Review paper

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Critical factors responsible for the therapeutic effect of mesenchymal stem/stromal cells in central nervous system disorders

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Abstract

Nowadays it is observed that the number of stem-cell based experimental therapies in neurodegenerative disorders is massively increasing. Most of the clinical trials registered to date have been based on autologous mesenchymal stem/stromal cells (MSC) obtained from somatic tissues. In the conducted clinical trials neither serious side effects, nor statistically significant improvement were observed. The lack of statistical significance could result from a relatively small number of patients involved in clinical trials or highly incoherent study protocols. However, most clinical groups describe a trend towards improvement in MSC-treated patients. Hence, the question arises which factors associated with MSC-based therapy may be the key and result in better therapeutic response. In the presented paper, we summarize, in our opinion, the most important factors that could increase the effectiveness of this therapy.

Key words: *mesenchymal stem/stromal cells, CNS disorders, intrathecal injection, immunomodulatory properties, therapeutic effect, cell therapy.*

Introduction

Although some of the first results demonstrated that it is not only possible to obtain unlimited numbers of cells *in vitro* but also cells able to differentiate towards most somatic cell types, including the neural cells (e.g. neurons, astrocytes and oligodendrocytes) [2], it is now known that the way to introduce effective cell therapy is not so simple. Translation of preclinical results to the first clinical trials has proved that cell therapy is not a panacea for every disease, and the cells must be properly prepared, in validated process, and repeatedly used in order to obtain a therapeutic effect.

Most of the ongoing clinical trials focus on the transplantation of heterogeneous mesenchymal stem/stromal cell (MSC) fractions [13]. They are obtained in a non-invasive way and are relatively easily accessible from different tissue niches, such as bone marrow, adipose tissue or afterbirth tissues. Unfortunately, after several decades of research, there are only a few papers demonstrating the ability of transplanted MSC to integrate into the injured tissue architecture and take over functionally the role of dead cells *in vivo* and in animal models [5]. The potential of MSC for multidirectional, mature differentiation is being questioned. Nevertheless, the results of

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clinical trials indicate a certain improvement in the functioning of the injured organs or the inhibition/retardation of the ongoing disease processes.

The solution to the abovementioned problem seems to be the discovery of methods leading to dedifferentiation of the somatic cells to their original state of pluripotency, so-called induced-pluripotent stem cells (iPSCs). These cells show a differentiation potential comparable to that of embryonic stem cells (ESCs). This unique feature, however, is closely related to the unlimited proliferation potential of these cells, which, along with genetic instability and the instability of the directions of differentiation increase the probability of ectopic undesirable tissues development, including neoplastic ones. Due to these risk factors, MSC continue to be the most often used cells in cell therapy.

In this review, we would like to focus on some of the factors that may underlie the effectiveness or lack of the effect of MSC-based therapy in patients with central nervous system (CNS) pathology. We have summarized our pre-clinical and clinical experiences from 2015 and compared them with the experience of other groups working with MSC. Furthermore, we have analysed procedures, including the route of cell administration, the number of cells administered, determining the safety of the therapy in specific applications, and correlation of clinical data (cerebrospinal fluid analysis) with transplanted cell features.

In this paper, we do not discuss the effects of cell therapy, but focus on the mechanisms that can be activated by delivering MSC to the central nervous system. Our decision was made due to the fact that despite the numerous clinical trials, most of them were carried out according to different criteria, using different material qualitatively or quantitatively, and the effects were evaluated differently. The problem connected to the incoherence of stem cell based clinical trials was devoted to one other work [5,7].

Route of cell administration

In patients with neurological diseases, cells were administered: intravenously, intrathecally, intracerebrally or intranasally. Intracerebral or intraspinal administration route requires a specialized neurosurgical centre and may cause complications like local structural injury. Intravenous cell injection is disputable due to the blood-brain barrier (BBB) that limits the migration of stem cells. Many studies have observed that intranasal application (INA) can bypass the BBB and enable the delivery of growth factors, chemokines or cytokines to the CNS. However only a small number of cells could reach the brain parenchyma after INA. Intranasal administration may be the best route for administration the exosomes derived from MSC [22]. It seems that the optimal method of cell administration is the intrathecal injection.

