



# Potential Therapeutic Features of Human Amniotic Mesenchymal Stem Cells in Multiple Sclerosis: Immunomodulation, Inflammation Suppression, Angiogenesis Promotion, Oxidative Stress Inhibition, Neurogenesis Induction, MMPs Regulation, and Remyelination Stimulation

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Multiple sclerosis (MS) is an inflammatory and degenerative disorder of the central nervous system with unknown etiology. It is accompanied by demyelination of the nerves during immunological processes in the presence of oxidative stress, hypoxia, cerebral hypo-perfusion, and dysregulation in matrix metalloproteinases (MMPs). Human amniotic mesenchymal stem cells (hAMSCs) as pluripotent stem cells possess some conspicuous features which could be of therapeutic value in MS therapy. hAMSCs could mimic the cascade of signals and secrete factors needed for promoting formation of stable neovasculature and angiogenesis. hAMSCs also have immunomodulatory and immunosuppressive effects on inflammatory processes and reduce the activity of inflammatory cells, migration of microglia and inhibit recruitment of certain immune cells to injury sites. hAMSCs attenuate the oxidative stress supported by the increased level of antioxidant enzymes and the decreased level of lipid peroxidation products. Furthermore, hAMSCs enhance neuroprotection and neurogenesis in brain injuries by inhibition of inflammation and promotion of neurogenesis. hAMSCs could significantly increase the expression of neurotrophic factors, which prevents neurons from initiating programmed cell death and improves survival, development, and function of neurons. In addition, they induce differentiation of neural progenitor cells to neurons.

hAMSCs could also inhibit MMPs dysregulation and consequently promote the survival of endothelial cells, angiogenesis and the stabilization of vascular networks. Considering the mentioned evidences, we hypothesized here that hAMSCs and their conditioned medium could be of therapeutic value in MS therapy due to their unique properties, including immunomodulation and inflammation suppression; angiogenesis promotion; oxidative stress inhibition; neurogenesis induction and neuroprotection; matrix metalloproteinases regulation; and remyelination stimulation.

**Keywords:** angiogenesis, anti-inflammation, antioxidant, amniotic membrane, mesenchymal stem cell, multiple sclerosis, neurogenesis

## INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system which is accompanied by neural demyelination, axonal loss, and disability. Although the main etiology of MS is unknown, genetic, environmental and infectious agents may be among the factors that play a role in the development of MS (1). The main pathologic processes of MS include inflammatory and degenerative phases (2). The presence of inflammatory cells and their secreted molecules in the demyelinating lesions supports the notion that pathogenic T cells that react with myelin antigens play a key role in MS, which then results in the degeneration of neurons (3). In addition, the migration of autoreactive T cells to the central nervous system results in destroying the central neurons and their myelin sheaths, which is mediated by matrix metalloproteinases (MMPs) (4). Furthermore, oxidative stress, hypoxia and cerebral hypo-perfusion may lead to increased demyelinating lesions in MS (5, 6).

Although various pharmacological and non-pharmacological therapeutic approaches have been used in the treatment of MS, no definite cure of MS has been discovered to date. Moreover, all the approved therapeutic approaches are expected to be life-long, while their potential adverse effects may compromise their safety or patients' adherence to treatment. Therefore, it is imperative yet challenging to find new safe therapies with fewer delivery concerns and less adverse effects that are more effective in slowing or preventing MS progression, have the potential to reverse patients' disability, and consequently improve patients' adherence.

Cell-based therapy by mesenchymal stem cells (MSCs) is one of the therapeutic approaches that has drawn attention as a potential approach to address these challenges, considering initial promising research results (7). Nevertheless, there are uncertainties regarding the use of MSCs in MS treatment including tissue source, route of delivery, cell number, dosing, adverse effects, safety monitoring, and duration of action (8).

Human amniotic membrane is considered a potential source for MSCs which possesses unique biological properties, including anti-inflammatory, anti-fibrosis, anti-scarring and low immunogenicity characteristics (9, 10). Human amniotic mesenchymal stem cells (hAMSCs) are pluripotent stem cells that could be isolated from the fibroblastic layer of human amniotic membrane.

