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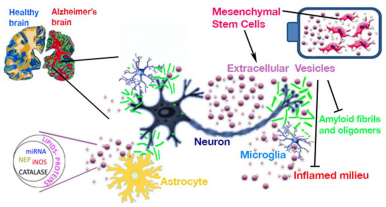
S. Coco*

Extracellular Vesicles from Mesenchymal Stem Cells Exert Pleiotropic Effects on Amyloid-β, Inflammation, and Regeneration: A Spark of Hope for Alzheimer's Disease from Tiny Structures?



Extracellular vesicles from mesenchymal stem cells represent a novel tool for the treatment of different diseases, showing a positive action in detrimental inflammatory conditions. Based on careful reviewing of the most recent literature their therapeutic role in Alzheimer's disease is hypothesized.

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Extracellular Vesicles from Mesenchymal Stem Cells Exert Pleiotropic Effects on Amyloid-β, Inflammation, and Regeneration: A Spark of Hope for Alzheimer's Disease from Tiny Structures?

Chiara A. Elia, Morris Losurdo, Maria L. Malosio,* and Silvia Coco*

No cure yet exists for devastating Alzheimer's disease (AD), despite many years and humongous efforts to find efficacious pharmacological treatments. So far, neither designing drugs to disaggregate amyloid plaques nor tackling solely inflammation turned out to be decisive. Mesenchymal stem cells (MSCs) and, in particular, extracellular vesicles (EVs) originating from them could be proposed as an alternative, strategic approach to attack the pathology. Indeed, MSC-EVs—owing to their ability to deliver lipids/proteins/enzymes/microRNAs endowed with anti-inflammatory, amyloid-β degrading, and neurotrophic activities—may be exploited as therapeutic tools to restore synaptic function, prevent neuronal death, and slow down memory impairment in AD. Herein the results presented in the most recently published studies on this topic are critically evaluted, providing a strong rationale for possible employment of MSC-EVs in AD.

1. Introduction

Myriads of complex factors contribute to Alzheimer's disease (AD), promoting the deposition of amyloid-β (Aβ) peptides into plaques, the main pathognomonic hallmark of AD (see Box 1).

"The amyloid cascade hypothesis"[10] has been for a long time the most accountable explanation for the disease because it was able to interpret disease onset, progression, and the underlying morphological, functional, and cognitive changes. Dissolving Aβ plaques has been the leading milestone of large phase III clinical trials run by big pharma companies, all of which have failed in their attempt to remit the pathology. Disappointing results from over 400 clinical trials performed between 2002 and 2012, testing amyloid-modifying therapies in individuals with AD dementia, suggest that either these interventions may be starting too late to alter the disease clinical course[11] or that acting only on Aβ deposition is not sufficient for reversing the disease by allowing the regenerative potential of the brain to take action.[12] Indeed, considerable evidence now indicates that soluble Aβ oligomers, monomers, and protofibrils, rather than amyloid deposits, are the main toxic agents in AD.[13,14] These forms can propagate between cells with greater efficacy compared to the larger aggregated forms.[4,5] If that is the case, it should not be surprising that targeting amyloid deposits in the brain has proven to be a faulty approach in AD therapeutics. Approaches are now being developed that target soluble Aβ oligomers in AD brains, but these still need to be rigorously tested.[15]

In addition a large body of recent evidence has identified inflammation as a key additional process involved in the pathogenesis of AD. Inflammation in the context of the "amyloid cascade hypothesis" has been restricted, for a long time, to a mere consequence of plaque deposition. However, more recent indications highlighted it as a key player in neuronal death, being active already before the manifestation of symptoms,[16] possibly associated with Aβ oligomers, monomers, and protofibrils acting on microglia and neurons.[13,14,17] This is further supported by numerous genetic, preclinical, and clinical studies focusing on the relationship between AD and alterations of the immune system. The discovery of gene variants in myeloid lineage cells, such as TREM2[18] and CD33,[19] related to a high risk of developing AD (see also Box 1), has led to a reconsideration of previous findings regarding high concentrations of inflammatory cytokines and

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Box 1
Alzheimer's Disease

AD incidence and prevalence have been rising over the last 50 years, so that it has evolved from a rare disease to a global human problem, affecting roughly 50 million people worldwide in 2018, who will prospectively become 152 in 2050. AD is the leading cause of dementia in the elderly contributing to 60–70% of cases, with a prevalence of >10% in people aged 65–74 years, approximately doubling every decade up to age 85+ years, at which it is around 40%.[1] In 2015, East Asia was the region with most people affected by dementia (9.8 million), followed by Western Europe (7.5 million), South Asia (5.1 million), and North America (4.8 million). According to the World Health Organization (WHO), AD is a public health priority, leading to a growing awareness of the global impact of this neurodegenerative disease worldwide.

