Chapter

Advances in Cellular and Cell-Free Therapy Medicinal Products for Huntington Disease Treatment

Irina Kerkis, Rodrigo Pinheiro Araldi, Cristiane Valverde Wenceslau and Thais Biude Mendes

Abstract

Huntington's disease (HD) is a neurodegenerative disorder caused by the expansion of CAG repeats in the huntingtin gene. The disease causes the progressive degeneration of neurons affecting particularly the medium spiny neurons (MSNs) within the striatum. The mHtt inclusions promote neurodegeneration. However, the mHtt can spread to different brain areas through exosomes. For this reason, it is not surprising that HD causes motor, cognitive and neuropsychiatric dysfunctions. To date there is no treatment able to modify the natural history of the disease. In this sense, the advanced cellular therapy, based on the therapeutic use of mesenchymal stem cells (MSCs) emerges as a potential candidate for HD treatment. This is because, the MSCs produce many critical therapeutic molecules which act in multiple cellular and molecular targets. Moreover, in addition, advanced cell therapy is a unique approach that could provides neuroprotection and neuroregeneration. However, the current discovery that the MSC mechanism of action is mediated by exosomes, have encouraged scientist to explore the therapeutic potential of the cell-free therapy. Based on this, we revisited the HD pathophysiology, areas. Providing evidence that MSC and MSC-derived exosomes can be used to change the natural history of HD.

Keywords: Huntington's disease, stem cells, therapeutic cells, exosomes, cell-free products

1. Introduction

Huntington's disease (HD, OMIM 143100) is a rare and incurable hereditary autosomal dominant neurodegenerative disorder, affecting 5–10 individuals per 100,000 in the Caucasian population. In certain regions, such as Australia, North America, and Western Europe, including the United Kingdom, the prevalence of the disease has increased over the past 50 years [1, 2].

HD is characterized by the loss of specific neurons within the striatum. The most sensitive cell population is the gamma-aminobutyric acid (GABA)ergic medium spiny neurons (MSNs). Neuropathologically, the disease leads to about 57% loss of

cross-sectional area from the caudate nucleus and about 65% loss of the putamen (in *postmortem* samples). The progression of HD also leads to loss of cortical volume (particularly in cases with more advanced disease), affecting the large pyramidal neurons predominantly in layers III, V, and VI. In these cases, the loss of neurons in the thalamus, substantia nigra pars reticulata, and the subthalamic nucleus can also be observed. The neurodegeneration of these brain areas results in progressive motor (chorea, saccadic eye movement abnormalities, ataxia of speech, dysphagia, etc.), cognitive (dementia), and neuropsychiatric disturbances (depression, anxiety, apathy, etc.). These HD symptoms usually develop between ages 30 and 50 (adult-onset Huntington's disease—AOHD, which is verified in 95% of cases), but they can appear as early as age 20 (juvenile-onset Huntington's disease—JOHD). However, neurological loss and metabolic alterations generally occur in the adult HD brain before symptoms, but the precise timetable for the neuronal degeneration remains unknown [3].

HD is caused by the expansion of trinucleotide Cytosine-Adenine-Guanine (CAG) repeat, located in the first exon of the HD gene, also known as HTT or IT15 gene (locus 4p16.3, OMIM 613004), which encodes the huntingtin protein (Htt). Since the discovery of HTT gene mutation (in 1993), it has been recognized that larger CAG expansions are associated with early-onset in HD, especially for AOHD. Generally, unaffected individuals have less than 35 CAG repeats (common rage in humans: 17–25), while affected individuals have 36–250 CAG repeats. The CAG repeat range of 36–39 might be found in affected individuals and asymptomatic individuals (reduced penetrance alleles), whereas individuals with over 40 CAG repeats always develop the disease (fully penetrance alleles) [4].

The wild-type allele of the HTT gene (i.e., <35 CAG repeats) typically segregates and stably as a polymorphic locus. However, the allele carrying higher-normal CAG repeats (27–35 repeats) has increased instability. For this reason, individuals with 27–35 CAG repeats have a high risk of passing on repeats in the affected size range to their offspring. HTT gene encodes the huntingtin protein (Htt), a sizeable soluble protein (350 kDa), consisting of 3114 amino acids, which is expressed in all metazoans, is highly conserved among vertebrates. Although, all tissues ubiquitously express the HTT gene, Htt protein is found higher expressed in the brain, represented by all neurons and glial cells [4].

1.1 Htt protein characterization and function in HD

The Htt protein is crucial for developing and maintaining central nervous systems (CNS) homeostasis since the protein is engaged in many cellular and biological functions, including transcription, transport, vesicular trafficking, and coordination of cell division, energy metabolism, and antiapoptotic activity. For this reason, it is not surprised that Htt co-localizes with many organelles, such as the nucleus, endoplasmic reticulum, Golgi complex, endosomes, mitochondria, and synaptic vesicles. Furthermore, cells expressing mRNA of the HTT gene were described by in situ hybridization in the usual human 20 to 23-week fetal brain, suggesting that huntingtin protein is crucial for the development of the CNS. Studies also demonstrated that the deletion of the mouse homolog of the HTT gene is lethal in the embryo before the brain is formed. By contrast, heterozygote mice for the HTT gene usually develop but exhibit motor deficits and cell loss in basal ganglia. Altogether, these data confirm that the Htt protein is mandatory for CNS development and function [5].

The Htt protein is characterized by the presence of (i) the N-terminal 17 amino acids (or N17 region), which is followed by (ii) the polyglutamine (poly Q) tract

(encoded by the CAG repeats), (iii) a proline-rich region (PRR), (iii) clusters of Huntingtin, Elongation factor 3, PR65/A regulatory subunit of PP2A and target of rapamycin 1 (HEAT) repeats (α-helix-loop-α-helix motif), and (iv) caspase and calpain cleavage sites (in higher vertebrates). The N17 region has been identified as a critical region that plays a role in Htt localization, aggregation, and toxicity. It is subject to several post-translational modifications, including acetylation, SUMOylation, phosphorylation, and ubiquitination. The polyQ tract is encoded by the CAG trinucleotide repeats, which code for the glutamine (Q) amino acid. PRP region is exclusively found in mammals and is essential for the Htt interactions with proteins containing tryptophans or Src homology 3 domains. In addition, PRP encodes the polyproline (polyP) region, which interacts with polyQ, increasing the Htt protein stability and solubility. HEAT repeats consist of around 50 amino acids and contains two antiparallel α-helices forming a hairpin, which acts as a scaffold for various protein complexes and mediates inter and intramolecular interactions. Sixteen HEAT repeats organized into four clusters were identified in the Htt protein. Htt protein also has several proteolytic cleavage sites, including proline, glutamic acid, serine, and threonine domains. These domains are found in both Htt and mHtt proteins. Thus, these proteins can be cleaved by caspase 3 at amino acid 513 and 552, caspase 1 at amino acid 572, caspase 2 at amino acid 552, and caspase 6 at position 586. In addition, two calpain cleavage sites are located at amino acid 469 and 536, and the metalloproteinase (MMP)-10 cleaves Htt our mHtt at amino acid 402 [5].