Altogether, it seems that hAMSCs have potential therapeutic features that could be promising in MS therapy. There is some evidence for some hAMSCs properties including immunomodulation and inflammation suppression (11); angiogenesis promotion (12, 13); oxidative stress inhibition (14); neurogenesis induction and neuroprotection (15); matrix metalloproteinase regulation (16); and remyelination stimulation.

## SUPPORTING EVIDENCE FOR HYPOTHESIS

### Immunomodulation and Inflammation Suppression

Inflammation in the central nervous system is one of the major pathogenic processes in MS. Although the trigger of the inflammatory response in MS is still not clear, it is suggested that MS is developed when auto-reactive T cells target proteins which exist predominantly in myelin and on axons (17, 18). Besides T cells, B cells, macrophages, activated microglia, and dendritic cells act as key players in the pathogenesis of multiple sclerosis (19). When dendritic cells are exposed to myelin-derived antigens, they secrete cytokines which induce the differentiation of naive T cells into effector T cells in secondary lymphoid tissues (2). Inflammatory cells migrate across the blood-brain-barrier to the central nervous system and thus cause the inflammatory lesions which are characterized by an area of demyelination of nerves and axonal loss. In addition, poor T-regulatory functioning enhances the expansion of inflammatory responses (20).

Efficient treatment of MS depends on developing a therapeutic method that can specifically target and regulate immune responses. A number of immunomodulatory or immunosuppressive drugs including Interferon- $\beta$ , Glatiramer acetate, Natalizumab, and Fingolimod have been designed to target the immune component of MS. Although these drugs have displayed beneficial effects for halting MS, they have shown little impact on its progression (21). On the other hand, mesenchymal stem cells could be applied as therapeutics in MS through imposing their immunomodulatory effect by inducing a shift in T cells from a pro-inflammatory to an anti-inflammatory state (22); inhibiting naive and

memory T cell proliferation and maturation (23); inhibiting proliferation, secretion of proinflammatory cytokines, and cytotoxicity of natural killer cells and natural killer T cells; inhibiting B cell proliferation, and antibodies production; inhibiting the initial differentiation of monocytes to dendritic cells and impairing their activation, and antigen presentation (24); and inhibiting the chemotactic activity of neutrophils (25). In addition, they promote the generation of regulatory T cells (23), preserve neutrophil viability and function (26), and regulate macrophage recruitment (27) and function (28).

hAMSCs could lead to a decrease of peripheral blood mononuclear cells, interferon-gamma and interleukin-17 production (29). They might also reduce migration, recruitment, and activity of a broad range of immune cells, including T cells, natural killer cells, natural killer T cells, dendritic cells, B cells, neutrophils, monocytes, and macrophages at injury sites (30). The secretion of nitric oxide by hAMSCs could be the main cause of their immunosuppressive effect (11). Currently, several soluble factors, either produced constitutively by hAMSCs or as a result of cross-talk with target immune cells, have been shown to exert the immunomodulatory properties of MSCs, including indoleamine 2,3-dioxygenase, prostaglandin E2, interleukin-10, interleukin-6, HLA-G, transforming growth factor- $\beta$ 1, and hepatocyte growth factor (23, 31–33). In addition, hAMSCs have shown a positive effect on immunosuppression in mice models of MS (2, 18).

## Angiogenesis Promotion

Cerebral hypoperfusion as well as vascular factors are involved in neurovascular dysfunction, vascular oxidative stress, and relative tissue hypoxia, which could increase the risk of developing demyelinating lesions as observed in MS. Therefore, cerebral hypoperfusion may represent pathologic factors or neuroprotective processes involved in recovery or progression of MS (6).