The circulating cerebrospinal fluid (CSF) helps to distribute the injected cells and their products throughout the subarachnoid space. Moreover, the injection into the CSF by lumbar puncture is a low-risk medical intervention. In 2010 we published the results from the first intracerebroventricular transplantation of cord blood-derived neural progenitors in a child with severe global brain ischemic injury. At that time, we had the possibility of labelling the transplanted cells with iron oxide nanoparticles (SPIO) and following their fate by magnetic resonance imaging (MRI). A 16-month-old child at 7 months after the onset of cardiac arrest-induced global hypoxic/ischemic brain injury, resulting in a permanent vegetative state, was subjected to intracerebroventricular transplantation of the autologous neutrally committed cord blood cells. These cells were tagged with SPIO nanoparticles and grafted monthly by three serial injections (12×10^6 cells/0.5 ml) into the lateral ventricle of the brain. MRI examination revealed the presence of cells in lateral ventricles for approx. 4 months [12]. The presence of injected cells in the intrathecal space was also confirmed by other groups. In 2019, during an MRI examination, Singer *et al*. described nerve root thickening, clumping, nodular thickening of nerve roots and enhancement after intrathecal injection of adipose-derived autologous MSC to patients with early multiple system atrophy – MSA [29]. The described changes may be related to the immune (inflammatory) response [10] to the cell presence in CSF, but may also indicate the deposition of these cells on cauda equina. Hurst *et al*. described a woman who received intrathecal neural stem cell therapy. In MRI examination, the authors marked enlargement of lumbosacral roots of the cauda equina, which was not seen before the stem cell treatment. Electrodiagnostic studies confirmed chronic multiple lumbosacral radiculopathies. Biopsy of a lumbar dorsal sensory root showed myelinated fibre degeneration and loss, with endoneurial

inflammation. The hypertrophic inflammatory cauda equina syndrome was potentially triggered by the prior intrathecal neural stem cell injection. It is important to underline that the inflammatory reaction could be the result of allogenic transplantation. In 2018, Israel and colleagues published the results from animal model indicative of survival and distribution of astrocytes derived from human embryonic stem cells (hESCs) injected intrathecally. The injected cells detected along the meninges, attached to the pia mater were analysed in different spinal levels and their number ranged between 17% (distal areas from the injection site) and 80% (at vicinity of the injection site) after 4 weeks, between 13% and 97% after 17 weeks and between 21% and 96% after 39 weeks [11]. The cells were almost uniformly seen along the meninges, attached to the pia mater. The place of cell injection is a critical factor, which was shown in Panayiota Petrou's research, where different routes of MSC administration were compared in treatment of active progressive multiple sclerosis (MS): intrathecal vs. intravenous [23]. Independently of the way in which MSC had been transplanted, patients showed improvement when it comes to EDSS (Expanded Disability Status Scale) parameters in progressive multiple sclerosis compared to the control group. However, MSC transplanted intrathecally exhibited more potential in treatment of multiple sclerosis than MSC transplanted intravenously. This may be due to the neuroprotective and neurotrophic properties of MSC. The authors speculate that the intrathecal injection brings a higher proportion of the injected cells into close proximity with damaged sites of the CNS, compared to intravenous injection.

Frequency of cells application

A repetitive therapy instead of one single application is the other critical factor. A phase II clinical trial showed that transplanting 1-4 doses of MSC by intrathecal injections to patients with amyotrophic lateral sclerosis did not cause serious adverse events [24]. Additionally, 7 out of 19 patients showed clinical improvement after first transplantation and this effect was noted for 5 patients after the second dose. Violaine K. Harris' team also reached similar conclusions on safety of multiple MSC administration [8]. After transplanting 2-5 doses of neural progenitors derived from bone marrow mesenchymal stromal cells to patients with MS, they observed clinical improvement. However, during long-term observation it had been noticed that 1 out of 6 patients showed evidence of disease progression. Despite this, the other patients did not show disease progression or significant abnormalities. The clinical trial was conducted by Fedor Hlebokazov's team [9]; in the trial they compared single and repeated dose of bone marrow mesenchymal stem cells (BM MSC) in epilepsy patients. Six months after the first dose of BM MSC, a significant decrease of average monthly seizure count was observed in a patient who received correlated therapy consisting of BM MSC with levetiracetam compared to the control group. Due to the positive response to BM MSC therapy, this group was selected to receive a repeated dose of MSC and was divided into two different groups: with levetiracetam or without levetiracetam treatment. Additionally, independently from the supportive treatment of levetiracetam, the first dose of MSC had already decreased the paroxysmal activity. After 12 months, patients who received either single or double dose of MSC, both showed a decreased average monthly seizure count. Patients, who received a double dose of MSC with levetiracetam treatment, showed a significant decrease in paroxysmal activity compared to patients treated with a single dose of MSC. The team of Panayiota Petrou (2020) received similar results. They observed that a double dose of MSC confirmed disability improvement (CDI) compared to a single dose. Additionally, patients who received a double dose, did not show disability progression [23].

