90 while negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR) and they have the ability to differentiate into adipocytes, chondrocytes, and osteoblast in specific culture conditions [16].

As mentioned above, the self-renewal and multilineage differentiation properties of MSCs are interesting points for basic and translational investigation, but also for clinical studies on several pathologies, such as cardiology, neurology,

orthopaedics, among others areas [17–20], as they promote tissue repair and regeneration (**Figure 1** and **Table 1**) [21–23]. For instance, MSC-based therapies are generating increasing interest in the current pandemic situation with the Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Several research groups have reported the beneficial effects of MSC application for pulmonary complications of COVID patients. Shetty and collaboration have demonstrated that intravenous application of human MSCs (hMSCs) produced improvements in 7 patients with COVID-19 pneumonia for 14 days compared to 3 placebo-treated patients. It was suggested that this could be due to reduced hyperactivation of the immune system and increased endogenous repair due to the paracrine effects of MSCs [24]. They observed that the administration of hMSCs originated changes in inflammatory markers, such as a significant increase in IL-10 and a decrease in TNF-α. In addition, computed tomography images showed that MSCs reduced the lesion area in the lungs at the end of the treatment in a critically ill patient with COVID-19 [24,25].

Furthermore, MSCs possess intrinsic tropism toward damaged tissues that is mediated by chemotaxis signalling pathways [13], being the C-X-C motif chemokine ligand 12 (CXCL12) – C-X-C chemokine receptor type 4 (CXCR4) axis one of the key players [26]. The CXCL12 is found in different tissues and is released in high concentrations during injury [27]. Importantly, it has been demonstrated that MSCs express the CXCR4, one of the receptors to which CXCL12 binds to mediate migration towards injury tissues [28,29].

The low quantity of MSCs in their multiple sources creates the need to expand them *in vitro* to obtain sufficient cells for therapeutic application [30]. MSCs cultures are not subject to standardized protocols. There are unequal culture media and different methods to isolate the cells, such as the explant culture method [31,32] or the enzymatic method [32,33]. In addition, MSCs can be expanded

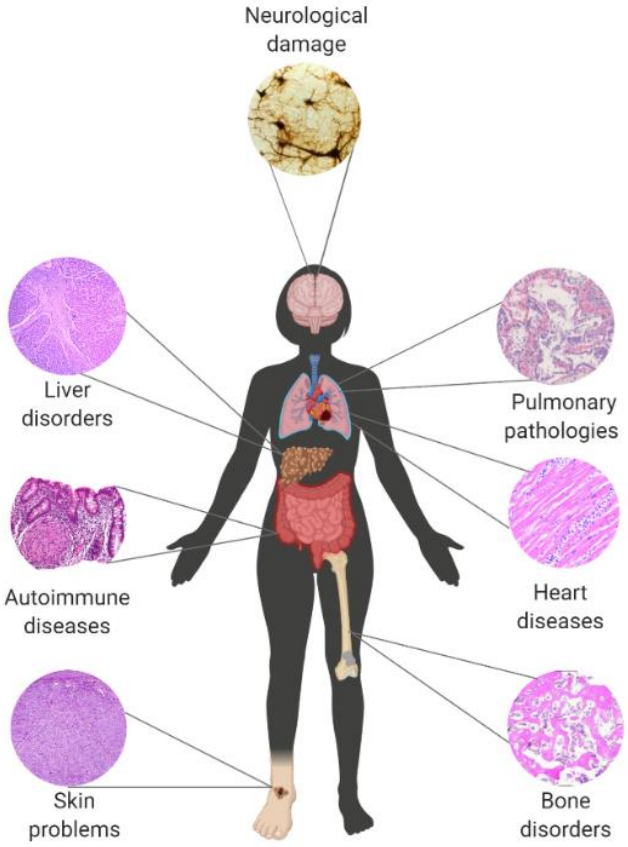

FIGURA 1. ***Representation of some MSCs therapeutic applications***. MSCs as treatment in pathological conditions: liver disorders (e.g. cirrhosis), autoimmune diseases (e.g. Crohn's disease), skin problems (e.g. skin ulcers), bone disorders (e.g. imperfect osteogenesis and osteonecrosis), heart diseases (e.g. myocardial infarction and cardiac ischemia), pulmonary pathologies (e.g. pulmonary COVID-19 infection) and neurological damage (e.g. Parkinson's disease and spinal cord injury).

on different plastic surfaces, which have peculiar hydrophobicity characteristics affecting cell growth [14,34]. The lack of common rules generates a huge variety of results when MSCs are used in pre-clinical therapies [35]. It is suggested that alterations in therapies could be associated with modifications during MSC culture, such as distinct morphologies, different membrane receptors and modifications in their secretome [30,36].

The major goal of this review is to provide a general overview about the modifications occurring in cultured MSCs that may lead to inter-laboratory variability observed when working with this cell type. In addition, we discuss alternatives *in vitro* conditions that may help to obtain more effective MSCs for cell-based therapies. The significance of this review lies in the importance of MSCs as therapeutic tool for the treatment of a wide range of pathologies due to their benefits on tissue repair and regeneration.