Although various therapeutic approaches have been utilized to promote angiogenesis, most approaches still cannot fully mimic the process of natural vessel development. The use of hAMSCs has been explored to mimic the cascade of signals needed for enhancing viability and promoting formation of stable neovasculature (16). We also showed the inducing effects of hAMSCs conditioned media on the sprouting of endothelial cells (12, 13). MSCs' potential for angiogenesis relies on their ability to differentiate to smooth muscle cells and endothelial cells as well as their paracrine effects by angiogenic factors. Angiogenic factors secreted by MSCs vary based on the source and include VEGF, bFGF, MCP-1, SDF-1, angiopoietin, monocyte chemoattractant protein, interleukin-6, placental growth factor, and cysteine-rich angiogenic inducer 61, which could regulate vascular network remodeling (34). Moreover, hAMSCs could promote angiogenesis by inducing the extracellular signaling-regulated kinase 1/2-MAPK signaling pathway (35). Therefore, conditioned medium from hAMSCs could be beneficial for promoting angiogenesis and would probably enhance tissue repair.

## Oxidative Stress Inhibition

The inflammatory processes play a significant role in neural tissue injury (36) and in MS pathogenesis critically involve Reactive Oxygen Species (ROS). Infiltrated immune cells, macrophages and activated microglia could generate immense amounts of oxidizing radicals including superoxide, hydrogen peroxide and nitric oxide. In addition, the activation of immature myeloid cells induce synthesis of nitric oxide and reactive oxygen species (5). Free radicals can also activate nuclear transcription factor-kappa B (NF- $\kappa$ B). NF- $\kappa$ B upregulates the expression of genes involved in MS, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), nitric oxide synthase (iNOS), intracellular adhesion molecule 1 (ICAM-1) and vascular-cell adhesion molecule 1 (VCAM-1) (37).

Treatment of mice model of MS with antioxidant enzymes markedly suppressed the severity of MS (38). In addition, mitochondrial stabilization and ROS-mediated phagocytosis of myelin may reduce axonal damage in mice models of MS (39–41).

The utilization of MSCs in mice models of MS can inhibit the production of inflammatory factors, including nitric oxide (NO), tumor necrosis factor, IL-1- $\beta$  and reactive oxygen species by activated microglia and preventing neuronal damage. The intravenous injection of MSCs showed a strong antioxidant effect in a mice model of MS through the high expression of antioxidant enzymes including catalase, superoxide desmutase and poly (ADP-ribose) polymerase-1 during MSC treatment (42).

In addition, hAMSCs transplantation into transgenic mice increased the level of antioxidant enzymes and decreased the level of lipid peroxidation and oxidative stress (14).

## Neurogenesis Induction, Neuroprotection, and Remyelination Stimulation

Neurodegeneration is considered as a major contributor to neurological disability in MS and might be the dominant underlying process of progressive MS. Whether the neurodegeneration is an independent process or due to inflammatory processes remains unknown (43). There are studies that report the mechanisms of neurodegeneration in MS, including the accumulation of amyloid precursor protein in neurons and a reduction in the N-acetyl-aspartate/Creatine ratio (2). In addition, damage to mitochondrial DNA and mitochondrial enzyme complexes may lead to neurodegeneration (44). Axonal density reduction in the white matter and spinal cords of MS patients is another probable mechanism of neurodegeneration (45).

Another process involved in pathogenesis of MS is demyelination. Demyelination causes myelin-producing oligodendrocytes to undergo apoptosis and thus results in myelin loss (46). In response to demyelination, activated resident oligodendrocyte progenitor cells proliferate, migrate to affected areas, and differentiate to replace lost oligodendrocytes, which might lead to myelin reconstitution and functional recovery (47, 48). However, remyelination is typically incomplete or defective and many lesions remain demyelinated (48, 49). This could be either due to the limited ability of mature oligodendrocytes to compensate for myelin loss (50) or to the failure of oligodendrocyte progenitor cells to successfully

generate new myelinating cells (48). Therefore, oligodendrocyte progenitor cells cannot often compensate myelin loss on their own.