The Htt protein interacts with over 200 other proteins, many of them involved in microtubule-mediated axon traffickings, such as the Huntingtin-associated protein 1 (HAP1), which mediates the interaction between Htt protein with microtubule motor proteins and their co-factors (kinesin, dynactin subunit p150, and dynein). Htt protein also mediates long- and short-range axonal transport and vesicle trafficking. This is because the Htt protein binds to the endocytic pathway-related proteins (clathrin and dynamin), as well as endocytic organelle trafficking proteins (α-adaptin, Hip1, Hip14, HAP40, PACSIN1, SH3GL3/endophilin 3). Htt protein is enriched at synaptic terminals and interacts with cytoskeletal and synaptic vesicle proteins to regulate synaptic activity in neurons. However, by exhibiting a C-terminus containing a nuclear export signal (NES), Htt protein can traffics between cytoplasm and nucleus. In addition, the N17 region also interacts with a nuclear pore protein (TRP), which has nuclear translocation activity. The N-terminal domain also forms an amphipathic alpha-helical membrane-binding domain that reversibly mediates association with the endoplasmic reticulum (ER), endosomes, and autophagic vesicles. Thus, it is not surprising that Htt protein also interacts with various transcription factors and transcriptional regulatory proteins, acting as a positive regulator of brain-derived neurotrophic factor (BDNF) transcription (a protein in which expression levels are found reduced in individuals with HD), stimulating the BDNF vesicular trafficking in neurons.

However, by increasing the number of glutamine residues in poliQ, the CAG trinucleotide expansion, verified in HD, reduces the solubility of mutated huntingtin protein (mHtt), resulting in intracellular aggregates (inclusions) in the brain, particularly in GABAergic medium spiny neurons (MSNs), located within the striatum. This event occurs because the expanded polyQ sequence in mHtt protein undergoes conformational changes to form a B-pleated sheet prone to aggregation. In addition, the early phases of aggregate formation appear to accelerate the hydrophobic interactions with an amphipathic α-helical structure of N17. Under physiological conditions, proteostasis balances protein synthesis, folding, trafficking, and degradation. The impairment of the proteostasis systems aggravates the aggregation of the misfolded

mHtt. In addition, posttranslational modifications influence the mHtt toxicity, aggregation propensity, and intracellular localization. For example, proteolytic cleavage of mHtt generates N-terminal fragments with an increased tendency to aggregate. Furthermore, the mHtt inclusions can block the axonal transport between the cell body and the synaptic cleft and recruit other polyQ-containing proteins, which interact with mHtt, leading to loss of biological function, therefore, cell death. In addition, mHtt also silences the activity of RE1-Silencing Transcription Factor (REST), increasing the binding of REST to RE1/neuron restrictive silencer element, producing transcriptional dysfunction [6, 7].

1.2 Htt protein and mitochondrial dysfunction

The mHtt inclusions promote mitochondrial dysfunction, decreasing the activity of mitochondrial respiratory complexes II, III, and IV, which was already verified in *postmortem* brain samples of HD patients. Furthermore, the activity decrease of these mitochondrial complexes was also reported in asymptomatic HD carriers, indicating that mitochondrial defects may initiate disease onset. Experimental results also demonstrated that the ectopic expression of mitochondrial complex II subunit in striatal neurons expressing mHtt exon 1 restores complex II respiratory activity and protects against cell death. Confirming this data, it was extensively demonstrated that the rats treated with the neurotoxin 3 -nitropropionic acid (3-NP)—a selective inhibitor of succinate dehydrogenase and complex II—recapitulates the loss of MSNs in the substantia nigra, resulting in HD-like symptoms. In addition, studies showed that humans exposed to 3-NP exhibit motor dysfunction similar to HD patients [8–11].

Moreover, the mHtt can be cleaved by caspase 6. The fragments of cleaved mHtt protein bind to several transcription regulators, including the tumor suppressor, p53, thus regulating genes involved in mitochondrial function. Therefore, the mHtt increased the levels of p53, which in turn increased Bax and Puma expression, resulting in mitochondrial dysfunction and neuronal loss. These actions increase the reactive oxygen species (ROS) production, justifying the oxidative damage commonly observed in the plasma of HD patients, HD *postmortem* brain tissue, lymphoblasts, and cerebrospinal fluid. In addition, markers of oxidative damage, including heme oxygenase (an inducible isoform that occurs in response to oxidative stress), 3-nitrotyrosine (a marker for peroxynitrite-mediated protein nitration), and malondialdehyde (MDA), are elevated in human HD striatum and cortex as compared with age-matched control brain specimens. Consistent with these data, an increase in 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, in both plasma and urine of HD patients is observed [8–11].

Cumulative evidence has also demonstrated that mHtt protein causes a reduction in TORC1, the most potent transcriptional activator of (peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1 (PCG-1α) [12–14]. In addition, the mHtt protein also increases transglutaminase (Tgase) activity, which impairs the transcription of PCG-1α. Thus, mHtt downregulates the expression levels of PCG-1α [14]. The last is recognized as a critical transcriptional coactivator, which interacts with a broad range of transcription factors within a variety of biological processes. In addition, PCG-1α is involved in the regulation of mitochondrial biogenesis, OXPHOS, antioxidant defense, adaptive thermogenesis, and glucose/fatty acid metabolism. Under physiological conditions, the PGC-1α forms heteromeric complexes with nuclear respiratory factors (NRF-1 and NRF-2), and with the nuclear

receptors (PPARα, PPARδ, PPARγ and estrogen-related receptor α (ERRα)). These heterodimers regulate the expression of many nuclear-encoded mitochondrial genes, including cytochrome c, complexes I–V, and the mitochondrial transcription factor A (Tfam), as well as antioxidant genes, including superoxide dismutase (SOD) and glutathione peroxidase (GPX). Thus, the mitochondrial dysfunctions promoted by the mHtt-mediated PCG-1α downregulation lead to an increase in oxidative stress [12, 13, 15].