TABLE 1. *Clinical trials of Mesenchymal Stem Cells therapy for different types of pathologies.*

CLINICALTRIALS IDENTIFIER	PATHOLOGY	DISEASE NAME	TIME	N	MSC TYPE	ADMINISTRATION	PHASE	COUNTRY
NCT00420134[37]	Liver	Cirrhosis	24 wk	30	BM-hMSC	Intravenous	I/II	Iran
NCT01220492[38]	Liver	Cirrhosis	48 wk	45	UC-hMSC	Intravenous	I/II	China
NCT01454336[39]	Liver	Liver fibrosis	48 wk	3	BM-hMCS	Intravenous	I	Iran
NCT01591200[40]	Liver	Alcoholic Cirrhosis	96 wk	40	BM-hMCS	Intraarterial	II	India
NCT01157650[41]	Autoimmune	Crohn's disease	144 wk	15	Ad-hMSC	Unknown	I/II	Spain
NCT01659762[42]	Autoimmune	Crohn's disease	48 wk	16	BM-hMSC	Intravenous	I	EE. UU.
NCT03778333[43]	Autoimmune	Multiple Sclerosis	48 wk	7	BM-hMSC	Intravenous	I	Sweden
NCT01873625[44]	Autoimmune	Rheumatoid Arthritis	48 wk	60	BM-hMSC	Intraarticular	II/III	Iran
NCT02824393[45]	Skin	Chronic autoimmune urticaria	48 wk	10	Ad-hMSC	Intravenous	I	Turkey
NCT03887208[46]	Skin	Cutis laxa senile and scars	27 wk	100	Ad-hMSC	Subcutaneus	I/II	Poland
NCT02685722[47]	Skin	Skin ulcers	24 wk	20	UC-hMSC	Topic	I	China
NCT02491658[48]	Skin	Vulgar Psoriasis	48 wk	30	UC-hMSC	Intravenous	I/II	China
NCT01513694[49]	Bone	Intervertebral Degenerative Disc disease	24 wk	15	BM-hMSC	Implantation	I/II	Spain
NCT01605383[50]	Bone	Osteonecrosis of the Femoral Head	48 wk	23	BM-hMSC	Implantation	I/II	Spain
NCT02172885[51]	Bone	Osteogenesis imperfecta	96 wk	2	MSC	Intravenous	I	Spain
NCT00186914[52]	Bone	Osteodysplasia	Unknown	8	BM-hMCS	Intravenous	I	EE. UU.
NCT01739777[53]	Heart	Cardiopathy	48 wk	30	UC-hMSC	Intravenous	I/II	Chile
NCT01449032[54]	Heart	Chronic myocardial ischemia	24 wk	60	Ad-hMSC	Intramyocardial	II	Denmark
NCT02387723[55]	Heart	Severe Heart Failure	24 wk	10	Ad-hMSC	Intramyocardial	I	Denmark
NCT02467387[56]	Heart	Non-Ischemic Heart Failure	64 wk	23	BM-hMSC	Intravenous	II	EE. UU.
NCT04366323[57]	Pulmonary	COVID-19	48 wk	26	Ad-hMSC	Intravenous	I/II	Spain
NCT04288102[58]	Pulmonary	COVID-19	12 wk	90	UC-hMSC	Intravenous	II	China
NCT02594839[59]	Pulmonary	Progressive Interstitial Lung Disease	48 wk	20	BM-hMSC	Intravenous	I/II	Russia
NCT01919827[60]	Pulmonary	Idiopathic pulmonary fibrosis	48 wk	17	BM-hMSC	Endobronchial	I	Spain
NCT02668068[61]	Pulmonary	Pneumoconiosis	24 wk	80	UC-hMSC	Lavage	I	China
NCT01056471[62]	Neurological	Secondary Progressive Multiple Sclerosis	48 wk	30	Ad-hMSC	Intravenous	I/II	Spain
NCT02611167[63]	Neurological damage	Idiopathic Parkinson's Disease	52 wk	20	BM-hMSC	Intravenous	I/II	EE. UU.
NCT01325103[64]	Neurological	Spinal Cord Injury	24 wk	14	BM-hMSC	Intralesional	I	Brazil
NCT02249676[65]	Neurological	Neuromyelitis Optica	48 wk	15	BM-hMSC	Intravenous	II	China

**Abreviations:* Mesenchymal Stem Cells (MSC), Bone Marrow human Mesenchymal Stem Cells (BM-hMSC), Umbilical Cord human Mesenchymal Stem Cells (UC-hMSC), Adipose derived human Mesenchymal Stem Cells (Ad-hMSC).