Treatment of MS with hAMSCs could significantly increase the expression of neurotrophic factors including NGF, CNTF, and BDNF (51). Neurotrophins prevent neurons from initiating programmed cell death and improve survival, development, and function of neurons (15, 52, 53). In a study, hAMSCs promoted neurological recovery in rats after intracranial hemorrhage. It was concluded that the mechanism of action was mediated by inhibition of inflammation and apoptosis, increasing neurotrophic factors expression, and promoting neurogenesis and angiogenesis (54). hAMSCs induce differentiation of progenitor cells to neurons (52). In addition, hAMSCs have the ability to differentiate neural and glial cells in response to induction medium (55). The level of neurotrophins significantly decreases in the CNS of MS patients and is correlated with neuron damage. Therefore, increasing the levels of neurotrophins—or at least maintaining their physiological levels—in MS patients might be of therapeutic value (56, 57). The therapeutic effects of hAMSCs were able to improve the motor functions of neurodegenerative diseases in mice models significantly (58), which indicates that hAMSCs have the potential to differentiate into neural cells. Therefore, hAMSCs could potentially promote neurogenesis, neuroprotection, and remyelination.

## Matrix Metalloproteinase Regulation

MMPs are a family of a large number of proteolytic enzymes that have received much attention in neuro-inflammatory diseases. Leukocyte infiltration through the blood-brain-barrier is dependent on several factors including secretion of tumor necrotizing factor- $\alpha$ , gelatinase B/MMP-9 and gelatinase A/MMP-2 (59). It has been shown that the expression of MMPs leads to degradation of extracellular matrix proteins of the basal lamina which surrounds blood vessels (60). MMP-9 is a major matrix metalloproteinase in the pathogenesis of multiple sclerosis and experimental autoimmune encephalitis, which could enhance leukocyte migration, blood-brain-barrier disruption and myelin lysis (4). Different attempts have been made to develop inhibitors of MMPs for the potential treatment of diseases in which MMPs play a major role. In one study, treatment of MS patients with Natalizumab decreased the risk of progressive multifocal encephalopathy by inhibition of MMP-9 (61).

Mesenchymal stem cells application in inflammatory diseases resulted in decreased levels of MMPs or reduced MMPs activity. In another study, a conditioned medium of MSCs decreased disease severity by inhibition of the MMPs activity rate in inflammatory arthritis (62). In another study, MSCs were introduced as robust sources of MMPs inhibition that were mediated by Tissue Inhibitors of Metalloproteinase (TIMPs). This may have therapeutic effects in inflammatory and vascular diseases (63). It has been shown that treatment with mesenchymal stem cells inhibited dysregulation of both MMPs and TIMPs after focal ischemic stroke, which facilitated neurological and functional recovery after stroke (64).

## HYPOTHESIS

Considering the supporting evidences, we hypothesize that hAMSCs have potential therapeutic features in multiple sclerosis via angiogenesis promotion, inflammation suppression, oxidative stress inhibition, neurogenesis induction, neuroprotection, MMPs regulation, and remyelination stimulation (**Figure 1**).

## Evaluation of Hypothesis

The hypothesis will be evaluated by conducting *in vitro* and *in vivo* experimental studies to address the building block questions of the hypothesis.