1.3 Htt protein and neuroinflammation

In addition, the mHtt accumulation in neurons promotes microglial activation, increasing oxidative stress. In addition, microglial cells that express mHtt show significant elevations in nuclear factor kappa B (NF-κB). This elevation occurs because the mHtt interacts with the IκB kinase (IKK) γ subunit, promoting the assembly and activation of the IKK complex (comprised by IKKα and IKKβ subunits). The IKKβ kinase phosphorylates IκBα causes the liberation of NF-κB, promotes the gene expression of the pro-inflammatory cytokine, including interleukin (IL)-6, resulting in neuroinflammation [16, 17]. The neuroinflammatory cytokines produced in response to mHtt protein accumulation leading to the activation of microglial cells considered the brain's resident immune cells. Under physiological conditions, i.e., in the absence of inflammatory stimulus, microglia are in a surveilling state, being responsible for maintaining synapses and synaptic plasticity. In addition, Microglia also facilitates the growth and development of surrounding neural networks by secreting neurotrophic factors, such as BDNF, nerve growth factor (NGF), and insulin-like growth factor (IGF-1) [18]. Moreover, significant evidence suggests the microglia promotes neurogenesis by phagocytosing apoptotic neural cells, facilitating the migration and differentiation of neural progenitor cells, and secreting soluble factors related to neurogenesis. However, microglia become activated upon detecting inflammatory stimuli, such as the increase in ROS or cytokine production [19]. When activated, microglia can adopt different polarization states, such as M1 and M2. Interestingly, microglia can alternate between these states. For this reason, recently, studies have suggested using M1/M2 terminology to categorize activated microglial cells. M1 microglia exhibit a proinflammatory phenotype, the significant initiators of innate and adaptive immunity in the brain. In addition, these cells elicit a phagocytic function and release cytotoxic factors, including nitric oxide and ROS. M2 microglia also carry out phagocytosis, but contrary to the role of M1 microglia, M2 microglia exhibit an anti-inflammatory phenotype, releasing anti-inflammatory cytokines such as interleukin (IL)-4, IL-13, and transforming growth factor-beta (TGF-β), which suppress inflammatory responses. The continued activation of microglia, stimulated by the inclusions of mHtt, prolonged the production of inflammatory mediators, resulting in chronic inflammation. The last is implicated in further tissue damage, justifying the microglia activation in striatal GABAergic neurons verified by Positron Emission Tomography (PET) in HD patients. Interestingly, studies based on PET also reported the presence of microglia activation in striatal GABAergic neurons in presymptomatic HD gene carriers, suggesting that microglial activation is an early characteristic of HD before symptom onset. However, the activation of microglia increases oxidative stress, resulting in both nuclear and mitochondrial DNA oxidative damages and protein and lipid oxidation. These damages lead to progressive cell death, particularly of MSN's [20, 21].

1.4 Subventricular zone involvement in HD physiopathology

Studies based on animal models of HD demonstrate that cell death in the striatum serves as a potent stimulator of progenitor cell proliferation (which are resident into the subventricular zone – SVZ), neuroblast migration, and neurogenesis. This is because, in the transgenic mouse model of HD (in which there is minimal cell loss in the striatum), the SVZ is unaltered, while in rat striatal-lesion models of HD (in which there is a cell loss in the striatum), there is a marked increase in SVZ progenitor cell proliferation and neurogenesis. The SVZ of the lateral ventricle is the resident niche of stem cells. These stem cells give rise to proliferative progenitor cells during brain development, which migrates to the cortex or the basal ganglia, where they differentiate into neurons. SVZ preserves its critical developmental characteristics in the adult brain, responsible for the continuous generation of migrating neuroblasts destined for the olfactory bulb or other areas of cell death in the brain. Thus, the maintenance of SVZ is crucial for neuron replacement along adulthood [22–24].

Supporting the involvement of SVZ with the physiopathology of HD, several studies revealed that the SVZ of HD patients is enriched in endogenous factors and receptors that actively regulate the cell cycle and the differentiation of precursors, such as the neuropeptide Y. Furthermore, studies already showed a significant increase of GABAA, receptor subunit γ2 (involved in the desensitization of the receptor complex to GABA) in SVZ in HD. GABA is an essential trophic factor for neurons during development. High levels of GABA are found in the normal SVZ and the SVZ of HD patients, suggesting that the SVZ maintains a germinal capacity for proliferation and neurogenesis in response to neurodegenerative cell death in adult life. However, it was proved that, while the Htt protein interacts with cAMP response element-binding protein (CREB) and specificity proetin1 (Sp1), conferring anti-apoptotic action, the mHtt protein triggers a pathogenic cascade involving Sp1 transcription factor activation, which leads to repressor element-1 silencing transcription factor (REST) upregulation, repressing neuronal genes [22–24].

2. Exosomes

With the progression of HD, others brain areas, besides the substantia nigra, are subjected to neuronal loss, leading to cognitive and neuropsychiatric dysfunctions. This occurs because the mHtt (as the Htt) is widespread to different brain areas through extracellular vesicles (EVs).The EVs comprise a heterogeneous group of phospholipid bilayer-enveloped particles that are naturally produced and secreted into the extracellular environment by almost all cell types. According to their size, biogenesis, and content, these vesicles are classified as (i) microvesicles, (ii) exosomes, and (iii) apoptotic bodies. Among these vesicles, exosomes are the most investigated. This is because, due to the repertoire of bioactive molecules carried by these vesicles (coding and non-coding RNA, proteins, lipids, and metabolites), the exosomes play an important role in cell-to-cell communication and intercellular signaling, regulating both physiological and pathophysiological processes. Moreover, in the function of their nanosize (30–200 nm), exosomes easily cross the blood-brain barrier [1, 25].

The growing interest in this class of EV has been reflected in the creation of distinct databases that compile data on exosome content, such as Exocarta (http://www.exocarta.org/), EVpedia (http://bigd.big.ac.cn/databasecommons/database/

id/4354) and Vesiclepedia (http://microvesicles.org/), which are constantly updated with released studies.

Exosomes are formed by endocytosis and released by exocytosis. During the biogenesis of these vesicles, the inward budding of the plasma membrane results in small intracellular vesicles. These small vesicles fuse, forming early endosomes. The invagination of the early endosome membrane results in the formation of intraluminal vesicles (ILVs) within large multivesicular bodies (MVBs). In contrast, cytoplasmic molecules such as coding and non-coding RNA, proteins, lipids, and metabolites are engulfed and enclosed into the ILV lumen. Along with the maturation of early endosomes to late endosomes, some proteins are directly integrated into the invaginating membrane. However, this process depends on the endosomal sorting complexes required for transport (ESCRTs), which are comprised of four proteins (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) that work cooperatively to facilitate the MVB formation, vesicle budding, and protein cargo sorting [1, 25]. The exosomes biogenesis also occurs through an ESCRT-independent pathway mediated by tetraspanins and ceramide-enriched lipid rafts. Tetraspanins are recruited at early steps to endosome membranes before ILV formation, and at least CD9, CD63, CD81, and CD82 are found in endosome and exosome membranes [1, 25].

Ceramides and their derived metabolites are organized in raft-based microdomains that interact with proteins, such as flotillins. The lipid-enriched structures are involved not only in endosomal membrane invagination for ILV formation but also in cargo loading. The selective cargo loading occurs during exosome biogenesis through tetraspanins-dependent and/or ESCRT-dependent mechanisms [1, 25]. Although, the biological cargo of exosomes varies widely according to their cell type of origin, they mainly consist of proteins, nucleic acids (particularly RNA) and lipids. More than 2400 different RNAs and, 4000 proteins were already identified and characterized in exosomes [1, 25].