AD is characterized by a slow but inexorable neuronal death that tends to involve a vast area of the central nervous system (CNS), including hippocampus, amygdala, and several cortical areas, such as para-hippocampal, entorhinal, associative, frontal, temporal, parietal, and occipital,[2] thus highlighting its complex and heterogeneous nature.

The diagnosis of AD can only be confirmed by assessing the post mortem presence of amyloid plaques, neurofibrillary tangles, neuronal and synaptic loss, and brain atrophy in specific brain areas, such as the hippocampus and the cortex.

Amyloid plaques are extracellular aggregations of the $A\beta_{1-42}$ peptide, originating from altered processing of the amyloid precursor protein (APP). Toxicity of the $A\beta_{1-42}$ peptide is mediated supposedly by the oligomeric form, prior to fibrillar aggregation, which has been shown to be toxic on neuronal cells when administered in the range from 5 μM to 15 μM.[3] Microglia is also thought to actively contribute to the production of toxic Aβ forms, favoring the generation of soluble forms[4] and the formation of damaging truncated forms derived from $A\beta_{1-42}$ peptide.[5] Neurofibrillary tangles consist of intracellular hyperphosphorylated forms of the microtubule-associated protein tau (p-tau), a brain-specific cytoskeletal protein. The presence of extracellular $A\beta_{1-42}$ oligomers and truncated forms along with amyloid plaques, together with the intra-neuronal accumulation of p-tau, has been generally proposed to lead over time to synaptic dysfunctions and neuronal loss. Recently, neuroinflammation has acquired a leading role in AD etiology—to be clarified whether it represents a contributing factor or an initiator of the pathology, or both—through microglia and astrocytes activation,[6] with the participation of the innate immunity receptors, such as the complement receptors CR1 and CD33.[7] Moreover, a novel player, triggering receptor expressed on myeloid cells 2 (TREM2), present on microglia membranes, has recently emerged and shown to bind with high affinity to soluble $A\beta_{1-42}$ oligomers inducing their scavenging by phagocytosis. The AD-associated variants of TREM2 reduce the amount of internalized $A\beta_{1-42}$ oligomers, thus disabling the detoxifying function of microglia.[8]

So far all the available pharmacological treatments provide only symptomatic relief for a limited time, but none of them targets underlying etiological mechanisms or disease processes.[9]

chemokines identified in tissues and body fluids of AD patients preceding the overt symptoms appearance.[20] Clinical and preclinical studies[20,21] focusing on the timing of inflammatory changes in the disease have identified, in fact, early involvement of the immune system. What is the trigger of this early inflammation and which relationship is there between inflammation and plaque and tangle formation? Certainly, extracellular plaques and neurofibrillary tangles have been shown to chronically stimulate an inflammatory response, which determines the activation of microglial cells.

Given the discouraging results of the last 20 years of preclinical and translational research based on dissolving amyloid plaques, which failed to produce a significant advancement in the treatment perspectives of AD patients, researchers worldwide are moving on to examine the "oligomer hypothesis" of AD, which argues that oligomers, monomers, and protofibrils are the proximal neurotoxins in AD. Moreover, the scientific community is realizing that novel therapeutic perspectives should consider the complexity of the disease. A certainly challenging yet viable research perspective is to study both the role of oligomeric and monomeric Aβ forms in well-established cellular toxicity processes and inflammation they procure.[13] Modulating both components can provide a more successful treatment. Herein we present the attractive hypothesis that extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) can represent a novel therapeutic perspective to be explored for AD treatment.