### *In vitro* Studies

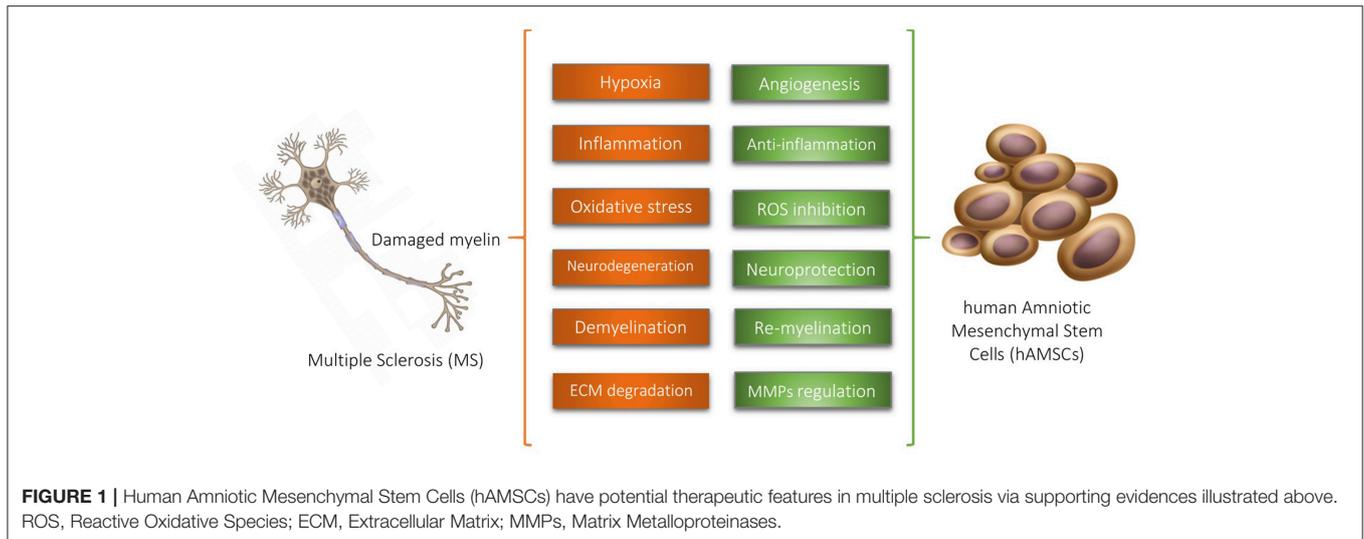
To evaluate the *in vitro* effects of hAMSCs conditioned medium, oligodendrocyte cell lines such as the OLN-93 will be cultured. To determine neuroprotection and oxidation inhibition of hAMSCs conditioned medium, first oxidative stress and cell death will be induced by using  $H_2O_2$  or cuprizone. Afterwards, viability, qualitative and quantitative levels of apoptotic markers as well as neurotrophin levels will be measured and mitochondrial assays will be conducted before and after treatment with hAMSCs conditioned medium. The concentration-dependence to exert the immunomodulatory actions of hAMSCs will be investigated by mixed lymphocyte reaction assay.

### *In vivo* Studies

To evaluate the *in vivo* effects of hAMSCs conditioned medium, the toxic model of multiple sclerosis will be created in C57BL/6 mice by cuprizone. To measure the motor coordination and balance among mice, rotarod performance testing will be carried out before and after treatment with hAMSCs conditioned medium. To determine the effect of hAMSCs conditioned medium on angiogenesis, VEGF levels will be evaluated by ELISA kit. To determine inflammation suppression by hAMSCs conditioned medium, inflammatory and anti-inflammatory cytokines will be measured before and after treatment with hAMSCs conditioned medium by ELISA kits.

To evaluate direct injection of hAMSCs, the cells would be locally injected in the affected regions of the brain tissue. In addition, as MSCs express a variety of chemokine receptors including CXCR4 and CCR2, and cell adhesion molecules including CD44, integrins  $\alpha 4$  and  $\beta 1$ , and CD99 (65), they could be delivered via intravenous administration. Therefore, we aim to compare the effectiveness of the ways of administration of hAMSCs in MS models in future studies. In addition, to evaluate the effect of the number of cells administered in future studies,  $1 \times 10^6$  hAMSCs (66) will be injected into the tail vein of rat and the number of cells will be adjusted if necessary. Results will be evaluated after 6 weeks for investigation of the acute phase of inflammation and 12 weeks to evaluate the chronic phase of the MS model.

To evaluate oxidative stress inhibition by hAMSCs, the reactive oxygen species and glutathione reduction is measured before and after treatment with hAMSCs conditioned medium by glutathione assay kit.



To evaluate the effects of hAMSCs conditioned media on OPC differentiation for neurogenesis and remyelination, oligodendrocyte precursor marker, Olig2, and adult myelin markers, PLP, MBP, MOG, will be measured by real-time PCR and Western blot analyses. To further investigate the effects of hAMSCs, cellular and molecular tests using immunohistochemistry, Western blot and real time-PCR analyses on the myelin genes and proteins as well as microscopic examinations will be carried out. Finally, to determine the effect of hAMSCs conditioned medium on MMPs regulation, MMPs activity will be determined by MMPs activity assay.