Adjuvant vs. restorative therapeutic effect

Indirect evidence of cell survival after transplantation and their therapeutic effect is the cytokine release and the kinetics of their concentration in CSF after cell administration. Clinical improvement after autologous bone marrow-derived lineage-negative (Lin-) cells injection in patients suffering from amyotrophic lateral sclerosis (ALS) was correlated with the level of selected trophic, proinflammatory factors, expression profiles of miRNA in cerebrospinal fluid (CSF) and plasma by multiplex Luminex and q-PCR in different time points. The cells were intrathecally administered three times at six-week intervals to 42 sporadic ALS patients. Patients were examined for articulatory functions using subjective

(VHI) and objective (FDA) scales. Authors observed partial speech improvement in a group of patients, expressed in better VHI scores and laryngeal time according to FDA, sustained until 4 weeks post Lincell administration. The improvement correlated with the neurotrophin release. The transient therapeutic effect indicated the injected cells death, and traced to the necessity to repeat the cell-based therapy to maintain its effect [30]. We have analysed CSF from two groups of patients (adults suffering from ALS and children suffering from drug-resistant autoimmune epilepsy) treated intrathecally with ADRC three times, every three months. Our observations were coherent with those described by the other groups. After cell administration, a significant increase in selected cytokine and neurotrophin levels was observed, which slowly decreased over time to reach baseline levels after approximately 3 months. To sum up, previous results indicate that to maintain the beneficial effect of cell therapy in CNS, repeated application is necessary. In a study carried out by Ki-Wook *et al*., the concentration of cytokines was measured in CSF obtained from ALS patients, who were first treated with autologous MSC – the patients received two intrathecal injections (the second one after a month). It has been shown that the mean levels of transforming growth factor (TGF)-β1-3, interleukin (IL)-6, and IL-10 proved to be significantly increased between before the first and second MSC injections. The mean levels of tumour necrosis factor α (TNF-α) and MCP-1 were significantly decreased. It appears that one-month post-injection, thanks to the secreted cytokines, the microenvironment presents an anti-inflammatory character [20,21]. In another study, Krull *et al*. performed protein quantitation on CSF samples from ALS patients, who were being treated with autologous adipose-derived MSC. The analysis was performed before and after MSC administration (subjects received an intrathecal dose of 1×10^7, 5×10^7, or 10×10^7 cells). Significant levels of vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and growth/differentiation factor-15 (GDF-15) were detected one-week post-injection. The authors observed a dose-dependent response trend. High-dose intrathecal MSC injections resulted in increased levels of growth factors in CSF samples [14]. When it comes to brain injury, transplanted MSC proved to have neuroprotective properties. In CSF obtained from patients, who suffered from brain injury, significant levels of IGFs, FGFs, NGF, TGF-β, GDNF, brain-derived neurotrophic factor (BDNF), VEGF were detected. These factors play a crucial role in growth and viability of neurons, proliferation of neural stem cells and regeneration [33].