2. Changes induced in MSCs during cell culture.

Each step in cell culture is parallel to MSCs morphological disorders, changes in their markers profile and physiological perturbations. Furthermore, the alterations are determined by many variable conditions such as donor age [66], tissue source [67], passages number [68], oxygen levels [69] or medium composition [70] (**Figure 2**). Zaim et al. demonstrated that donor age affects differentiation of bone marrow hMSCs (BM-hMSCs). BM-hMSCs from children between 0-12 years old showed more adipogenic, neurogenic and osteogenic differentiation potential and more proliferation than BM-hMSCs from adults between 25-50 years old or from elderly over 60 years old in the same passage [66] . Another study demonstrated that the source of MSCs affects later differentiation. The use of BM-hMSCs presented a greater differentiation potential to osteogenic cells, while MSCs derived from adipose tissue (Ad-hMSCs) revealed a greater differentiation potential to adipogenic cells [67]. Furthermore, the passage number of the cell culture is another significant point. Tan and co-workers showed that surface markers of bovine synovial membrane-derived MSCs (SD-MSCs) change in passages (P) 4 [68]. There was an increase in the expression of CD73 between P1 and P2, whereas CD73 levels had a significant reduction in P3. In this report, they suggested that the decrease on CD73 expression could be responsible for the changes in migration. When they applied a direct current electric field (DC-EF), 85% of cells moved towards the positive pole in P1, while a 75% of cells migrated towards the negative pole in P4. This variations in the direction of SD-MSCs migration correlated with the changes in CD73 expression [68]. All these investigations evidence how the cell culture conditions influence SD-MSCs. Throughout this section we will focus on describing morphological alterations, changes in the markers profile and physiological perturbations that MSCs undergo during culture.

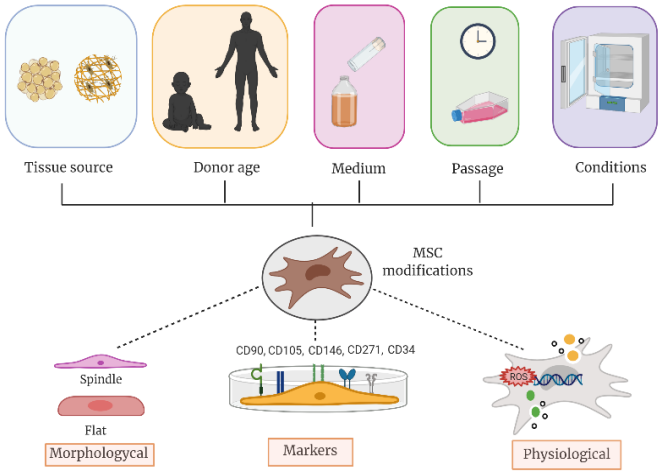

FIGURE 2. ***Representation of influencing factors on morphological and physiological characteristics, in addition to surface markers.*** Factors responsible for changes in cultured MSCs include tissue origin, donor age, medium and supplements, passage number, and incubation conditions such as oxygen levels. All these parameters cause morphological alterations in MSCs, which change from a spindle form to a flattened form. Moreover, there are changes in surface markers such as higher expression of CD146, CD105 and CD271 and a lower expression of CD34 and CD90. Physiological changes include the generation of free radicals that directly affect the amino acid profile and lipid peroxidation. Abbreviations: Cluster of Differentiation 146 (CD146), Cluster of Differentiation 105 (CD105), Cluster of Differentiation 271 (CD271), Cluster of Differentiation 34 (CD34), Cluster of Differentiation 90 (CD90), Reactive oxygen species (ROS).

2.1 Morphological alterations.

The MSCs normally have a spherical form *in vivo* [71]. When MSCs are seeded as adherent cells, they usually acquire spindle form [72]. However, the MSCs morphology can change in response to medium supplements [73,74], passage number [75] and/or oxygen conditions [76]. One of the most common supplements used in cell culture is fetal calf serum (FCS) as a nutrition source, protein and growth factors [74]. Chase et al., demonstrated that the use of FCS affects MSCs morphology. Using light microscopy, they observed that BM-hMSCs cultured in a medium with FCS had a flattened shape, while BM-hMSCs cultured in a serum-free medium had their characteristic spindle morphology [73]. Another influential factor affecting cell morphology is the passage number, which refers to MSC aging. A study grew BM-hMSCs in two different media, Minimum Essential Medium Eagle - Alpha Modification (α-MEM) and Dulbecco's Modified Eagle Medium (DMEM), and observed that MSCs acquired atypical and flat shapes by P6 [75]. In addition to medium supplements and aging, another factor to consider is the oxygen concentration. Holzwarth's team demonstrated how low oxygen levels and donor affect cell morphology. They cultured BM-hMSCs from 10 donors under two conditions 21% and 1% oxygen. When examining the cells under the light microscope, they observed that BM-hMSCs from most donors showed the same spindle morphology and all the cells appeared as a monolayer at 21% and 1% oxygen after one or three weeks. Conversely, BM-hMSCs from 7 donors did not adopt the typical spindle shape and they did not create monolayers at 1% oxygen in the two measures of time [76]. Examples such as these demonstrate that MSCs undergo dynamic changes. The question is, which are the molecular mechanisms underlying the morphological alterations?