Due to their endosomal origin, the exosomes are enriched in several proteins engaged in the biogenesis of MVBs, including clathrin, which can bind to hunting protein. Moreover, exosomes contain CD9, CD63, CD81, CD82, CD54, and CD11b tetraspanins, which serve as specific molecules. In addition, the exosomes contain heat shock proteins (HSP90, HSP70, and HSP60), which act as chaperones and play an essential role in cellular responses related to environmental stress. Besides this, exosomes also carry mRNA and a multitude of long non-coding (lnc) RNA and small RNA (particularly miRNA) that can be transferred into recipient cells, inducing cellular responses [1, 25–27].

The interaction of MVBs with actin and microtubules is essential for their transport to the plasma membrane. The translocation of MVB toward the plasma membrane depends on several molecules via the cytoskeleton. Rab GTPases such as RAB11, RAB27A/B, and RAB35 are mediators of selective sorting of MVB to the plasma membrane and exosome release. The MVBs are decorated with tethering protein complexes, such as HOPS and SNAREs, that mediate the fusion of these vesicles with the plasma membrane, The presence of tetraspanins and lysosomal-associated membrane proteins LAMP1 and LAMP2 in late endosomes also facilitate the fusion of MVB with the plasma membrane [1, 25].

After secretion, the exosomes will dock into the membrane of the target cells and activate signaling events or be internalized through specific receptor-ligand interactions. The transmembrane proteins present in the surface of exosomes (tetraspanins) can be recognized by signaling receptors in the target cells, resulting in activation of transduction pathways and modulation of the intracellular process without entering the target cells. Exosomes can merge with the target cells' plasma membrane, releasing its

cargo directly into the cytosol by a low pH-dependent mechanism. However, the main route for exosome uptake can occur by clathrin-mediated or caveolin-dependent endocytosis, and the presence of lipid rafts in the membrane facilitates the process [1, 25].

After internalization, exosomes are sorted into MVB with two possible fates: (i) to be released again to neighboring cells or (ii) to be degraded after fusion of LE/MVB with lysosomes [1, 25]. The uptake of exosomes by brain cells seems to be cell type-dependent. For instance, neurons and glial cells seem to uptake exosomes by clathrin-mediated endocytosis. Some neurons can also use specific receptors from the SNARE family, such as SNAP25, for exosome uptake. Interestingly, the uptake of exosomes seems to be a selective pathway. Exosomes derived from cortical neurons were primarily internalized by hippocampal neurons, whereas astrocytes and oligodendrocytes took up exosomes released by neuroblastoma cell line N2A. Exosomes derived from oligodendrocytes are mainly internalized by microglia but not by neurons or astrocytes. In addition, the uptake of exosomes was also more active in pre-synaptic regions, which might indicate that these vesicles use constitutive endocytosis processes at these regions for neuronal cell entrance [1, 25].

2.1 Exosomes as a key mediator of HD pathophysiology

Initially, exosomes were considered vehicles for the elimination of cellular components. However, current studies have provided evidence that exosomes play multiple physiological roles in the nervous system. Exosomes are released by neural cells, including neurons, astrocytes, microglia, and oligodendrocytes, playing essential physiological roles in neurogenesis, synaptic activity and plasticity, myelination, and protection and regeneration neurons after injury and disease. Thus, it is not surprising that exosomes mediate the pathogenesis of neurodegenerative disorders, such as HD. This is because the misfolded proteins related to these disorders can be selectively integrated into ILVs of MVBs, and subsequently released into the extracellular environment within exosomes [28].

In HD, cumulative evidence has demonstrated that exosomes are implicated in the physiopathology of HD, serving as a vehicle for the expanded polyglutamine tract of HTT RNA and protein (mHtt), as well as mHtt aggregates transport to different brain areas. Supporting this evidence, it was verified that exosomes could deliver expanded trinucleotide repeat RNAs among cells and facilitate the propagation of mHtt protein [29–32]. It was shown that the injection of fibroblast-derived exosomes from an HD patient into a newborn mouse brain ventricles triggered the manifestation of HD-related behavior and pathology [31]. Moreover, it is known that the Htt protein regulates anterograde and retrograde transport of endocytic vesicles by interacting with several mediators, such as α-adaptin, Hip1, Hip14, HAP1, HAP40, SH3GL3, clathrin, and dynamin [29, 30]. This process is coordinated by the phosphorylation of Htt, which serves as a molecular decision marker for the anterograde or retrograde direction of vesicle transport. Thus, while the Htt promotes axonal BDNF vesicle trafficking, mHtt interacts with HIP1 and dynactin, leading to de-railing of molecular motors from microtubules tracks and cessation of transport [33].

3. Animal models for Huntington's disease

Animal models for HD have been successfully used for more than three decades to identify pathways involved in HD pathology or for preclinical testing

of therapeutic strategies. These models are divided into (i) monogenetic and (ii) genetic murine models. However, none of these models can mimic the main feature of HD since no rodent model develops the chorea. For this reason, herein, we summarize the pros and cons of each animal model, considering their utility for preclinical test purposes [34–36].

3.1 Monogenetic models

Historically, monogenetic models have dominated the field of HD disease. These models are based on the use of toxins that typically induce cell death either by excitotoxic mechanism or by disruption of mitochondrial machinery. Among the excitotoxicity toxins used to obtain murine models for HD are quinolinic acid (QA) and kainic acid (KA). These neurotoxins induce cell death by binding to their cognate receptors, N-methyl-D-aspartic acid (NMDA) and non-NMDA, respectively, on striatal neurons. The QA or KA rat models exhibits motors (hyperkinesia, apomorphine-induced dystonia, and dyskinesia) and cognitive symptoms of HD (visuospatial deficits, procedural memory deficits, and poor memory recall). However, for various reasons, QA became the preferred excitotoxin for use in HD studies. The QA is formed from the metabolism of tryptophan via the kynurenine pathway, which is found in high quantities in the urine of rats that received a diet high in tryptophan. Interestingly, the tryptophan crosses the blood-brain barrier (BBB) using transporters shared by other neutral amino acids. In the brain, tryptophan is taken up by astrocytes, macrophages, microglia, and dendric cells and converted into kynurenine. In the presence of the enzymatic 3-hydroxyanthranilic acid oxygenase, a series of enzymatic reactions converts kynurenine to QA. Thus, the expected level of QA does not cause damage, but only small increases in QA levels cause toxicity. Moreover, it was verified that the administration of QA in the mouse models promotes the upregulation of Htt protein, linking the levels of this neurotoxin with HD pathogenesis. However, the QA is incapable of crossing the BBB. For this reason, the QA has been administrated directly within the brain [37–39].

Unlike the QA, the mitochondrial toxin 3-nitropropionic acid (3-NP) crosses the BBB and could be systemically administrated through intraperitoneal or subcutaneous injection [40–42].