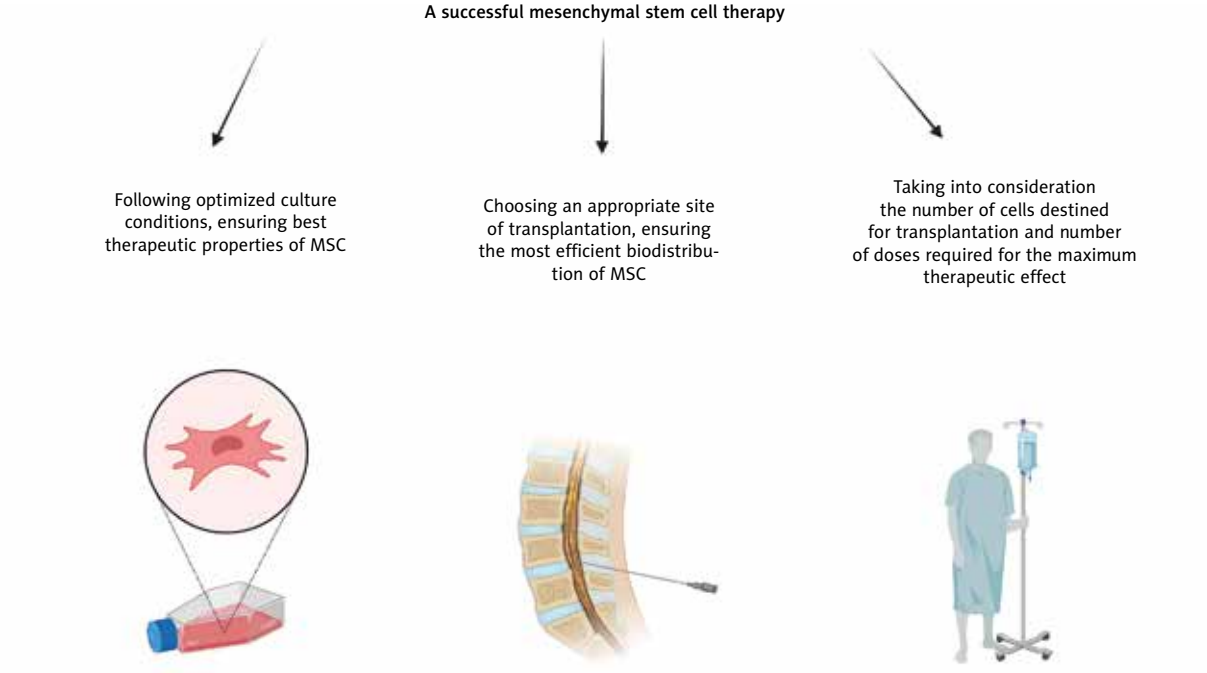

Fig. 1. Critical factors responsible for efficiency of MSC therapy.

Heterogeneous (freshly isolated) vs. homogenous (cultured) cell population

According to the position of the European Medicines Agency (EMA), the production of a pharmaceutical-quality cellular drug is associated with obtaining a consistent and repeatable final product. In this case it may consist of terminally differentiated cells derived from stem-cells, of undifferentiated stem cells or even of a mixture of cells with a varying differentiation profile.

For a cellular drug, "impurities" in the final product may lower pharmaceutical quality in terms of uniformity, and affect its therapeutic efficacy. Concerning cell isolation from adipose tissue, researchers might either receive a freshly isolated heterogeneous adipose-derived stromal vascular fraction (AD-SVF) or homogenous population of adipose-derived mesenchymal stem/stromal cells – AD-MSC (after cell cultivation under strictly defined conditions). The AD-SVF consists not only of AD-MSC, but also of HSC, Treg Cells, Pericyte-EC, mast-cells, complex microvascular beds (fibroblasts, WBC, dendritic cells etc.), and extracellular matrix. The use of AD-SVF in cell therapy may offer some advantages, as the components of this fraction support many regenerative mechanisms and tissue homeostasis. To date, the treatment with SVF has been evaluated in clinical trials, including diseases such as post-infarct remodelling, ischemic heart disease, type I diabetes, and liver failure [1].

Heterogeneous (freshly isolated cell population)

The results of these and many other clinical trials confirm the regenerative and immunomodulative properties of SVF. Nevertheless, researchers and clinicians are much more interested in the isolation of ASC from SVF – the fraction of mononuclear cells showing adhesion to plastic surface. Clinically desirable repopulatory, paracrine and thanks to the secreted factors also immunomodulatory and regenerative properties, are mainly associated with mesenchymal stem/stromal cells of adipose tissue. Nevertheless, the use of heterogeneous SVF is justified. The main advantage of SVF over ASC in the aspect of clinical application is the lack of manipulations required for obtaining the final product (those manipulations may reduce the repair potential of transplanted cells), no influence of xenobiotics and, above all, the possibility of delivering a cellular prod-

