Effects of labeling human mesenchymal stem cells with superparamagnetic iron oxides on cellular functions and magnetic resonance contrast in hypoxic environments and long-term monitoring

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Abstract:

Ischemia, which involves decreased blood flow to a region and a corresponding deprivation of oxygen and nutrients, can be induced as a consequence of stroke or heart attack. A prevalent disease that affects many individuals worldwide, ischemic stroke results in functional and cognitive impairments, as neural cells in the brain receive inadequate nourishment and encounter inflammation and various other detrimental toxic factors that lead to their death. Given the scarce treatments for this disease in the clinic such as the administration of tissue plasminogen activator, which is only effective in a limited time window after the occurrence of stroke, it will be necessary to develop new strategies to ameliorate or prevent stroke-induced brain damage. Cell-based therapies appear to be a promising solution for treating ischemic stroke and many other ischemia-associated and neurodegenerative maladies. Particularly, human mesenchymal stem cells (hMSCs) are of interest for cell transplantation in stroke, given their multipotency, accessibility, and reparative abilities. To determine the fate and survival of hMSC, which will be imperative for successful transplantation therapies, these cells may be monitored using magnetic resonance imaging and transfected with superparamagnetic iron oxide (SPIO), a contrast agent that facilitates the detection of these hMSCs. This review encompasses pertinent research and findings to reveal the effects of SPIO on hMSC functions in the context of transplantation in ischemic environments and over extended time periods. This paper is a review article. Referred literature in this paper has been listed in the references section. The data sets supporting the conclusions of this article are available online by searching various databases, including PubMed. Some original points in this article come from the laboratory practice in our research center and the authors' experiences.

Keywords:

Cell tracking, human mesenchymal stem cells, hypoxia, ischemia, magnetic resonance imaging, superparamagnetic iron oxide

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Introduction

Mesenchymal stem cells (MSC) are progenitor cells that are widely available from various tissues, multipotent, and able to proliferate and increase in quantity,[1-3] making them ideal for regenerating and mending adipose, cartilage, bone, and other mesenchymal-derived tissue in cell-based and tissue regeneration therapies.[4,5] In addition, MSC release anti-inflammatory

factors that abate inflammation and growth factors to repair brain injury.[6] In lieu of using cell differentiation to replace degenerated neural cells, MSC may utilize trophic factors to promote a microenvironment conducive to regenerating cells.[7] MSC are a great candidate for treating neuronal damage as they are capable of crossing the blood–brain barrier[8,9] and thus, could possibly ameliorate diseases characterized by neurodegeneration or cerebral ischemia, including Parkinson's disease, amyotrophic lateral sclerosis, and ischemic stroke.[8,10-18] In fact, animals with cerebral ischemia inflicted locally through the middle cerebral artery occlusion (MCAO) experimental stroke model[13,19-23] or globally through cardiac arrest[24] demonstrate diminished lesion volumes and increased functionality upon MSC administration. It is uncertain how MSC exert these effects but is speculated to be associated with MSC's ability to restore and regenerate neurons through neuroprotection,[14,20-22,25-27] anti-inflammation,[10,28,29] and angiogenesis.[11,13] Of note, MSC transplantation therapy in humans is hindered by the ischemic microenvironment generated following stroke comprising reactive oxygen species, inflammatory factors, toxic components, and minimal nutrients, all detrimental to the survival of transplanted MSC grafts.[30]

Monitoring the fate of transplanted cells will be imperative to ensure successful administration and cell survival in cell-based therapies. Magnetic resonance imaging (MRI) can noninvasively observe cell transplants, possesses other favorable benefits, and has been previously used with MSC, making it a viable alternative to traditional histological analysis.[31-37] In general, MRI requires a contrast agent within the intracellular space of the cells to be visualized, which are usually paramagnetic agents related to superparamagnetic iron oxide (SPIO) nanoparticles. SPIO typically act as R_2 or R_2^* agents, given that they lead to dephased proximal spins and negative contrast, thus elevating MR sensitivity. Moreover, while SPIO are deemed to be safe and express inconsequential effects on human MSC (hMSC),[38-40] varying transfection methods, times, and dosages may possibly produce unfavorable changes, as exhibited by inconclusive evidence regarding SPIO influence on the bone-related differentiation of hMSC.[41-43] SPIO's dose-dependent and long-term effects on hMSC differentiation, as well as their influence on hMSC graft survival and hMSC's potential to differentiate into various cell lines in ischemic stroke, will be crucial to ascertain in future studies.