The 3-NP is a plant (Indigofera endecapylla) and fungal (Aspergillus flavus, Astragalus, Arthrinium) toxin, which acts as an irreversible inhibitor of succinate dehydrogenase. It inhibits both the Krebs cycle and the mitochondrial complex II of the electron transport chain. The toxin also induces caspase-9 activation, which in turn requires the simultaneous presence of Apaf-1, cytochrome c, and ATP, suggesting that neuronal death may occur in the presence of intense ATP depletion. Moreover, the 3-NP induces oxidative and nitrate stress due to excessive ROS/RNS production and lack of the antioxidant system [40, 43–45]. Interesting, numerous studies demonstrated that the chronic systemic administration of 3-NP in rats impairs energy metabolism and results in striatal lesions, inducing a spectrum of HD-like pathology in rat striatum. In addition, in 1993, Beal et al. [41] showed that the 3-NP model causes selective striatal lesions characterized by the loss of medium spiny neurons (MSNs) and astroglial proliferation, replicating the histological and neurochemical features of HD. Although, the loss of MSNs in 3-NP rat models causes motor and cognitive symptoms analogous to those verified in HD, this model does not exhibit chorea.

However, the 3-NP model is capable of mimicking both hyperkinetic and hypokinetic symptoms of HD depending on the time course of administration. Thus, while

the administration of 3-NP in two individual doses causes hyperkinetic movements analogous to those observed in early to mid-stage HD, the administration of more than four injections of 3-NP causes hypokinetic movements similar to those that appear in late-stage HD [40, 43–45]. Nevertheless, the response to the 3-NP changes according to the murine (CD1, C57BL/6, BALB/c, Sebster/Swiss and 129sEMS) or rat strain (Fischer, Lewis, and Wistar). In this sense, it is recognized that rats are most vulnerable to the toxic action of 3-NP treatment than mice. Fisher rats are the most susceptible to the 3-NP toxin but display significant variability in response to the toxin due to the difficulty of controlling damage caused by this toxin. In contrast, Lewis rats are less susceptible to 3-NP but respond more stably and consistently to 3-NP in behavioral alterations and lesions. Wistar and Sprague-Dawley rats are also sensitive to the 3-NP, developing lesions and behavioral modifications of extraordinary value for studying possible routes involved in HD and testing new therapeutic strategies. Although, the 3-NP model leads to a (i) massive cell death induced by the toxin, (ii) serving as a helpful model for (ii) analyzing and studying neuroprotective and (iii) neurorestorative therapies for HD patients, (iv) allowing to study the mechanisms involved in HD pathogenesis, including energy deregulations and ROS production, this model does not express the mHtt protein.

3.2 Genetic models

The genetic or transgenic animal models emerge as an alternative to nongenetic models since they express the mHtt protein [46, 47]. Transgenic models are divided into (i) those expressing transgenes with a truncated section of human HTT carrying the CAG repeats or full-length human HTT gene, and (ii) those with long CAG repeats replacing mouse Htt. Instability of the CAG repeat has been observed in many of the mouse models and was noted in the first HD model (R6 series). Although, different rodent models have been used to understand the biology of HD or employed in preclinical trials to investigate the therapeutic potential of products candidates to alleviate HD symptoms, they are limited in their ability to provide evidence of the effects of genetic modifiers of disease. In addition, there are many differences among the transgenic rodent models that can lead to different results, especially for preclinical trials.

In this sense, in two independent studies, it was demonstrated that a version of the R6/2 mouse with 90 CAG repeats (R6/2(CAG)90) shows earlier mHtt nuclear aggregation when compared to the R6/2 mouse with 200 CAG repeats (R6/2(CAG)200). Moreover, the R6/2(CAG)90 brains contain nuclear aggregates with a diffuse punctate appearance which remained partly detergent soluble, which correlated with the onset of transcriptional changes. In contrast, the R6/2(CAG)200 brains contain cytoplasmic aggregates that gave larger inclusion bodies related to behavioral changes. These data indicate that CAG length gives different phenotypes [48–50].

Several models encoding glutamine but using a mixed CAACAG rather than a pure CAG tract were developed to prevent germline and somatic expansion of CAG trinucleotide. An example of these models is the BACHD models with 97 glutamines encoded by a diverse CAACAG tract. These mice have five copies of the transgene integrated into their genome and express BACHD HTT, an estimated three-fold level of the transcript, and 1.5 to 2-fold protein level (mHtt).

BACHD rats show string impairment in muscle endurance at 2 months of age. Altered circadian rhythmic and locomotor activity are also observed in these animals

[51–53]. However, the BACHD model is not commercially available, difficult to access this model.

4. Therapeutic cells: perspectives of HD treatment

When discovered, stem cells—therapeutic cells gain exceptional attention due to their capacity to produce precursors and differentiated cells. Propose, therefore, was to use stem cells in tissue regeneration [26, 27]. Stem cells showed differentiation potential *in vitro* and *in vivo* (animal models). Thus, we know two principal types of stem cells: adult and pluripotent. Pluripotent cells are embryonic stem cells or induced pluripotent stem cells, which are adult stem cells reversed in stem cells similar to embryonic [28]. However, this chapter will focus on adult stem cells isolated from different tissues like bone marrow, adipose tissue, umbilical cord, and dental pulp. Adult stem cells, especially mesenchymal stem cells (MSC), differ from their pluripotent counterparts, and being more mature, they cannot differentiate *in vivo* into appropriate tissue. However, these cells present specific characteristics that are of great interest in treating neurodegenerative diseases.

MSC secretes a large number of biologically active molecules, growth factors, hormones, interleukins, etc. [29]. These biomolecules can be found in free form or contained in exosomes, which are recognized as a key component in paracrine regulation [1, 25]. These molecules provide beneficial effects on injured tissues. For example, they induce angiogenesis and tissue regeneration and inhibit fibrosis, apoptosis, and inflammation [30–32]. In addition, which is essential for HD disease, MSCs and MSC's secretomes provide neurogenic, neuroprotective, and synaptogenic effects [33]. They improve the abnormal dopamine transmission and inflammatory reaction in the transgenic HD model [34]. Animal models showed that they produce factors protecting retinal ganglion cells against glutamate excitotoxicity, neurotrophins expressed by MSCs inhibit pro-inflammatory cytokine secretion, MSCs fight oxidative stress and others [35, 36]. Due to the characteristics above, MSCs called medicinal signaling cells or simply therapeutic cells [37].

4.1 Therapeutic cells

Medicinal signaling cells (MSC) have been used in a variety of preclinical studies, which were focused on behavioral and memory outcomes, reduction of brain damage and minimization of striatal degeneration. "Native" MSC isolated from different adult tissues such as bone marrow, adipose tissue and umbilical cord were used in these studies. Due to their ability to adhere to plastic, MSC can be easily isolated and expanded *in vitro* [38]. They are isolated and cultured using similar protocols and culture medium reagents. However, different research groups usually introduce a few modifications in the protocol, which may affect the MSC quality and efficiency. MSC can differentiate *in vitro*, especially into mesoderm derivatives, but not *in vivo*. After isolation, these cells express similar markers and share similar morphological features. However, different MSC populations can be isolated from the same or different tissues. These populations differ in self-renewal, plasticity and therapeutic potential [29, 39, 40]. To standardize the concept of MSC used in different studies and by various scientific groups the International Society for Gene and Cell Therapy (ISGCT) established the minimum criteria for defining these cells and populations isolated

[41]. Despite MSC populations' heterogeneity, these cells share regenerative, anti-apoptotic, anti-fibrotic anti-inflammatory, immunosuppressive, immunomodulatory, and angiogenic properties [42].