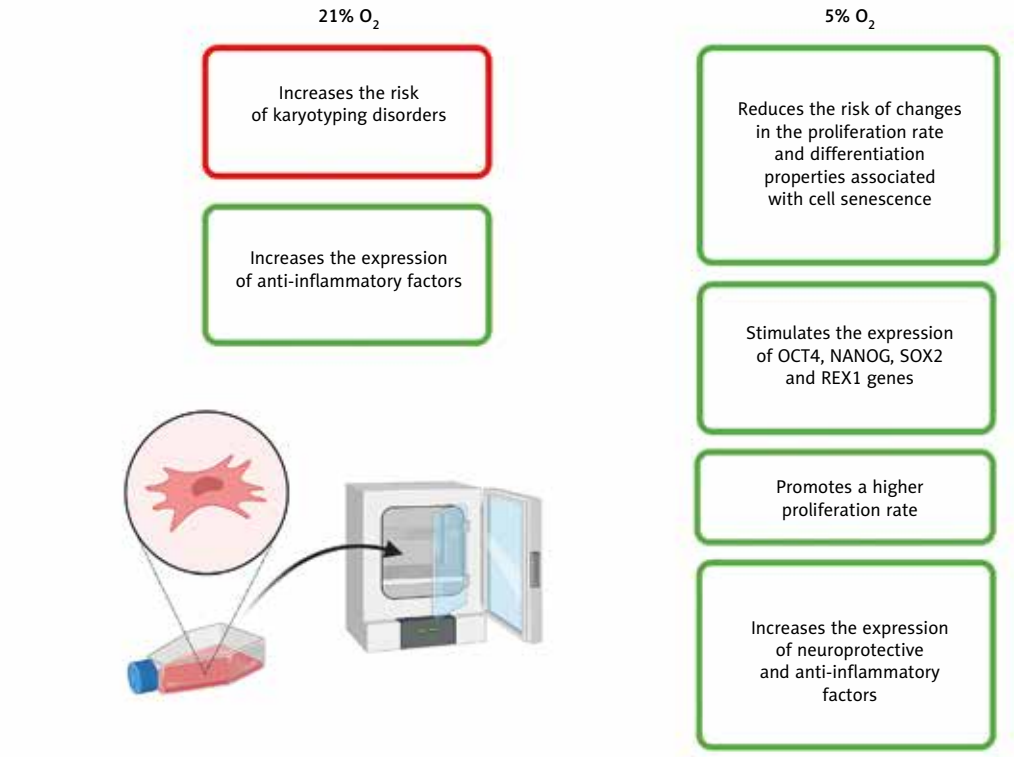

Fig. 2. The influence of different oxygen concentration on the therapeutic properties of MSC.

uct during one surgical procedure [1]. Nevertheless, the literature provides evidence for effectiveness of the highly heterogeneous stromal vascular fraction. A Semon *et al*. study has confirmed the comparable effectiveness of BM-MSC, ASC and SVF in the treatment of MS, expressed as a similar reduction in inflammatory infiltrates, tissue damage and serum interferon γ (IFN-γ) and IL-12 levels. It is noteworthy that IFN-γ levels fell to comparable levels between all treatment groups, while IL-12 levels were significantly lower in SVF-treated mice than in BMSC or ASC-treated mice [27].

Besides the heterogeneity within the cell population, this aspect also has to be considered in the area of donor-to-donor transplantation. An example of such variability is the presence of cells carrying the CD34 antigen. The number of $CD34^+$ cells may be determined, for example, by the age of the donor, and their number decreases in direct proportion to the time of culture. Traktuev *et al*. published studies demonstrating the bidirectional paracrine interaction of endothelial cells and SVF-contained $CD34^+$ cells. The following factors were identified in the media conditioned by $CD34^+$ cells: angiogenic factors (VEGF, hepatocyte growth factor [HGF], basic fibroblast growth factor [FGF]), inflammatory factors (IL-6 and IL-8 and monocyte chemoattractant protein-1 and -2) and mobilization factors (macrophage colony stimulating factor and granulocyte/macrophage colony stimulating factor) and a strong mitogenic $CD34^+$ response to factors produced by vascular endothelial cells (primary FGF, epidermal growth factor and platelet-derived growth factor BB) has been demonstrated. This study confirmed that adherent $CD34^+$ cells are resident pericytes that are responsible for vascular stabilization through mutual structural and functional interactions with endothelial cells. This finding explains the biological basis for the ability of the cell subpopulation most abundant in SVF to promote vascularization and accelerate tissue perfusion in the context of ischemia [31]. Finally, numerous regulatory T cells (Treg) are present among the SVF. These cells not only enhance regenerative properties but also promote tissue tolerance [1].