2. MSC Therapeutic Potential in AD

Age-related alterations are associated with the decline of neural functions, which besides impacting structural aspects, such as cortex thickness and gray matter loss, comprise the reduction of self-repair abilities. Cell-based therapies for the treatment of neurodegenerative diseases have recently gained a great deal of attention because they offer the potential of cell replacement and neuroprotection.[22] Stem cell therapy, in particular, is considered an appealing therapeutic avenue to be pursued in neurodegeneration. In the case of AD, the possible exploitation

Box 2
Mesenchymal Stem Cells

MSCs are multipotent non-hematopoietic adult stem cells resident in different tissues, where they provide functional support. MSCs phenotypic profile and differentiation potential can vary according to the tissue of origin.[23] Minimal criteria to define adult MSCs relative to other types of adult stem cells (i.e., hematopoietic stem cells) were set by the International Society for Cellular Therapy in 2006 and subsequently implemented over the years (see **Table 1** for human and murine surface markers, the most characterized up to now), especially in relation to MSC tissue of origin.[24–26]

One of the main features of MSCs is their ability to migrate and mediate repair of injured tissues at sites where they do not normally reside (homing).[27] Initially investigated for possible effects in cardiovascular diseases, renal injury, osteogenesis imperfecta, and graft vs host disease, lately the enthusiasm was aimed at the possible use of MSCs in neurodegenerative diseases, including AD. Among their features, MSCs exert immunoregulatory activities on innate and adaptive immune responses,[28] which are largely mediated by the release of a highly proactive secretome[29] rather than proliferation and differentiation. In fact, MSCs exert a paracrine activity that involves the secretion of soluble factors, the transfer of mitochondria (mostly occurring by way of tunneling nanotubes), and the release of exosomes or microvesicles (MVs; see Box 3) containing RNA, proteins, and other molecules, which target neighboring cells.[30,31]

The successful differentiation of MSCs from human iPSCs and their comparable or even better performances in terms of proliferation, immunomodulation, cytokine profiles, production of EVs, and bioactive secretome[32] are opening a novel perspective in terms of personalized medicine.[33]

of embryonic stem cells (ESCs), brain-derived neural stem cells (NSCs), induced pluripotent stem cells (iPSCs), and MSCs has been considered; the former mostly because of their reprogramming potentialities and MSCs for their well-characterized immunoregulatory and trophic properties.

MSCs, due to their accessibility and relative ease of handling, are being extensively investigated and their actions in the context of AD therapy can be resumed in three main effects: 1) immunomodulation, 2) reduction of Aβ plaque burden by internalization and degradation of Aβ oligomers via the endosomal–lysosomal pathway, and 3) neurotrophic/regenerative potential (see Box 2). Systemic injection of green fluorescent protein (GFP)-labeled bone marrow-derived MSCs has been demonstrated to be able to reduce Aβ plaque size in the hippocampus of AD animal models[34] and to act in an immunomodulatory fashion. Intracerebroventricular (ICV) transplantation of placenta-derived MSCs in $A\beta_{1-42}$-infused mice has been described to produce beneficial effects, including i) functional improvement in memory deficits, ii) reduction of $A\beta_{1-42}$ levels, iii) decreased APP and BACE1 expression levels, iv) decreased α- and β-secretase activity, and v) a considerable reduction of gliosis.[35] Evidence for MSCs supporting local stem and progenitor cell growth and differentiation has been provided following administration of MSCs in an AD animal model, by inducing differentiation of neural progenitor cells (NPCs) into mature neurons in the hippocampus, via the activation of the Wnt pathway.[36] In another study, human MSCs transplanted in aged rats have been shown to reach the brain and to differentiate into neural cells, restoring locomotor and cognitive activity.[37] Its worth noting that encouraging clinical outcomes attained in different pathological conditions and preclinical results with MSCs in AD animal models[38] have fostered the start of clinical trials with MSCs in AD patients (https://clinicaltrials.gov using as keywords Mesenchymal Stem Cells and Alzheimer's Disease), of which one has already concluded the phase I, confirming feasibility and safety of MSC injection into the human brain of nine patients.[39]

2.1. MSC Therapeutic Potential in AD Mainly Acts through the Secretome

The emergence of MSCs as efficacious therapeutic agents spotlighted the importance of secreted factors in stimulating

Table 1. MSC surface markers. Consensus view of surface markers of MSCs deduced from Dominici et al.[24] and following studies.[26,86,114–117]

	MSCs	
Features	Positive	Negative
Stemness	SCA1, Oct 4, Oct 4A, Nanog, Sox-2	CD117, CD34
Hematopoietic cells		CD19, Cd45, CD14, CD11b, CD79α, HLA-DR
Mesenchymal	CD73, CD105, CD90[a)]	CD31
Other markers	Collagen I, fibroblast surface antigen, smooth muscle α-actin, vimentin, CD10,[b)] CD166, ICAM-1, CXCR4, CD13, CD106, CD44, CD29, CD49, CD146	MHC class II[c)]