Transplantation of Superparamagnetic Iron Oxide-Labeled Human Mesenchymal Stem Cells

hMSC have the potential for treating ischemic stroke. These cells can be transfected with an SPIO to determine how the SPIO affects hMSC function during ischemia,

and subsequent MRI can evaluate how long these SPIO-labeled hMSC can be detected. Ischemic regions possess an array of toxic factors and limited nutrients and oxygen, which may affect the MRI detectability and viability of SPIO-incorporated hMSC. In addition, hMSC can survive for several days after transplantation, warranting the evaluation of their long-term detection and viability after SPIO uptake. Understanding how SPIO transfection influences hMSC and tracking the survival and fate of grafted hMSC over an extended time period will be critical for developing successful hMSC transplantation therapies for stroke and other diseases involving ischemic outcomes. During *in vitro* culturing of hMSC, and in *in vivo* animal models, SPIO exerts minimal effects on cell differentiation and proliferation. Higher initial SPIO exposure levels enhance MRI relaxation rates and contrast but are not ideal for detection over longer durations. In addition, greater SPIO doses make hMSC more susceptible to ischemia-induced damage. This review examines relevant investigations and resulting evidence that demonstrate how labeling hMSC with SPIO is suitable for MRI detection over longer periods of time and has negligible effects on which cell lineage hMSC commit to, but survivability of hMSC in ischemic and hypoxic environments may decrease with high levels of SPIO exposure.

Discussion

hMSC have previously been labeled with SPIO with no substantial changes to their differentiation and proliferation.[31,35,38,44,45] Currently, studies involving SPIO-labeled hMSC have only tracked these transplanted cells for brief periods of time, around 0–3 days following transfection with SPIO.[31,35,45-47] Given the possibility that hMSC can survive for longer than a week, it will be critical to evaluate their long-term detection.[20,48] hMSC are ideal for healing ischemia-induced damage to neural tissue, as they promote anti-inflammatory events[10,28,29] and angiogenesis.[11,13] However, because ischemic regions are hypoxic and lack nutrients, it will be important to determine through *in vivo* and *in vitro* studies how this affects MRI detectability and the survival of transplanted hMSC transfected with SPIO, in addition to monitoring the detection and survival of these transplanted SPIO-transfected hMSC past seven days.

As evaluated by Prussian blue staining and ICP-MS, hMSC uptake of SPIO increases in a linear fashion when directly incubated in media comprising SPIO. Cell-penetrating peptides (CPP) facilitate transfection and may result in the maximum internal concentration manifesting during the highest initial concentration.[40] Chemical manipulation may not be

necessary for transfecting SPIO in hMSC, as SPIO uptake increases with additional exposure time.[33,40,49,50] SPIO can be altered with antibodies and other receptors selective for certain cells,[38,51-53] or the nonspecific CPP poly-L-lysine (PLL),[54] to help hMSC integrate SPIO. hMSC differentiation, proliferation, and viability are unaffected by iron in a concentration of 100 µg/ml. In addition, hMSC demonstrate successful detection by a 1.5-T scanner and after 24 h, incorporate 23 pg of iron per cell.[31] CPP or other agents that assist with transfection have insignificant improvements on SPIO uptake,[31] but dextran,[55,56] liposomes,[57] lectin,[58] chitosan, starch, and polystyrene[59] can coat SPIO and increase efficacy. It is possible that adding PLL or other CPP can also coat other cells and compromise hMSC activity, although PLL can help hMSC internalize SPIO.[60,61]