Semon and colleagues investigated the impact of SVF and ASC transplanted into C57Bl/6J mice with experimentally induced autoimmune encephalitis (EAE) [28]. The researchers observed a reduced progression of EAE in both SVF and ASC-treated mice. However, in mice treated with SVF the first symptoms of EAE appeared much later (14 DPI – day post-induction) than in the control group (9 DPI) and ASC-treated group (9.3 DPI). This may be the evidence of potential neuroprotective properties of SVF. Moreover, Semon *et al*. observed fewer demyelinated regions, myelin breakdown products and inflammatory cell infiltrates in mice receiving either SVF or ASC. The authors also examined the cytokine level responsible for Th1 cell stimulation which participated in pathology of MS and EAE. SVF and ASC treatment decreased levels of IFN-γ and IL-12, although in SVF-treated mice the level of IL-12 decreased more significantly than in ASC-treated mice. Another research group analysed the neuroprotective potential and paracrine activity of Wharton's jelly (WJ) fragments and Wharton's jelly mesenchymal stem cells (WJ-MSC) in co-culture with rat hippocampal slices (OHC – organotypic hippocampal culture) in an oxygen-glucose-deprived (OGD) stroke model [3]. A significantly decreased maximum death value (MDV) was observed in co-culture consisting of either WJ fragments or WJ-MSC and injured tissue slices in the OHC-OGD model. However, an increased level of hVEGF (human VEGF) was only observed in co-culture with WJ fragments in the OHC-OGD model (hVEGF is one of the factors secreted during tissue repair). Additionally, the presence of WJ fragments in culture increased the secretion of EGF, GDNF, VEGF, FGF, Bax and Bcl2 in hippocampal slices. When it comes to co-culture with WJ-MSCs, an increased secretion of GDNF and FGF was observed. Wharton's jelly fragments exhibit stronger neuroprotective properties in co-culture with OHC compared to WJ-MSCs – a similar dependence was noted for SVF compared to ASC when it comes to therapeutic potential. Hiroki Uchida's team inspected human Muse cells for repair effect in the lacunar stroke model. Eight weeks after transplantation, Muse cells expressed neuronal markers (NeuN, MAP2) and oligodendrocyte marker (GST-pi) [32]. Additionally, the authors observed a connection between the transplanted cells and host cells in the place of injury. Furthermore, 10 months post-transplantation Uchida *et al*. did not notice human specific Alu sequence in other areas except for the injection site. Obtained results confirmed the safety and positive therapeutic effect of Muse cells in treatment of lacunar stroke.

Most of the clinical studies we have undertaken involved the use of a heterogeneous population of cells, transplanted immediately after isolation, in

a single medical procedure. The targeted mechanism responsible for the therapeutic effect was based on the factors secreted by these cells.

In this review, it is impossible not to mention the homogeneous cell fractions with differentiation potential broader than typical MSC. These cells are obtained thanks to the modifications of isolation methods, which enabled the researchers to bypass the use of genetic manipulations. The first type of the abovementioned cells is Muse cells (multi-lineage differentiating stress enduring cells) with primary origins, which exhibit features resembling pluripotent cells. The subpopulation of these cells is isolated by cell-sorting on the basis of the presence of two markers: stage-specific embryonic antigen 3 (SSEA-3) and typical marker for mesenchymal cells: CD105 [15]. Moreover, the use of the so-called ceiling or membrane culture allows to obtain a homogeneous fraction of cells derived from the adipose tissue, called DFAT – dedifferentiated fat cells [19]. As Muse cells exhibit a non-tumorigenic character and can not only be used in allogenic transplantation but also migrate to the site of injury, they had already undergone clinical trials [4,5]. Muse cells have been used in the treatment of acute myocardial infarction, stroke, epidermolysis bullosa, spinal cord injury, amyotrophic lateral sclerosis, acute respiratory distress syndrome (ARDS), neonatal hypoxic-ischemic encephalopathy and stroke (www.clinicaltrials.gov).