2.2. Modifications in the markers profile.

There is not a set of definitive markers which define a unique phenotype in MSCs due to their variability (origin, conditions, age, isolation method). However, there are common receptors for growth factors, chemokines, cytokines, matrix proteins, cell-cell receptors and inmuno-modulating receptors [26]. Most membrane markers have been determined *in vitro*, hence there is a limited knowledge of their properties *in vivo* [77]. These antigens are not exclusive of MSCs as Schrage et al. showed in their study by demonstrating that CD46 is also an endothelial marker (antibody ME-9f1) [78]. Moreover, MSCs can have a different expression receptor fraction according to their source, for example Stro-1 appears in BM-hMSCs but there is a lack of this antigen in Ad-hMSCs [79]. As previously discussed, the ISCT indicated that the positive MSCs surface markers are CD73, CD90 and CD105. Furthermore, Maleki et al. compared hMSCs from ovary, testis, hWJ-MSCs and hair follicle, and they found that all these hMSCs also shared Stro-1, CD44, CD166 and CD106 [80]. On the other hand, Ly and co-workers in their review added three repetitive membrane markers, e.g. Stage-specific

Embryonic Antigen-4 (SSEA4), CD271 and CD46 [81]. Several investigations have demonstrated that the markers profile changes when MSCs are cultured *in vitro*. For example, Braun and collaboration have shown that clusters of differentiation, such as CD146, CD105 and CD271 of adventitial stromal cells (AST), were over-expressed after four days *in vitro*. However, CD34 had less expression between the fourth and sixth day [72]. Another example of marker that changes *in vitro* was identified by Yu et al., who demonstrated high expression of Stro-1 in dental pulp stem cells (DPSCs) of rat and human at P9, as compared to P1 [82].

Moreover, it has been shown that BM-hMSCs seeded in a 3D alginate culture or under mechanical stimulation present low CD90 expression [83,84]. This protein is involved in the regulation of cell-cell contact and cell-matrix junctions. The lack of this marker has consequences such as impaired cell migration, affected actin filaments and loss of cell-cell and cell-matrix junctions, which could alter cell morphology [85].

2.3. Physiological perturbations.

Cell culture conditions, such as oxygen concentration, passages, supplements, contribute to maintain cellular homeostasis. The oxygen levels applied in culture are usually those that we have in the atmosphere (20%), but this point is questionable since cells in the body are indeed exposed to 2-7% oxygen. This is an important issue because high oxygen levels generate metabolism perturbations and oxidative stress, generating one of the most notorious consequences that is the increase of the radical oxidative species (ROS) concentration, one of the typical senescence marks in cultures cells. In addition to the activation of the senescence process, high oxygen levels reduces cell survival and proliferation, which is a bottleneck to use MSC in therapy [86]. ROS are small molecules usually generated in mitochondria and they can react easily due to their free electron (superoxide anions [O^{2-}], hydrogen peroxide [H_2O_2] and hydroxyl radicals [OH^-]). ROS can coordinate different cell levels because of its ability of regulating redox state of proteins and lipids, among others, generating cellular perturbations[87]. Shin et al. demonstrated that increased ROS levels relate to changes in amino acid profile and lipid peroxidation. BM-hMSCs with high ROS levels due to serum starvation exhibited alteration of amino acids like lysine, tyrosine, and γ-aminobutyric acid (GABA) accumulation. At the same time, there is a gain of lipid peroxidation giving rise to a decreased membrane permeability and fluency [88].

3. Consequences of the *in vitro* MSCs modifications for their use in cell therapy.

The changes occurring in cultured MSCs, such as morphological alterations, the different profile of surface markers and the physiological modifications, limit their widely use in clinical application. For example, the increased size of MSCs *in vitro* influences their therapeutic effect

after cell delivery. In consequence, transplanted MSCs may exhibit a reduced migration to the target tissue [89,90] or may originate adverse events during cell therapy that compromise patient safety [91,92]. On the other hand, changes in the surface markers occurring in cultured MSC are related to the immune response, giving rise to rejections of transplanted cells [93]. Moreover, physiological modifications of cultured MSC derived from patients with pathologies can develop an altered phenotype. For instance, MSCs derived from diabetic patients with critical limb ischemia were found to display a prothrombotic phenotype [94].

The most frequent administration routes are intravenous [89,96,97], intranasal [98–100], intramyocardial [101,102], intramuscular [103,104], intracoronary [105–107], intrathecal [108,109], intra-arterial [110–112] (**Figure 3**). However, MSC delivery is affected by the increased size of these cells. Most of the infused cells are found in the lungs one hour after BM-hMSCs intravenous administration in mice [89]. Schrepfer and co-workers showed, through fluoresce microspheres of different dimensions, that lung capillaries have an average diameter between 4-15 µm in mice. It is suggested that the retention of BM-MSCs in the lungs could be due to the increased size of cultured MSCs, which reach an average diameter of 15-19 µm [90]. The retention of MSCs in non-target organs is associated with a reduced efficacy of the cell therapy. On the other hand, studies have shown that the increased size of the MSCs is unsafe for intracarotid administration [91,92]. Ge at al. showed that intra-carotid injection of large placental-derived hMSCs (average diameter 29 µm) in rats caused severe vascular obstructions and strokes in comparison with those rats that were injected with small placental-derived hMSCs (average diameter 13-17 µm) [91]. Moreover, another study with rats demonstrated that the intracarotid administration of large MSCs (average diameter 25 µm) resulted in a reduction in cerebral blood flow at doses of $2x10^6$ cells related to cerebrovascular accidents [92].