HLA-DR, human leukocyte antigen-DR isotype; ICAM-1, intercellular adhesion molecule 1; MHC, major histocompatibility complex; SCA1, spinocerebellar ataxia type 1.
[a)]CD90 positivity has been verified in human MSCs but it is controversial in mouse MSCs.
[b)]CD10 expression was demonstrated for MSCs derived from human adipose tissue.
[c)]MHC class II expression has been often observed upon application of inflammatory stimuli (interferon-γ [INF-γ]). Other markers include antigens that can vary their expression according to cell source and culture conditions.[118]

proliferation, neuronal differentiation, and survival in endogenous neurogenic niches,[40,41] and in cellular models of AD.[42] MSC secretome was shown to reduce cell death,[42,43] Aβ deposits, and plaque formation[34,35,44] and to stimulate neurogenesis, synaptogenesis, and neuronal differentiation,[36,42,45] eventually rescuing spatial learning and memory deficits in vivo.[35,43–45] MSC immunoregulatory properties also result in the upregulation of anti-inflammatory cytokines, such as interleukin-10 (IL-10), and reduction of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-1β.[35,43–45]

Based on this amount of evidence and in consideration of the safety concerns associated with stem cell therapies, which in humans remain consistently high, the possibility of using MSC-derived secreted material for therapeutic purposes is extremely attractive, provided all therapeutic benefits are maintained. Among the secretome, MSC-derived EVs (see Box 3) are the best cell-free candidates for promoting a reparative process, activating—via intercellular communication—positive responses in the brain microenvironment.

EVs are in fact endowed with several advantageous properties and possess a higher safety profile with respect to the whole cells; since they do not induce vascular obstruction, they can pass the blood–brain barrier easier than the entire cells, have low immunogenicity, and could be engineered for personalized treatments.[77]

The protective potentials of MSC-EVs in the brain have been recently demonstrated in both the regulation of the inflammatory response mediated by microglial cells and in the regenerative and maintenance effects of tissue homeostasis.[78] In particular, it has been shown that the administration of MSC-EVs promotes neurogenesis, angiogenesis, remodeling of nervous processes with the formation of new synapses, and induce axonal plasticity.[77,79,80] In addition, MSC-EVs have been shown to exert immunomodulatory effects[81] (see Box 3).

3. MSC-EVs in AD Carry Enzymatically Active Molecules and Could Act as Aβ Scavengers

The impact of MSC-EVs was first investigated in a cellular model of AD by using EVs isolated from adipose tissue MSCs.[54] The transfer of MSC-EVs to N2A neuroblastoma cells reduced both secreted and intracellular Aβ peptide levels. In an attempt to unveil the mechanisms underlying such effects, Katsuda and co-workers performed a characterization of EVs and a large amount of neprilysin (NEP) was detected, the most prominent enzyme involved in the degradation of Aβ peptide in the brain. These data might indicate that the processing of APP could be a possible target of the pharmacological treatment of EVs, thus supporting the possibility of using MSC-EVs as therapeutic tools in AD.

An interesting report supporting the role of EVs as Aβ scavengers showed that mouse intracerebral injection of neuroblastoma-derived exosomes reduced $A\beta_{1-42}$ burden by its binding to glycosphingolipids on exosome surface and conveying Aβ peptides to microglia for phagocytosis.[82] Hence, it is possible that MSC-EVs could also directly bind to Aβ, promoting its clearance and exploiting the Aβ-binding ability of glycosphingolipids, membrane components highly abundant in exosomes.

4. The Extracellular Environment Influences the Type of Released EVs

Given the plasticity, typical of stem cells, endowing MSCs with the ability to adapt to the microenvironment where they are transplanted into, several studies have highlighted the possibility of preconditioning MSCs in vitro, to make them more suitable in tackling specific pathological mechanisms.[83,84] The optimization of MSCs preconditioning protocols, therefore, could represent a key element for the enhancement of the immunosuppressive properties of released vesicles. According to Cui et al.,[85] exosomes extracted from MSCs preliminarily subjected to a hypoxic pretreatment enhanced the therapeutic properties of the vesicle derived therefrom. The authors demonstrated for the first time that exosomes from normoxic versus hypoxic MSCs had a different effect on learning and memory improvement of APP/PS1 AD mice. Interestingly, AD mice treated with exosomes from normoxic MSCs showed improved learning compared to untreated AD mice, but the treatment with exosomes derived from hypoxia-preconditioned MSCs exerted an even better action in rescuing learning memory impairments,[85] in line with the previous description of an enhancement of MSC therapeutic effects by hypoxia preconditioning.[86–88] This is fairly plausible, considering that physiologically MSCs reside in niches characterized by a low oxygen tension (hypoxia),[87] albeit inhibitory effects of hypoxia on the proliferative and differentiation abilities of MSCs have been demonstrated in vitro.[89,90] In addition, exposing MSCs to different metabolic conditions, such as diabetes, which is a pathological condition predisposing to AD, can modify characteristics of the obtained EVs.[91,92] Therefore it should be carefully considered that the in vitro and in vivo states of MSCs could determine different effectiveness of EVs.