Initial hMSC interaction with SPIO is correlated with R_2 and R_2^* relaxation, and R_2 and R_2^* decrease as cells divide and consequently dilute SPIO concentration. Tracking transplanted cells over time *in vivo* with SPIO relies on contrast dilution, which can be modeled with *in vitro* MRI. With high-resolution and high-field MRI, hMSC detection is still possible over 14 days of culturing, even with relatively meager uptake of SPIO.[62,63]

SPIO dilution does not necessarily correlate with the rate of R_2 and R_2^* relaxation. In a 14-day study with cultured hMSC and SPIO, while SPIO transfection rates by hMSC and hMSC proliferation rates in culture increase linearly, R_2 and R_2^* relaxation rates are nonlinear for the group exposed to the highest amount of iron, 56.0 µg. Between days 7 and 14, the percentage of hMSC containing SPIO significantly decrease relative to other time points, and there were similar percentages of SPIO-transfected hMSC for the 56.0 µg and 22.4 µg groups on day 14, as indicated by Prussian blue staining.[63]

hMSC given lower doses of SPIO may experience a slower decrease in SPIO labeling over time than hMSC with the highest initial amount of SPIO uptake, even though elevated relaxation rates in MRI and greater initial contrast are produced by increased SPIO labeling. Initial proliferation of cells may be influenced by greater SPIO integration by hMSC. Cell maturation, spreading, and construction of focal adhesion momentarily decrease during incubation with endothelial cells for a six day duration with increased exposure to SPIO coated with dextran.[64] In stem-like neuroprogenitor cells, elevated SPIO exposure also temporarily increases the time necessary for cell doubling. Sub-24 h in culture, ferucarbotran SPIO modulate regulators of the cell cycle and curtail peroxide in the cell to increase hMSC proliferation without any transfection facilitators.[65] Thus, increased exposure to SPIO may impact effects cell growth more significantly. Greater initial SPIO exposure

promotes contrast but sacrifices MRI detection over longer durations.

Generating the ideal dosage and timing for labeling hMSC with SPIO requires understanding how these SPIO manipulate hMSC multipotent and proliferative capabilities. Long-term culturing yields no differences between iron-labeled hMSC and nonlabeled hMSC in regards to cell proliferation, and demonstrates that modifying SPIO doses has no substantial effects. In addition, doses of SPIO between 12.5 and 50 µg/mL in the presence or absence of CPP induce limited changes in hMSC growth.[40,66] CFU-F assays and RT-PCR outcomes indicate that even initially high doses of SPIO generate inconsequential changes to the hMSC phenotype. Various *in vivo* and *in vitro* experiments show that any alterations to hMSC stem cell-related properties induced by SPIO incorporation are negligible, although MSC surface markers were not examined.[67] Flow cytometry analyses indicate that with CPP-mediated high quantities of transfected SPIO, there are only slight modifications in MSC-negative surface markers and none in positive surface markers.[40,68,69]

In a study with SPIO-marked hMSC, hMSC expression of ALP diminishes until day 21 after a peak on day 14, with various quantities of SPIO. A separate investigation demonstrates that in osteogenic induction media, hMSC exhibit similar ALP expression patterns after culturing for a week,[70] while another illustrates that the decline in ALP expression is dependent on the dose of SPIO.[43] While it can promote osteogenic differentiation in hMSC, ALP expression cannot dictate the magnitude of this process.[71] SPIO internalization in hMSC has little influence on hMSC commitment to a specific osteogenic cell line, as demonstrated by similar expression of the Osterix and Runx-2 master osteogenic transcription factors for all doses of SPIO and following incubation for 14 days.[72,67] Levels of calcium deposits on day 21 appear to be affected by levels of SPIO uptake, which was not observed in previous research examining calcification only in time periods under two weeks. SPIO in hMSC can conduct a deposition of calcium over longer periods of time in response to osteoinductive stimuli but have insignificant power over hMSC commitment to a specific osteogenic lineage. Future studies can elucidate the mechanism underlying calcification, as it is pertinent to cell-based therapies for ischemia-inducing diseases.[59]