4.2 Therapeutic cells in preclinical HD studies

Stem cell-based therapies are important to reconstruct damaged brain areas in HD patients. These therapies have a dual role: stem cell paracrine action, stimulating local cell survival, and brain tissue regeneration through the production of new neurons from the intrinsic and likely from donor stem cells. Initially, preclinical studies were mainly focused on the neuroprotective function of MSC. Since these cells express a variety of neurotrophins and in particular brain-derived neurotrophic factor (BDNF), which is implicated in the survival of striatal neurons. BDNF expression is reduced in Huntington's disease (HD) contributing to striatal neurodegeneration.

Initially, preclinical studies mainly discussed the neuroprotective function of MSC. Since these cells express BDNF, which is implicated in the survival of striatal neurons, its expression is reduced in Huntington's disease (HD) contributing to striatal neurodegeneration. The regenerative approaches of MSC potentially can cause the (i) promotion of endogenous neuronal growth; (ii) amelioration of the synaptic connection from damaged neurons; (iii) decrease of apoptosis of endogenous neurons; (iv) reduction of the levels of free radicals; (v) immunomodulation. Our group widely discussed these MSC functions in animal models in previous publications (**Figure 1**) [43, 44].

Furthermore, MSC can transfer larger molecules and even organelles, therefore, their use as delivery vehicles for therapeutic RNA inhibition was suggested [45]. MSC can transfer larger molecules and even organelles; therefore, suggesting their use as delivery vehicles for therapeutic RNA inhibition [45]. *In vitro* model systems showed that MSC can transfer RNAi targeting both reporter genes and mutant huntingtin in neural cell lines. Thus, the flow cytometry assay demonstrated that MSC expressing shRNA antisense to GFP decreases expression of GFP when co-cultured with SH-SY5Y cells. Furthermore, these cells, which express shRNA antisense to HTT, decreased the levels of mutant HTT expressed in both U87 and SH-SY5Y target cells

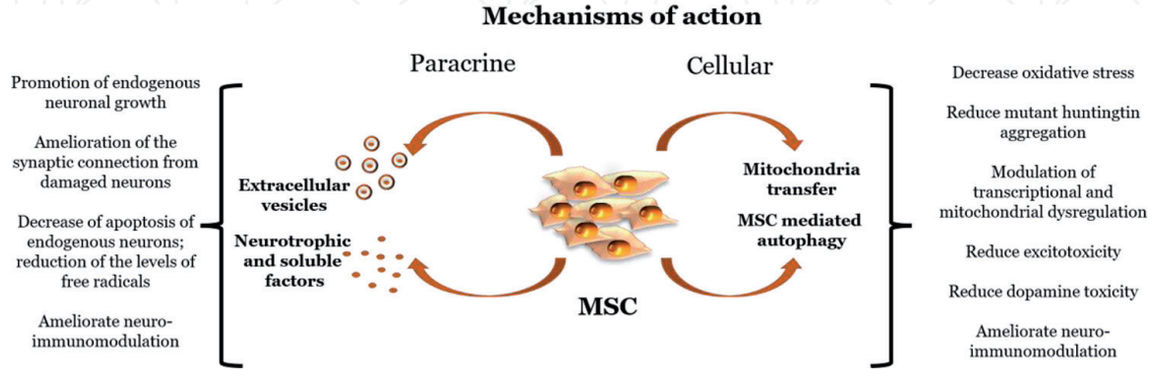

Figure 1.
Schematic representation of the paracrine and cellular mechanism of MSC action observed in pre-clinical studies in Huntington and other degenerative diseases. Paracrine action mainly includes neurotrophins and other soluble factors, including growth factors, small molecules, and cytokines, providing signals to cells and resulting in different cell actions such as survival, proliferation, and differentiation. The paracrine factors are secreted directly into the intercellular matrix or included in extracellular vesicles (exosomes) before secretion. The cellular effect includes mitochondria transfer and MSC mediated autophagy. MSC acting by paracrine and cellular mechanisms showed significant therapeutic potential.

as analyzed by Western blot and densitometry. These results are encouraging for expanding the therapeutic abilities of both RNAi and MSC for future treatments of Huntington's disease [46–48].

In addition, more recent findings suggest the potential therapeutic effect of MSC on different pathophysiological aspects of HD, such as (i) mitochondrial dysfunction; (ii) transcriptional dysregulation [49, 50]; (iii) altered axonal transport of critical factors [51, 52]; (iv) disrupted calcium signaling [53, 54]; (v) abnormal protein interactions [55]; (vi) impaired autophagy [56, 57]. However, here we will focus our review on HD mitochondrial dysfunction and MSC mitochondria transfer.

Figure 1 combines the well-known paracrine mechanism of MSC action and a novel cellular mechanism mediated by mitochondria transfer and autophagy. Both, paracrine and cellular, mechanisms provide clinical, cellular and molecular benefits in HD [43, 44, 49–59]. The complex mechanisms of MSC action and her multi-target orientation are the unique biological tool that could act on multiple pathophysiological aspects of HD cited above.

4.2.1 MSC and mitochondrial dysfunction in HD

Mitochondria roles in neurons differ from only a cell power source. Mitochondria are also dynamic organelles that fragment and fuse to achieve a maximal bioenergetics action. They are transported along microtubules, regulated intracellular calcium homeostasis through the interaction with the endoplasmic reticulum. In addition, they produce free radicals and participate in cell apoptosis [60]. These activities have been demonstrated to be changed in HD, potentially contributing to neuronal dysfunction in early pre-symptomatic HD phases. Thus, a polyglutamine-expansion disorder that primarily affects the striatum and the cerebral cortex has been described as mitochondrial dysfunction, an early pathological mechanism presenting selective HD neurodegeneration [61, 62]. One of the hallmarks of HD is an altered mitochondrial morphology that can be seen in different cell types and neurons, which are characterized by increased mitochondrial fragmentation [63]. The cells with altered mitochondrial morphology in HD cells showed a decrease in electron transport chain activity, oxygen consumption, Ca^{2+} buffering, and decreased ATP and NAD^{+} production [64]. It has been suggested that mitochondrial abnormalities can significantly affect MSNs due to the high-energy demand of this neuronal subtype [65]. Therefore, the mitochondria are a central regulatory organelle in HD-affected neurons.