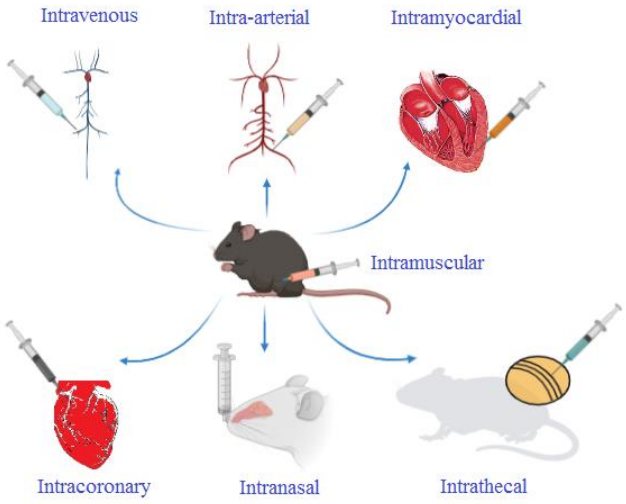

FIGURE 3. ***Representation of MSCs administration routes in mouse.*** Among the most used methods of cell delivery in the literature are intravenous, intra-arterial, intramyocardial, intracoronary, intranasal, intrathecal, and intramuscular administration.

The immunosuppression is another relevant property of MSCs that may be altered during cell culture, which should be taken into consideration during allogenic therapies. In this context, the expression level of human leukocyte antigen class I (HLA-I) in the surface of MSCs has been shown to change under specific cell culture conditions. For instance, MSCs treated with Interferon gamma (IFN-γ) exhibited reduced levels of HLA-I and

elicited a weaker immune response than non-treated cells [93].

Apart from the lack of an unanimous protocol to culture cells, their use in several pathologies is debatable because of their incompatibility with the host, generating arrhythmia [113,114], increasing the aggressiveness of previous tumours [95,115], favouring thrombotic events [94] and atypical tissue differentiation [116]. For instance, Djouad et al. demonstrated that co-injection or local separated injection of two types of MSCs (line C3H / 10T1 / 2 and primary BM-MSCs of two mice) with B16 melanoma cells in the same treatment resulted in a higher tumours incidence compared with B16 melanoma cells injected as control. This team suggested that the most accentuated tumours with MSCs could be due to the MSCs immunosuppressive activity, giving rise to the inhibition of the immune response on the tumour [95]. In addition, MSCs are characterized by releasing factors such as vascular endothelial growth factor (VEGF) involved in angiogenesis that could favour the development of tumours [95]. On the other hand, the pathological condition of the donor may influence the therapeutic properties of MSCs, as demonstrated by Capilla-González et al. [94]. They demonstrated that Ad-hMSCs derived from two diabetic patients with critical limb ischemia (CLI) showed a reduction in proliferation and migration, as well as an altered differentiation ability compared to Ad-hMSCs from healthy individuals. This defective phenotype was associated with a deficient platelet-derived growth factor (PDGF) signalling pathway. More importantly, Ad-hMSCs from diabetic patients exhibited a pro-thrombotic profile, which could be responsible for distal microthrombosis after intra-arterial administration of autologous Ad-hMSCs in these two patients [94]. Therefore, there is an urgent need to develop new strategies to improve the therapeutic properties of MSCs.

4. Strategies to potentiate the therapeutic properties of MSCs.

Criteria such as optimum basal medium [117], cell density in passages [118], plastic surface quality [119] and medium supplements [120] affect to the research findings and investigators should take into considerations these effects when making conclusions. Sotiropoulou et al., demonstrated that BM-hMSCs improved their proliferation by changing parameters as cell density, medium conditions, and growth surface (i.e. flasks). They showed that proliferation presented a higher rate with an initial low density in contrast with high density, they obtained an optimal value with an initial rate of 1000 cell/cm^2. Moreover, Eagle's minimal essential medium-alpha modification with Glutamax (α-MEM/GL) in a Falcon flask was the medium and plastic surface with best results in terms of proliferation. This research team suggested that these results could be due to the fact that Glutamax has a more stable L-alanyl-L-glutamine dipeptide compared to the L-glutamine presented in other culture medium. Furthermore, the increase in cell proliferation after a 4-weeks

period in culture in Falcon flasks could be explained with its peculiar manufacturing method. Falcon flasks acquire hydrophilicity on their surface through a treatment based on the corona effect carried out in closed chamber, while the rest of the flasks used in the study (Greiner, Nunc and Costar) acquire hydrophilicity by exposing the air that surround the flask to the environment without closed chamber, which could be the cause of less proliferation in these flasks [34].