## DISCUSSION

hAMSCs have unique properties including inflammation suppression, angiogenesis promotion, oxidative stress inhibition, neurogenesis induction, neuroprotection, MMPs regulation, and remyelination stimulation, which are of potential therapeutic value for MS therapy. The anti-inflammatory feature of hAMSCs, along with their ability to promote neurogenesis and differentiation of progenitor cells to neurons, could make them valuable sources for prevention and therapy in both early and advanced stages of MS. hAMSCs possess other conspicuous characteristics to make them practical sources for cell-based therapies against MS, which are discussed further here in brief. The amniotic membrane, which will be obtained from elective Cesarean sections to eliminate contaminations, is an attractive source of MSCs since large quantities of hAMSCs could be cost-effectively collected without invasive procedures and ethical concerns (67). Although hAMSCs are capable of self-renewal and differentiation to all the three embryonic layers, they are not tumorigenic and thus could be considered safe and easy-to-access sources for cell-based MS therapy (68). As we have shown previously, cryopreservation could be used as a viable system for banking human amniotic cells with low cost in terms of expense, time and personnel involved and its ease of implementation. Therefore, they are suitable for banking and

establishing ready-to-use sources for cell therapy (13). Since hAMSCs retain their reproducible biologic characteristics, they can be sufficiently expanded for use in regenerative medicine (69). In addition, the differentiation of hAMSCs to different cell lines relies on growth factors provided in the medium which can be altered in the laboratory. Amniotic stem cells can differentiate to neuron and glial cells (55); therefore, by regulating the growth factors, hAMSCs could potentially be applied in various neurological disorders. Neurological disorders occur among the elderly, and MSCs are affected by the aging process as indicated by the decrease in the bone marrow MSC pool and also reduced capacity to handle oxidative stress (70). Therefore, allograft transplantation of hAMSCs and the ability to provide large quantities of hAMSCs might make them the choice to substitute allogenic source of MSCs among the elderly. hAMSCs have low immunogenicity, which makes them suitable for transplantation (70). hAMSCs have the ability to pass the blood-brain-barrier and enter the central nervous system (71). Thus, they can be administered intravenously and there is no need for more invasive routes of administration. In addition, intravenous administration of hAMSCs enhances their global access to sites of inflammation and damage to secrete the required factors to alleviate MS considering that MS lesions are disseminated in space, meaning that patients have lesions in several areas of the CNS (72). A question that needs to be clarified is whether hAMSCs give the same effect regardless the degree of MS. The pathogenesis of MS is seemingly different in the early and late stages of the disease. There is less inflammation in the brain in primary progressive MS than in secondary progressive MS. In addition, increased blood T-cell reactivity to myelin sheaths was found in relapsing–remitting MS and secondary progressive MS (73). Therefore, anti-inflammatory and immunomodulatory qualities of hAMSCs could be effective in secondary progressive and relapsing–remitting MS. Acute axonal injury occurs focally in secondary progressive MS and diffusely in primary progressive MS (73). Therefore, neurogenesis, neuroprotection, and remyelination imposed by hAMSCs could be of value via

systemic administration in primary progressive MS and via local injection in secondary progressive MS. Taken together, the anti-inflammatory feature of hAMSCs, along with their ability to promote neurogenesis and differentiation of progenitor cells to neurons, could make them valuable sources for prevention and therapy in both early and advanced stages of MS.

To the best of our knowledge, no studies have focused on the unique properties of hAMSCs for MS therapy. Therefore, it is essential to investigate the potential therapeutic values of hAMSCs for MS.

## CONCLUSION

hAMSCs and their conditioned medium could be of therapeutic value in MS therapy due to their unique properties including immunomodulation and inflammation suppression; angiogenesis promotion; oxidative stress inhibition; neurogenesis induction and neuroprotection; MMPs regulation;

and remyelination stimulation. Therefore, it is required to evaluate the hypothesis in future *in vitro* and *in vivo* studies.

## AUTHOR CONTRIBUTIONS

MA-K, S-HG, FS, and HN hypothesized the idea, wrote the manuscript and revised it. SB revised the manuscript.

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