In addition, mitochondria act as a reservoir for pro-apoptotic factors, thus regulating cell death. The mitochondrial permeability transition pore (mPTP) is opened due to mitochondrial dysfunction, Ca^{2+} overload, and accumulation of reactive oxygen species (ROS). The transition pore opening initiates the intrinsic apoptotic pathway, which is connected with the mitochondrial outer membrane permeabilization, awakening cytochrome c release, and activation of caspase-3 [66, 67]. Bcl-2 inhibits the activation of proapoptotic factors such as Bcl-2-associated X protein (Bax) and Bcl-2-associated K protein (Bak), thus suppressing the release of cytochrome c from mitochondria. The Bax/Bcl-2 ratio imbalance often occurs during the process of apoptosis [68]. MSC mitochondrial transfer through regulation of the balance of Bax/Bcl-2 and reduction of the expression of caspase-3 can reduce apoptosis levels and promote cell viability in recipient cells [69, 70].

Recent studies have demonstrated that MSCs have the potential to transfer the defective mitochondria between MSCs and aging cells [71]. For the first time, the MSC mitochondria transfer was shown in A549 cells with mtDNA deletions after

their co-culture with human MSCs. This work demonstrated the recovery of function by mitochondrial activities such as increased oxygen consumption, membrane potential, and intracellular ATP levels [72].

It is worth mentioning that the transfer of dysfunctional mitochondria from damaged cells to MSC also can occur. Gozzelino et al. showed that mitochondria released from damaged cardiomyocytes or endothelial cells could be "swallowed" by MSCs. This event rigger increases the expression of Heme oxygenase-1 (HO-1), a protein that protects against programmed cell death, and increases mitochondria in MSCs, which in turn induces an adaptive reparative response [73, 74].

Fluorescence microscopy studies revealed MSC mitochondria transfer in astrocytes and neuron-like pheochromocytoma cells. MSC mitochondria transfer to astrocytes was more efficient when the astrocytes were subjected to ischemic damage associated with elevated ROS levels. The ROS accumulation in normal aging or disease leads to increasing the rate of mitophagy and decreasing the level of mitochondrial biogenesis, which reduces mitochondrial mass [75]. Such mitochondria transport re-established the bioenergetics of the recipient cells and stimulated their proliferation. Furthermore, the authors showed that MSCs mitochondria transferability may be enhanced by upregulation of Miro1 (adaptor protein participating in mitochondria moving along microtubules [76] therefore, this study showed that mitochondrial impairment in differentiated cells can be restored after MSC healthy mitochondria transfer and this approach may serve as a promising treatment for neurological diseases [77].

4.2.2 MSC mitochondria transfer and inflammation

Tissue injury or degeneration is usually followed by inflammation, which is a driving force for mitochondrial transfer. In HD, massive neuroinflammation in the striatum and caudate nucleus are already present before patients develop any symptoms [21, 78, 79]. The therapeutic effects of MSC are mediated mainly by its secretome/exosomes since in response to a combination of molecules present in the inflamed microenvironment, these cells undergo a process activation or "licensing," acquiring an anti-inflammatory phenotype and producing large amounts of immunomodulation factors, growth factors and specific chemoattractants, being able to modulate significantly innate and adaptive immune cells [38, 80].

The MSCs secreted cytokines that immunomodulate various immune cells, such as T cells, B cells, natural killer cells, and macrophages [81]. Recent studies demonstrated that between MSCs and immune cells MSC mitochondrial transfer can occur, such influencing the functions of the immune cells. Jackson et al. showed MSC mitochondria transfer occurs in an acute respiratory distress syndrome (ARDS) model. MSC provides mitochondria to host macrophages, thus enhancing the phagocytic capacity and bioenergetics of macrophages. This MSC mitochondria transfer leads to improved clearance of pathogenic bacteria [82]. Using the same model Morrison et al. showed that MSC exosomes mediated transfer of mitochondria, which can induce monocyte-derived macrophages to differentiate to an M2 phenotype with a high phagocytic capacity [58]. In addition, MSC mitochondria transfer regulates T cell differentiation. Some authors reported that when healthy donor-derived bone marrow-derived MSC (BMMSC) is cocultured with primary Th17 effector cells, the mitochondrial transfer occurs, increasing respiration in recipient Th17 cells [59].

HD demonstrates typical cellular and molecular features of inflammation, such as cytokine expression and microglia activation. However, no immune cell infiltration from the bloodstream was observed [83, 84]. Nevertheless, HD is characterized by a

chronic increase of systemic pro-inflammatory cytokine production. Microglia and astrocytes are non-neuronal cells in the brain that participate in tissue homeostasis and support neuronal function. Under pathologic conditions, these cells become 'activated.' They start to produce numerous mediators promoting inflammation. These cells change their morphology and, can divide, thus increasing cell numbers, an event named 'gliosis.' Recent studies suggest that cell-autonomous pro-inflammatory activation of microglia occurs due to the expression of mutant HTT, thus contributing to the progression of HD pathogenesis [21].

MSC's metabolic state is characterized by the balance between the intrinsic necessities of the cell and limitations imposed by extrinsic conditions. Under pathogenic conditions or inflammation, MSCs respond to reactive oxygen species (ROS), damage-associated molecular patterns (DAMPs), damaged mitochondria, and mitochondrial products, thus transferring their mitochondria to damaged cells. MSC therapies can protect the potentially damaged cells by regulating cellular metabolism in injured tissues, modulating ROS and endogenous MSCs.

Furthermore, to treat such complex diseases like Huntington's, we should develop new complex therapies acting on multiple targets. MSC, due to the wide range de therapeutic molecules they produced and the different mechanisms they used to fight the disease, these cells are a good candidate for the new class of such therapeutics.

5. Cell-free therapy: novel perspectives for the treatment of HD

For a long, it was considered that the therapeutic effects of the stem cells were associated with the replacement of dead cells [73, 74]. However, in a model of kidney injury caused by the injection of toxic doses of glycerol, it was verified that transplanted stem cells remain in the injury site for up few days and, subsequently, are not found in the tissue [73, 75, 76]. These data provide evidence that the therapeutic potential of MSCs is mediated by trophic factors naturally produced and secreted by these cells in an accessible form or into EVs [1]. However, whereas the bioactive molecules present in the extracellular medium are subjected to rapid hydrolysis and oxidative effects, the biomolecules present in EVs are more stable [73]. For this reason, the EVs (particularly exosomes) have been biotechnology explored in a novel therapeutic approach known as cell-free therapy [26, 77, 78].

Cell-free therapy possesses different advantages when compared with cell-based treatment. Among these advantages are: [1] EVs can be easily prepared, stored for a relatively long period without any toxic cry preservative such as dimethylsulphoxide (DMSO) and transported; [2] therapeutic application of exosomes have been demonstrated to be well tolerated; [3] the use of EVs instead of whole cells avoids possible complications associated with pulmonary embolism after intravenous infusion of MSCs; [4] avoids the risk of unlimited cell growth and tumor formation since EVs are not dividing; [5] exosomes from MSCs, and epithelial cells do not induce toxicity when repeatedly injected; [6] EV may be isolated from unmodified or genetically modified human stem cells; [7] evaluation of culture medium for safety and efficacy is much simpler and analogous to conventional pharmaceutical agents [1, 73, 79–82]. Further, the cell-free therapy allows biotechnologically exploring the use of the culture medium, which is generally discarded as a byproduct of the in vitro expansion of MSCs. This is because this culture medium—also termed conditioned medium (CM) [79]—is an essential source of bioactive molecules, which can find in an accessible form or an extracellular vesicle (EVs) [1].