Interestingly, not only the environment can affect MSCs and, in turn, their EVs, endowing them of specific properties, but also EVs themselves can be metabolically active and carriers of enzymatic activities, potentially contributing to changes in the cellular metabolic microenvironment.[93]

5. MSC-EVs Can Induce Anti-Inflammatory Effects

Immunomodulatory effects of MSC-EVs have been extensively described in diverse in vitro and in vivo models for several diseases. These effects turned out to be multifaceted and acting at multiple levels: cytokine release and enzyme activity modulation, gene expression regulation, and antioxidant effects.

5.1. Cytokine Regulation

The ability of MSC-EVs to exert both regenerative and immunoregulatory actions has been demonstrated in many studies. Particularly, MSC-EVs can contribute to antigen-specific and -nonspecific immune regulation and can modulate inflammatory and autoimmune diseases.[51,69,81,94] In the context of AD, MSC-EVs have been shown to reduce glial reactivity due to the increase of the anti-inflammatory cytokines (IL-10 and IL-4) and to the decrease of the pro-inflammatory ones (TNF-α and IL-1β). High levels of TNF-α and IL-1β are

Box 3
Extracellular Vesicles and Their Therapeutic Effects in Pathological Models

EVs are small membrane-delimited vesicles released by most, if not all, cell types into the body fluids. They encapsulate proteins, nucleic acids, proteins, and lipids, thus playing important roles in intercellular communication, both locally and systemically. The interest towards EVs considerably increased over the last years: they are involved in numerous physiological and pathological processes, including the regulation of immune activity, angiogenesis, tumor invasiveness, and wound healing[46,47] through paracrine and endocrine communication.[48,49] Indeed, EV-based therapeutics are being developed and clinically tested for the treatment of inflammatory diseases, autoimmune disorders, and cancer.[50]

Among the different types of EVs, MVs, and exosomes are widely studied as possible cell-free "natural" therapeutic effectors.[51] Collectively, they consist of nanoscale (30–1000 nm) lipidic organelles, released by cells under physiological and pathological conditions, flanking the action of soluble factors by allowing horizontal communication among cells.[52,53] The distinction between various groups of EVs, according to unambiguous markers, is still very difficult, and the identification of a univocal set of components for each subset still remains a major challenge. Currently, proteins are the best-characterized EV-enriched molecules, which we have tentatively classified as "expected" or "variable" (see **Table 2**).[54,55] EVs identified in a variety of body fluids including serum, plasma, urine, cerebrospinal fluid, saliva, ascites, and amniotic fluid are becoming more and more interesting as possible circulating biomarkers for different diseases.[56]

MVs, also referred to as ectosomes or shed vesicles, belong to a heterogeneous population of vesicles, whose size ranges from 40 nm to 1 μm and which are typically enriched in cell membrane proteins (including CD9[57] and annexin V, see Table 2). MVs sediment at $10\,000 \times g$ and their biogenesis starts from the formation of outward buds at specific sites of the plasma membrane, followed by a fission process and subsequent vesicle release into the extracellular space.[58] Exosomes are smaller vesicles (40–150 nm in diameter) thought to originate from the unconventional inward budding of multivesicular bodies (MVBs), which fuse with the cell membrane and release their content into the extracellular environment.[59] Exosomes typically sediment at $100\,000 \times g$ and are characterized by the enrichment of markers, among others, CD9, CD63, Tsg101, and CD81.