Following transplantation of hMSC grafts, hMSC death ensues due to a hostile microenvironment at the ischemic lesion which consists of pro-inflammatory factors and reactive oxygen species,[30] and insufficient oxygen and nutrients. *In vitro*, measuring LDH secretion can be used to evaluate the extent of SPIO-transfected hMSC death

after removing oxygen and serum, as a representation of hMSC viability under ischemic conditions *in vivo*. During the first 24 h, eliminating serum has minor effects on hMSC secretion of LDH/survival in circumstances with normal oxygen levels *in vitro*, but secretion sharply increases at three days. hMSC-mediated LDH release is significantly enhanced by low levels of both serum and oxygen, and the most LDH secretion following incubation for 24 h is observed in hMSC with the highest incorporation of SPIO. While the mechanism for how elevated SPIO exposure exacerbates ischemia-induced hMSC death *in vitro* remains uncertain, in *in vivo* settings with neuroprogenitor cells exposed to high amounts of SPIO, transfected dextran-coated SPIO increase reactive oxygen species by over 450% within 65 h and upregulate transferrin receptor-1 expression.[64] It is conceivable that transfection with SPIO renders hMSC more prone to injury from increased reactive oxygen species in ischemic-hypoxic settings, which can be probed in future research.

hMSC localize in lysosomes or endosomes in the perinuclear area, as indicated by MRI detection of hMSC containing SPIO conjugated with rhodamine.[40] Covalent coupling in the cytoplasm maintains the carboxyfluorescein succinimidyl ester (CFSE) label in these cells for longer durations. In the ipsilateral side to the SPIO-transfected hMSC injection and MCAO-induced stroke, accumulated iron and hMSC generate hypointense voids. MRI detection following 48 h is possible with hMSC exposed to only medium levels of SPIO, which evidently create sufficient contrast. These SPIO also produce gradients in the microscopic field, which expand signal voids to encompass numerous cells and not just single cells. Cerebral vasculature alterations induced by stroke and reperfusion-related blood flow from the site of injection to arteries in the ipsilateral hemisphere of the brain may prevent an increase in contrast in the brain's contralateral side.

Double labeling of hMSC with CFSE and SPIO conjugated with rhodamine demonstrate that the ipsilateral side of the brain where the stroke occurred contains hMSC incorporating both CFSE and SPIO, with 2.7 times more SPIO than in the contralateral side. It is probable that microglia and macrophages ingest SPIO that hMSC release on death, as unlabeled nuclei, also express rhodamine signals.[34,63] Similarly, as transplanted neuronal stem cells labeled with SPIO and PPL proliferate and migrate, asymmetric cell division releases internalized iron particles at six days following transplantation.[39] Thus, the decline in MRI contrast and the iron release is likely attributed to the death of hMSC instead of contrast dilution facilitated by increasing hMSC quantities, given that transplanted hMSC survive for 5–10 days and do not exhibit proliferation

in vivo. Systemic administration of stem cells through intravenous injection may cause more cells to be lost in various organs than if the cells were intra-arterially injected and followed a more direct avenue to the brain's ischemic site.[73,74] Delivering hMSC labeled with SPIO to the brain is not a completely efficient and consistent process,[75] although PPL could affect this and MRI signal voids can be augmented by an iron concentration of 15–20 pg per cell following exposure to SPIO for 24 h.

Overall, hMSC can still be detected in an agarose tissue mimic over 14 days with minimal changes to differentiation and proliferation, if the shorter incubation duration and lower SPIO exposure level are sufficient. hMSC transfected with SPIO are more vulnerable to damage from hypoxic and ischemic conditions than hMSC without SPIO, which will be imperative information for conducting *in vivo* experiments involving ischemia-associated maladies. The current efforts to elucidate mechanisms underlying hMSC function and increase hMSC survivability in toxic ischemic environments through preconditioning methods will help advance cell-based therapies for ischemic stroke and other related diseases.

Conflicts of interest
There are no conflicts of interest.

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