In addition to the selection or modification of the cell isolation technique used, the key role in the aspect of therapeutic effectiveness is played by the appropriate selection of environmental parameters in which MSC are cultured. The ability to multiply the genetic material *in vitro* allows for: obtaining a quantitatively effective dose of cells, repeating cell applications without having to re-collect the source tissue, as well as preparing the quality-controlled material before its use. Nevertheless, the problem of optimizing the conditions, or more precisely: attempts to recreate *in vitro* conditions characteristic for cell niches *in situ*, is still valid.

Long-term passage of MSC is associated with limiting the potential of cells to differentiate, proliferate, migrate, and lose the ability to self-renew – key features for the use of stem cells in regenerative medicine and tissue engineering. In the light of our results, as well as other research groups, the reduced oxygen concentration in culture to 5% reduces the extent of these changes [25]. The results obtained by our team showed that 21% oxygen concentration in culture is associated with an increased risk of karyotyping disorders in MSC [16]. In another paper we pointed out that 5% O_2 stimulates the expression of *OCT4*, *NANOG*, *SOX2* and *REX1* genes (stemness related transcriptional factors), which are responsible for maintaining MSC in an undifferentiated, phenotypically immature, parental state. Additionally, these conditions stimulate the cells to grow faster, with the production of numerous proliferation centres, considered to be another marker of undifferentiated stem cells [6,18]. In the aforementioned Drela *et al*. study, we also showed that low oxygen levels increase the expression of neuroprotective and anti-inflammatory factors. We discovered that WJ-MSC at the early stages in culture express neuro-ectodermal specific markers like Nestin or stage specific embryonic antigen (SSEA-4). In later passages, the WJ-derived cells not only express β Tubulin III (the early neuronal marker) and α-smooth muscle actin (α-SMA), but also more matured neuronal antigens, like heavy neurofilament NF-200 together with GFAP. When it comes to BM-MSC, low levels of SSEA-4, Nestin and α-SMA were detected in the early passages. The expression of these markers was not followed by the expression of NF-200 or GFAP. The level of β Tubulin III was similar to that expressed by WJ-MSC. Our results also showed that WJ-MSC revealed higher mRNA expression of VEGF (VEGF-A), GDNF (glial cell-derived neurotrophic factor), HGF, BDNF, NT3, NT4 than BM-MSC. The enhanced transcription for these factors confirmed that WJ-MSC possess neuroprotective properties. The expression of IGF (insulin-like growth factor), EGF (epidermal growth factor) and CNTF (ciliary neurotrophic factor) did not differ between those two types of MSC (Drela *et al*.). Moreover, a study carried out by Rodriguez *et al*. proved that the secretory profile of BM-MSC did not differ considering oxygen levels in cell culture. The authors showed that cells cultured either in 21% or 2% O_2 both expressed low levels of IFN-γ and IL-6, while expressing high levels of IL-1 and IL-1RA. The concentration of IL-8 was higher for cells cultured in 21% O_2 than for those cultured in 2% O_2 [26]. In our previous paper (Lech *et al*.) we demonstrated that 21% oxygen concentration directed cell differentiation towards glia phenotype (GFAP) while 5% oxygen concentration promoted neuronal differentiation (Nestin and NF-200 expression) [17].

Conclusions

Despite numerous questions about MSC repopulation abilities, most of the clinical trials concerning cell-based therapy describe its beneficial effect in neurological diseases. This is undoubtedly related to MSC adjuvant properties. It is therefore worthwhile to focus on these cellular mechanisms of action and set the therapy in such a way as to take full advantage of them. According to the published data, the intrathecal MSC administration ensures their survival up to 4-12 weeks (post-transplantation). During this period, cells secrete growth factors (responsible for neuroprotective and regenerative effect) and chemokines (which modulate inflammation processes). After this period, cells die and the therapeutic effect decreases. In order to maintain the effect, it is necessary to repeat the administration of cells.

The choice between transplanting a heterogeneous (freshly isolated) or a more homogeneous (cultured) cell population is still debatable. However, considering the restorative potential and neural differentiation of MSC (which is still controversial), administering a population of appropriately cultured and directed cells seems to be more appropriate.

Acknowledgments

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Disclosure

The authors report no conflict of interest.

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