Both MVs and exosomes mediate communication between neighboring cells. Depending on the cell of origin, their features and abilities vary according to their protein, nucleic acid, and lipid content, which can be influenced by several variables including cell physiological state and extracellular stimuli. Of note, MVs and exosomes purification methods can influence their characteristics, an important caveat to be kept in mind, which makes the broad definition of EVs more prudent.[55,60]

The effect of EVs on target cells can be modeled by three different mechanisms: i) internalization of the entire vesicle into recipient cells that can then follow two fates, a) fusion with target-cell endosomes undergoing transcytosis or b) maturation into lysosomes that undergo degradation;[61,62] ii) vesicle fusion with the recipient cells and direct release of its content into the cytoplasm, which directly regulates intracellular targets;[61] and iii) direct interaction between vesicle membrane proteins and cellular receptors, allowing a secondary cell response.[63] Upon receptors and/or bioactive molecule transfer, EVs exert functional modulation of target cells by delivering intracellular proteins or by operating genetic horizontal transfer between cells.[64] In particular, since the discovery of microRNAs (miRNAs) and their associated ribonucleoproteins inside EVs, many biological effects through inhibition of specific messenger RNAs (mRNAs) and modulation of gene expression have been reported.[57,63] How specific groups of miRNAs are selected and sorted into the lumen of EVs is still a matter of investigation.[65] Among different miRNAs identified up-to-date, a certain number of them have been found to be involved in cell development, survival and differentiation, and immune modulation.[66–68]

EVs from MSCs have been shown to exert a therapeutic effect in various diseases. Interestingly, Camussi,[69] Lim[70] and co-workers successfully pioneered the therapeutic use of MSC-EVs in mouse models of acute kidney and myocardial ischemia/reperfusion injuries, respectively. Interestingly, the therapeutic effect of MSC-EVs on tubular epithelial cells was RNA-dependent since it was significantly reduced after RNase incubation of EVs.[69]

Many studies followed addressing the therapeutic functions of MSC-EVs in animal models of heart, kidney, liver, and brain injuries.[51,71] A recent report presented a single administration of MVs derived from human Wharton's jelly MSCs protecting against oxidative stress induced by renal ischemia/reperfusion injury.[72] Interestingly, MSC-derived EVs have been shown to be effective in animal models of neurological diseases, e.g., ameliorating inflammation-induced cellular damage and improving long-term cognitive functions in a rat model of preterm brain injury.[73] MSC-EVs have also been found to be effective in traumatic brain injury models.[74,75] Despite intense investigation in several disease models (for a recent review see also Nooshabadi et al.[76]), the potential therapeutic effects of MSC-derived EVs in AD remain to be clarified.

Table 2. Extracellular vesicle markers. Expected proteins associated with EVs are transmembrane, membrane-associated, and cytosolic proteins of releasing cells. Some are highly enriched in MVs or exosomes. Variable proteins are found in EVs, depending on experimental conditions and cell type.[49,54–56,119,120]

	Extracellular vesicles	
	Microvesicles	Exosomes
Expected proteins	CD9, integrins (e.g., CD29, CD49, CD44), annexins (A1–5), RAB family proteins	
	SNARE proteins, Ago2, Stau1–2	CD63, CD81, flotillin 1, TSG101, Alix,
Variable proteins	Soluble secreted proteins, e.g. cytokines and interleukines, enzyme (e.g., NEP, CD73, GADPH, metalloproteinases, catalase), CD248, CD47, growth factor receptors	

Ago2, argonaute 2; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

indeed correlated with cognitive decline, typical of AD, while IL-4 and IL-10 are pleiotropic cytokines inducing inhibition of pro-inflammatory cytokine synthesis and release.[85]

5.2. Inducible Nitric Oxide Synthase (iNOS) Inhibition

Wang et al.[95] investigated the effects of MSC-derived EVs on cognitive behavior of APP/PS1 AD mouse model. Focusing on Aβ-mediated neurotoxicity that triggers nitric oxide (NO) production, a pathway active also in other neurological diseases such as multiple sclerosis, stroke, and Parkinson's disease,[96] the authors found that APP/PS1, ICV-injected, with 100 μg of exosomes (once for 2 days a week, for 2 weeks) showed amelioration of CA1 synaptic transmission and long-term potentiation, the neuronal mechanism underlying memory formation.[95] The mechanisms described so far support the idea that regulation of inflammatory processes can contribute to the behavioral improvements observed in animal models of AD and suggest the involvement of iNOS, a common target of many inflammatory pathways, as an important endpoint for the therapeutic effects of EVs.