As mentioned earlier, medium supplements are interesting since their use can lead to beneficial modifications in cell phenotype and physiology to face the negative effects of cell culture explained in previous sections. Under the terms of proliferation rate of BM-hMSCs, application of basic fibroblast growth factor (bFGF), Platelet-Derived Growth Factor (PDGF-BB) , Ascorbic Acid (AA) or the combination of all of them provide higher expansion until P6, without affecting the differentiation capabilities [120–122]. Furthermore, Capilla-Gonzalez et al. showed that the use of PDGF-BB can reverse the negative characteristics of Ad-hMSCs from diabetic patients previously mentioned. They demonstrated that Ad-hMSCs incubated with PDGF-BB improved migration, proliferation and pro-thrombotic phenotype in *ex vivo* studies. Furthermore, they demonstrated an improved homing of Ad-hMSCs incubated with PDGF-BB in a mouse model of cutaneous wounds[94]. Moreover, senescence is a process that cells undergo as they are grown *in vitro,* and ROS generation is one of its hallmarks. Several studies have shown the advantages of using bFGF in this process. The supplementation of culture media with bFGF have proved a high proliferation rate, a limited apoptosis, a reduced senescence and longer telomeres [34,123,124]. Apart from that, it has been suggested that diminished HLA class I marker in MSCs could be responsible for the inmune response [93]. Giuliani and co-workers have demonstrated that the addition of IFN-γ supplement to the culture for two days offers a remarkable results in the surface markers responsible for interactions between fetal, embryonic and BM-hMSCs with NK, including an up-regulation of HLA class I [125]. At this point, the discrepancy between adverse or positive effects in surface markers changes due to the composition of the medium, could be questioned. In this context, studies have also focused on the reversibility of these modifications. Gharibi et al. have demonstrated that the use of bFGF in BM-hMSCs causes changes in the CD146 marker. Moreover, CD146 returned to basal levels one week after the absence of bFGF [120] (**Figure 4**).

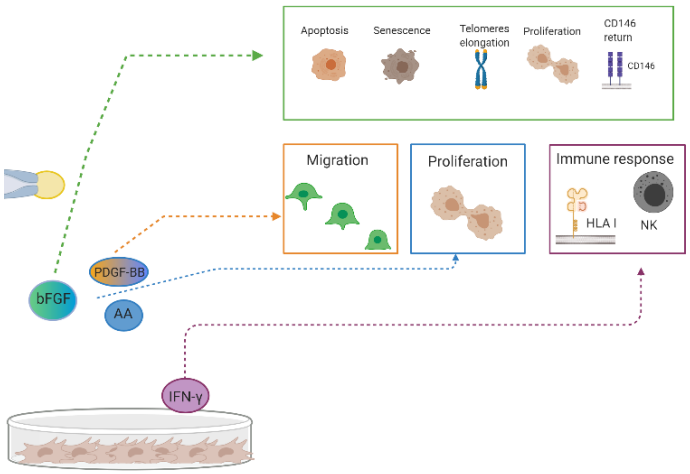

FIGURE 4. ***Medium supplements effects in MSCs***. The supplements use presents controversies in the results, the positive effects of factors such as bFGF, PDGF-BB, AA and IFN-γ are exposed in this representation. In green, bFGF presents results of improvement in the senescence process such as reduced apoptosis, lower senescence rate, more elongated telomeres, higher proliferation rate and reversible changes in the CD146 marker. In blue, bFGF, PDGF-BB, AA show a higher proliferation rate. In orange, PDGF-BB shows migration changes. In purple, IFN-γ causes an increase in the HLA class I marker, responsible for the interaction between MSCs and NK. Abbreviations: basic Fibroblast Growth Factor (bFGF), Platelet-Derived Growth Factor (PDGF-BB), Ascorbic Acid (AA), Interferon gamma (IFN-γ), Clustter of differentiation CD146 (CD146), Human leukocyte antigen class I (HLA I), NK (Natural Killer).

The route of administration can affect the therapeutic effects of MSCs since it may be relevant depending on the pathology to be treated. The increased size of MSCs *in vitro* gives rise to adverse effects in their administration, for example in intravenous administration, most cells are trapped in the lungs, so the number of cells is reduced in a distant target organ [90]. Therefore, the intravenous route would be appropriate for treatment of lung disease. For instance, Yip and collaboration demonstrated that a unique intravenous dose of MSCs is safe for the treatment of Acute Respiratory Distress Syndrome (ARDS), which is a pathology associated with COVID-19. This research included 9 patients that were divided in three groups receiving different doses of Umbilical Cord hMSCs (UC-hMSCs), $1x10^6$ cells/kg, $5x10^6$ cells/kg or $1x10^7$ cells/kg respectively. It was shown that the use of UC-hMSCs is safe and viable, and there is a lack of negative effects with the three different dosis. Moreover, this team identified changes in immunological biomarkers, they observed a reduction of dendritic cells one month later. They suggested that these modifications of immune response on account of UC-hMSCs treatment could be used to controlled the inflammatory response of ARDS and the results could be better if some doses are applied over time [126]. Furthermore, Zheng and collaboration have demonstrated the intravenous administration of Ad-hMSCs is safe in SARS-CoV-2. A group of 6 patients receiving a unique dose of $1x10^6$ cells/Kg showed lower levels of surfactant protein D (SP-D) in comparison with 6 control patients who was injected saline solution. The SP-D is a biomarker of the injured epithelial cells, which is symptom of COVID-19, so this reduction may account for the protective function of Ad-hMSCs. Moreover, this investigation verifies the lack of toxicity and adverse effects of MSC in therapy [127]. On the other hand, for neurological diseases, the blood-brain barrier is an obstacle for MSC homing to central nervous system. In this cases, the intrathecal or the intranasal routes would be a more effective alternative, as well as less invasive method than intraparenchymal or intracerebroventricular routes [100,128].