5.3. Gene Expression Regulation

Gene expression regulation has been observed as a downstream effect of the paracrine action of MSC-EVs, due to the delivery to target cells of mRNAs, miRNA, and noncoding RNAs, or transcription factors.[97–99]

Undoubtedly miRNAs are the most studied molecules since they can exert a great variety of regulatory actions.[100] In particular miR-21 could control the balance between initial pro-inflammatory and later immunoregulatory and anti-inflammatory responses.[101,102] In fact dysregulation of miR-21 plays a role in the balance between Th17 and Treg cells in chronic inflammatory and autoimmune diseases.[102,103] Moreover, miR-21 seems to be a key player in the beneficial EV actions, as it could dampen nuclear factor-κB (NF-κB) activation and signal transducer and activator of transcription 3 (STAT3) expression, both potentially relevant in the propagation of neuroinflammation in AD pathobiology.[85] MiR-21 could also promote c-Jun/AP1 activities, which control the anti-inflammatory response.[101] Indeed, the overexpression of miR-21 through engineered EVs not only decreased the plaque deposition but also downregulated the levels of TNF-α and IL-1β.[85] Interestingly, the expression of miR-21 in the brain of exosome-injected mice was higher than in control AD mice, suggesting that miR-21 might be elevated, through a transfer mechanism, determined by exosome action.

6. MSC-EVs Can Induce Antioxidant Effects

MSC-EVs have been observed to exert in vitro protection against neuronal oxidative stress, suggesting that they might contribute to the preservation of synapse integrity in neurons exposed to soluble oligomers of the amyloid-β peptide (AβOs).[104] AβOs have been described to trigger detrimental events determining neuronal oxidative stress[105] and synapse damage,[106] the mechanisms hypothesized to be leading to cognitive decline in AD. Along the same line of thoughts, it is plausible thinking that antagonizing the neuronal impact of AβOs may provide effective novel therapies for AD. The mechanisms proposed by de Godoy et al.[104] to explain, at least in part, neuroprotection by MSC-EVs pertain the content in antioxidant enzymes and in anti-inflammatory and/or trophic molecules. The authors could, in fact, demonstrate that EVs secreted by MSCs contain and carry endogenous catalase that endows EVs of reactive oxygen species (ROS) scavenging activity. Haney et al.[107] worked out exploitability of exosomes as carriers of antioxidant actions by showing that CNS delivery of macrophage-derived exosomes, loaded ex vivo with catalase, efficiently decreased oxidative stress and increased neuronal survival in an in vivo model of Parkinson's disease.

All of the studies reviewed so far report beneficial effects of EVs derived from MSCs, focusing on different possible mechanisms of action, indicating pleiotropic effects of MSC-EVs. Experiments carried out either in vitro or in vivo highlight the potential therapeutic effects of MSC-EVs in AD models. Moreover, since the efficacy of EVs has been appreciated in vivo upon either intranasal, intravenous, or ICV injection, it is conceivable to conclude that more than one route of administration might be effective. It has to be determined which one might be the best for possible treatment of patients.

7. MSC-EVs, Pleiotropic Relief against AD?

Overall, MSCs have been considered promising therapeutic tools due to their pleiotropic properties advantageous for pathological conditions affecting the brain, either of inflammatory, immunological, traumatic, or ischemic nature. Since MSCs-derived secretome retain many of the characteristics of the cells they originate from and EVs are the main components of the secretome, it is conceivable that MSCs-derived EVs be equally pleiotropic. Interestingly, the results obtained by different groups on the therapeutic effects of EVs seem to complement each other. In fact, if Cui et al.[85] showed an increase in the expression of synaptic proteins following treatment with EVs, Wang et al.[95] observed that EVs could rescue long-term potentiation (LTP) in AD mice. The increase in LTP could be linked to the decrease in AβOs, as suggested by in vitro experiments performed by de Godoy