Another alternative to improve the therapeutic potential of MSCs could focus on enhancing MSC migration. In a recent study, Xu and collaboration showed that the exposure of BM-hMSCs to hypoxic conditions (1%) for 4 and 6 hours improved their migration *in vitro*. This team suggested that these conditions come in parallel with the increase in the expression of hypoxia inducible factor 1-alpha (HIF-1α). They also demonstrated that, when HIF-1α is blocked, there was a reduction in the expression of the CXCL12 – CXCR4 axis, a key player in cell migration. Advances on this topic and more studies would be necessary to understand this phenomenon *in vivo* [129].

4.1. Biomaterials.

The use of biomaterials is an alternative to obtain better therapeutic effects of MSCs as they facilitate the retention of MSCs into the damaged tissues.

MSC function depends on biophysical conditions and bioactive signals. Among others, the influential factors affecting MSC processes include environment porosity, architecture, inflammation, the cellular matrix, and cell-to-cell contact. All these examples evidence the relevance of mimicking the *in vivo* environment during cell culture and administration [130,131]. Biomaterials are substances that simulate biological systems and act as a protective scaffold to maintain the properties of seeded cells, including MSCs [132]. A multitude of biomaterials have been successfully used in research, including gelatine, carboxymethyl cellulose (CMC), collagen type I, bone extracellular matrix (bECM) [132],elastin [133], chitosan [134], Matrigel [135], fibrin [136], fibronectin[136], protein-reactive nanofibrils[137], among others. Using an experimental rat model of myocardial infarction, Roche and colleagues demonstrated that the retention of hMSCs into infarcted heart was more efficient with the use of biomaterials, while the saline control group had less retention. They used four types of biomaterials based on hydrogels (alginate and chitosan / β-glycerophosphate) or epicardial patches (alginate and collagen). Twenty-four hours after implantation, the biomaterials showed between 50 and 60% retention of hMSCs, as compared to the control saline group that displayed 10% retention [138]. Furthermore, Lu and co-workers demonstrated that the use of biomaterials as scaffolding for delivering MSCs had improved results in an experimental model of traumatic brain injury (TBI) in rats. In this study, human marrow stromal cells were seeded in collagen scaffolds and then, deposited in the nucleus of the TBI (4 days after injury). hMSCs implanted with the collagen scaffolds showed higher migration and the lesion volume was lower, as compared to rats injected with saline, collagen or hMSC alone. Moreover, the scaffolding rats exhibited improved neurological functions when they were subjected to behavioural tasks, such as the *Maze Morris* test (**Figure 5**) [139]. Furthermore, it has been shown that Ad-MSCs presented beneficial effects for osteochondral defects when they were intramuscularly transplanted with a scaffold based on polymeric nanofibrils decorated with cartilage-

derived decellularized extracellular matrix[137]. They demonstrated that the scaffold with Ad-MSCs exhibited better chondrogenic regeneration potential, even without exogenous growth factors. Rats transplanted with Ad-MSCs seeded in scaffolds had better characteristics regarding cell morphology, matrix staining, and cartilage thickness in comparison with groups without Ad-MSCs [137].

All these investigations corroborate the importance of the use of biomaterials to serve as substrate, locators, and templates for transplanted MSCs [140]. In addition, they improve the survival and differentiation of MSCs [135,138,139].

4.2. Secretome.

Until now, the advantages and disadvantages of using MSCs, as well as some possible solutions, have been discussed. At this point, the question would be how MSCs provides therapeutic effects if they have a short lifetime after administration. The answer to this lies in the paracrine effects of MSCs, which are mainly responsible of their secretome. Despite transplanted cells have a short-life, MSC secretome induces lasting effects in the adjacent cells and the microenvironment. The secretome is the set of proteins released by the cells with an important role in regulating several cellular processes [141]. It is characterized by being composed of growth factors, cell adhesion molecules, cytokines, chemokines, lipids, exomes, hormones, microvesicles, among others [142,143]. Between the advantages of using the secretome in cell-based therapies are: 1) secretome is not a proliferative agent, unlike MSCs, reducing the possibility of inducing tumorigenic effects; 2) it is not toxic and it has a low immunogenicity; and finally, 3) its administration is safer than administration of MSCs [144]. All these advantages make the secretome an interesting therapeutic alternative.