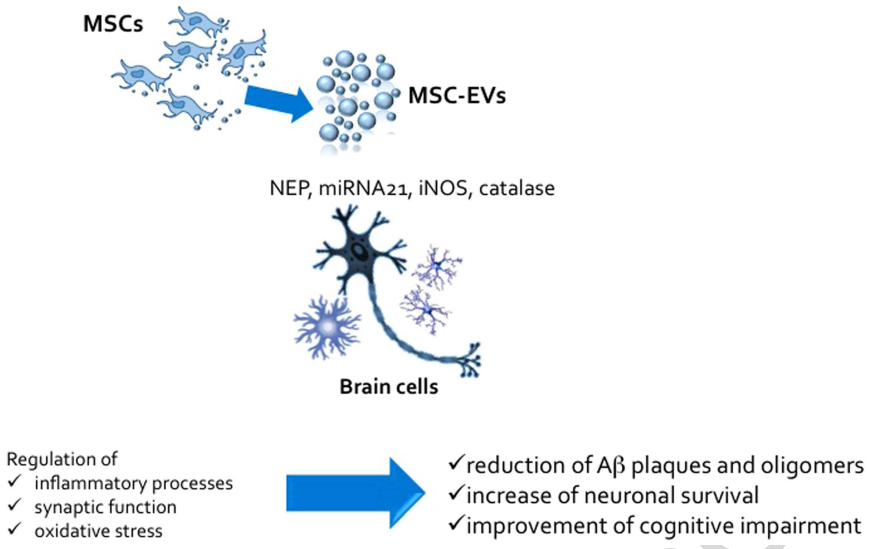

Figure 1. Schematic representation of the principal modes of action, described up to now, of EVs released from MSCs by which they could exert their pleiotropic effects in AD. MSC-EVs may act at multiple levels, by participating in the regulation of inflammatory processes (via increasing anti-inflammatory and reducing the pro-inflammatory cytokines), restoring neuronal survival (regulating gene expression in brain cells, clearing Aβ plaques, and oligomers) and exerting a general antioxidative effect. All these effects have been demonstrated to contribute in ameliorating cognitive symptoms of AD mice.

et al.[104] EV enzymatic activity, for instance, via NEP could promote Aβ clearance, thus possibly preventing also AβO's toxicity. Moreover, MSCs and their EVs not only prevented ROS increase induced by AβOs but also reduced basal ROS levels in neurons.[104] As a consequence, preventing the production of AβO-induced ROS may result in a restoration of synaptic activity and possible rescue of the detrimental effects on memory and learning processes in the hippocampus.[108]

Therefore, intervening on the regulation of inflammatory processes and of synaptic function, on oxidative stress, and on the prevention of AβOs toxicity, altogether, could be in the near future the most promising therapeutic strategies, worth being pursued for AD treatment by means of EV therapeutic actions.

Obviously, it is conceivable that the earlier we detect the signs of the symptoms, the sooner the pharmacological intervention should be performed. In the last few years, a progressively rapid and amazing technological development has been occurring for increasing accuracy and sensitivity in AD diagnosis and most of all to make it more reliable at early stages, taking into account multiple parameters, such as symptoms, biomarkers, and risk factors. EVs could be one of the players in this complex picture, contributing both as diagnostic and prognostic disease markers,[109] detectable in the cerebrospinal fluid (CSF) or blood of AD and MCI patients.[110,111]

8. Conclusions

The humongous efforts and resources invested in the last 20 years to find drugs to halt AD have not lived up to expectations, thus generating a general sense of frustration. On the one hand, we have learned that it is of pivotal importance to identify reliable early biomarkers for AD patients, who might be treated when symptoms are mild, to delay disease manifestation. On the other hand, the failure of therapeutic approaches centered around the amyloid hypothesis allowed us to identify other important etiological mechanisms. Preclinical results indicate the use of MSCs and MSC-derived EVs as promising therapeutic approaches in regenerative medicine. In particular, EVs isolated from MSCs of various origins carry biologically active molecules, which can be transferred to target cells to exert their therapeutic effects, through the inhibition of inflammatory responses, regulation of the immune system, and clearing Aβ plaques and oligomers (see **Figure 1**).

As a result, EVs represent an applicable, safe, and cost-effective approach in cell-free regenerative medicine and could become a suitable alternative to MSC therapy. Nonetheless, before passing from bench to bedside, a long way has still to be gone. Hopefully, a better understanding of EV actions and their biological functions will be reached in the next few years, and especially, studies allowing determining the best route of administration and the best dosage for each patient are needed.[112,113] Before being able to take full advantage of MSC-EVs in regenerative therapy,[51,76] it is imperative to set standard methods for EV isolation and characterization in order to provide reproducible, effective, safe, and powerful new therapies based on MSC-EVs.[85] To this aim, also the enhancement of the therapeutic actions of EVs derived from MSCs[104] by preconditioning could be conceived.

Eventually one could also think of a personalized treatment for achieving a targeted delivery of specific cargos to sick brains at an early stage in order to reduce inflammation, oxidative stress, or Aβ burden, possibly by engineering EVs derived from patients' MSCs. The gap between preclinical and human translation needs to be filled quickly! Consequently, if on one side we certainly are running out of time for curing AD, on the other several promising paths could be opened in the next future.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Q6 Alzheimer's disease, cell therapy, exosomes, extracellular vesicles, mesenchymal stem cells, microvesicles, neurodegenerative disease, personalized medicine

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