Texeira and collaborators demonstrated that the bilateral injection of the secretome derived from Human Umbilical Cord Perivascular Cells (HUCPVC) in the rat hippocampal dentate gyrus showed similar results than HUCPVC [145]. In particular, they observed similar improvements of endogenous cell proliferation, survival, and differentiation in the HUCPVC group and HUCPVC secretome group, in comparison with the control group (rats treated with Neurobasal A medium). Additionally, it was shown an increase in bFGF expression related to the early stages of neurogenesis, survival, and maturation [145]. Moreover, the use of the BM-hMSCs secretome showed improvements in brain structure and animal behaviour in a neurodegenerative disease model [146]. This research team generated a rat model of Parkinson's disease with a unilateral injection of 6-hydroxydopamine in the medial forebrain. After five weeks, unilateral injections of the secretome were applied to the substantia nigra and striatum of Parkinson animals. Animals injected with the secretome showed an increase in the density of cells capable of catalysing dopamine precursor dihydroxyphenylalanine (DOPA). In addition,

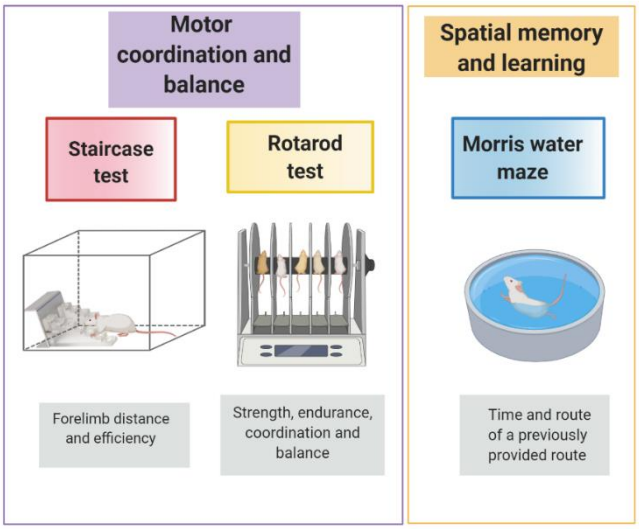

FIGURE 5. ***Motor coordination and balance, and spatial memory and learning some behavioural tests***. On the left, it is represented two behavioral tests related to motor coordination and balance. ***Staircase test***. In this test, two stairs are used with feed in each step on both sides of the transparent container, and between them a platform where the animal is placed. It can be measured the effectiveness and the distance reached by forelimbs depending on the feed consumed and observations. ***Rotarod test***. This methodology consists of a rotating cylinder with modifiable speed. Rodents are placers on the rod, and it can be measured their resistance, strength, balance, and coordination. On the right, there is a test connected with spatial memory and learning. ***Maze Morris test***. In this test, rodents are placed in a container of water in which there is a platform that can be viewed by individuals. After several repetitions, the platform is hidden to check the memory and learning of rodents. The length of the route, the time spent, the analysis of different quadrants can be measured.

secretome induced behaviour improvements *in vivo* through *Rotarod* and *Staircase* tests (**Figure 5**) [146].

5. Conclusions and future prospects.

Throughout this review, three main points have been addressed: 1) changes induced in MSCs during cell culture, 2) how these changes affect the use of *MSC-based therapies* and 3) strategies to improve the therapeutic effects of MSCs. MSCs have great potential for cell therapy and regenerative medicine due to their easy extraction from multiple sources [147], their ability to migrate to damaged tissues [148,149], their multilineage differentiation [150,151], their self-renewal [152], and the lack of ethical concerns [153]. All these advantages reinforce the use of MSCs to treat several diseases, such as myocardial infarction [154,155], cardiac ischemia [156,157], neurological disorders [100,158,159], imperfect osteogenesis [160], or more recently the COVID-19 [24,25,126,161,162].

Latest advances in this research area hold promises for the applications of MSCs in regenerative medicine. However, MSC-based therapies still present barriers that need to be overcome. In conclusion, all the procedures that are carried out during cell culture generate morphological and physiological modifications in MSCs that directly affect their clinical application. Moreover, the lack of a unanimous protocol originates discrepancies in the results obtained by researchers, and, in many cases, this makes difficult to reproduce the experiments. Therefore, it is important that the investigators make the effort to unify protocols to obtain conclusions that are more robust. On the other hand, strategies are being implemented to enhance the therapeutic properties of MSCs, but there is still much potential to be improved. The scientific community should further investigate how to develop strategies that significantly increase the efficacy of *MSC-based therapies* in a safety way, allowing the translation to humans.

As a critical analysis, the communication between the different research teams is required to the unification of protocols. The main obstacle to achieve this point is the lack of transparency in the publications, which often do not specify all the information in a detailed manner or even the obtained results. A universal protocol to culture MSCs should include an effective isolation method, the use of a specific cell culture vessel, the detailed media composition (including reagent references) and the standardized culture conditions, such as oxygen levels. Furthermore, the type of MSCs must also be taken into account since it has been shown that MSC properties may vary between source tissues. Apart from that, the experimental strategies to improve the therapeutic properties of MSCs are based on three main points: the minimization of the changes that MSCs undergo *in vitro*, the use of biomaterials to favor MSCs application, and the utilization of MSC secretome. The choice of the most appropriated strategy will depend on the specific aim of the therapy. For instance, the use of MSCs combined with biomaterials to facilitate their engraftment and survival, would be an appropriated option when doing local cell administration (e.g., intracranial injections). However, when a systemic administration is used in allogenic therapies, the MSC-derived secretome could be an interesting choice since it has a low risk of rejection, as well as a reduced tumorigenic potential, unlike live cells. In conclusion, the scientific community should make efforts in synergy to seek solutions to the aforementioned issues, bringing new perspectives for a personalized medicine that will allow us to successfully treat the specific pathological condition of each patient.

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