5.1 Perspectives and challenges in cell-free therapy

Although, different strategies have been successfully used to isolate exosomes, they represent the main obstacle to the therapeutic application of EV since these procedures are time-consuming and generally provide few quantities of EVs [1, 73]. However, novel methodologies have been proposed to solve these problems. Based on this, we aimed to summarize the pros and cons of each available method for isolating exosomes.

Ultracentrifugation (UC) and commercial kit rooted in polymer-based precipitation are the most well-established and standard methods used for isolating exosomes [74], being adopted as a strategy in about 81% of researches [78]. Ultracentrifugation-based methods can be divided into two major types of techniques according to the separation mechanism: (i) differential ultracentrifugation and (ii) density gradient ultracentrifugation [78]. For both methods, death cells, cellular debris, and large EVs (>200 nm) are separated using low centrifugal forces (300–2000 × *g*) for 10–30 min at room temperature, as verified in the most protocol, as already revised by us [1]. An additional filtration step using a 0.22–0.45 μm-membrane filter can increase the exosome purity. In differential ultracentrifugation, the particles are separated using a serial of differential centrifugal forces (100,000–120,000 × *g*) and time (70 min to 12 h). At the end of the process, the pellet of exosomes is washed with phosphate saline buffer (PBS) or 0.9% NaCl solution to remove remaining proteins co-isolated with the EVs. Differential ultracentrifugation provides pure EVs for both scientific and clinical purposes. However, the majorities of UC-based proposed methods are laborious, time-consuming, and unsuitable for mass-scale EV production, making it difficult for therapeutics [1]. In density gradient, ultracentrifugation (DGUC) is employed as a sucrose density gradient, which reduces the destructive effects of centrifugal force on exosomes [58, 78]. According to the exosome buoyant density in aqueous sucrose (1.10–1.20 g/mL), the exosomes can be easily isolated [59, 78]. Although, this method provides the highest efficiency for exosome purification, its suitability for clinical purposes is questionable due to the difficulty in upscaling and automating the process [83, 84]. Moreover, the wash step is mandatory for this method to remove eventual residues of CsCl or sucrose used to obtain the gradient density.

Another strategy commonly employed to isolate exosomes is coprecipitation. This method uses polymers, such as polyethylene glycol (PEG) 6000 or 8000, which can coprecipitate with hydrophobic proteins and lipid molecules present in exosome membranes [78]. Although, most simple and less expensive than the methods based on ultracentrifugation, the isolation using coprecipitation is not scalable, limiting its use for therapeutic purposes. Moreover, this technique requires sample incubation with the polymers for a long time (generally 12–16 h) [1].

The differential expression of specific surface biomarkers, such as CD9, CD63, and CD83, also provides an excellent opportunity to develop immunoaffinity-capture-based techniques for exosomes isolation using modified magnetic beads or microchannels surfaces with specific antibodies [1, 78]. Although, this technique allows isolating only exosomes, it works with few volumes, limiting its use for therapeutic purposes, which require scalable methods. Moreover, this method generally requires a pre-enrichment step, which is commonly performed using commercial kits based on coprecipitation, resulting in PEG contamination [1].

Another strategy used to isolate exosomes is the size-based isolation technique. This technique can be based on sequential filtration, size-exclusion chromatography (SEC), and size-dependent microfluids. These methods allow isolating the EVs

according to their size [78]. EVs are separated using membrane filters with different size or molecular weight exclusion limits in sequential filtration. First, the CM is filtered using a 0.22 μm membrane filter. Then, proteins with a 500 kDa molecular weight are purified using a dialysis bag. Finally, the samples are filtered with a 100 nm membrane filter [78]. The SEC is based on particle size filtration through a porous stationary phase composed of spherical gel beads with pores of specific size [78]. Large particles are eluted when the sample passes through the stationary phase, whereas small particles pass through the pores [78]. The size-dependent microfluidics uses a viscoelastic microfluidics device, composed of a microchannel, two inlets, and three inlets, to fractionate exosomes from other types of EVs [78]. These techniques are faster than those based on ultracentrifugation and do not require special equipment. Moreover, they avoid PEG contamination, frequently observed in coprecipitation-based methods. However, the size-based isolation techniques are relatively expensive and cannot separate exosomes from other EVs, requiring additional steps for exosome purification [1].

5.2 Cell-free therapy for the treatment of HD

Due to their ability to cross the blood-brain barrier and biocompatibility, exosomes are promising therapeutic drug carriers into the CNS. In HD, exosomes are exceptionally efficient in delivering specific microRNAs (miRs), short non-coding RNAs of about 22 nucleotides that regulate gene expression by suppressing the translation of mRNA, which are found deregulated in HD patients.

In this sense, several miRs had already been identified as deregulated in HD, including the miR-124, which was found downregulated in HD patients [85]. The decreased expression of miR-124 increases the levels of its target gene (REST), which acts as a repressor of BDNF [85]. By contrast, the expression of miR-124 induces adult neurogenesis in the subventricular zone (SVZ) and regulates the cell cycle in striatal neurons. Considering that the HD striatum exhibits decreased neurogenesis, which leads to brain atrophy, it was hypothesized that the delivery of miR-124 may be a feasible way to induce neural regeneration. However, naked miRs are vulnerable to degradation.

In this regard, exosomes emerge as candidates for the miR-124 delivery to recipient cells. Based on this, Lee et al. [85] injected exosomes derived from HEK 293 cells overexpressing miR-124 within (Exo-124) the striatum of 6-week-old R6/2 transgenic mice. Using ex vivo imaging, the authors demonstrated the presence and maintenance of the exosomes within the striatum even after one week later the Exo-124 administration. Furthermore, it was verified that Exo-124-treated R6/2 mice exhibited slightly higher levels of miR-124 when compared to the non-treated mice (control). However, no statistically significant differences between the treated and control mice were reported. By contrast, the Exo-124-treated R6/2 mice exhibited lower levels of REST protein concerning the control. Although, the study had provided a proof of concept for exosome-based delivery of miRNAs to the brain, the therapeutic efficacy of Exo-124 was modest, suggesting the need to increase the dose of miRNAs packed in the exosomes or to combine this miRNA with other candidate miRNAs such as miR-9, miR-22, miR-125b, miR-146a, miR-150, and miR-214.

In this sense, the exosomes derived from MSCs can be considered an important source of these miRs and other mRNAs and proteins deregulated in HD physiopathology. Supporting this, Lee et al. [86] showed that exosomes derived from an adipose-derived stem cell (ASC-exo) decreased mHtt aggregates in R6/2 mice-derived neuronal cells through the upregulation of PGC-1, phospho-CREB.

6. Conclusion

In this review, we demonstrated that Huntington's disease is devastating and affects brain cells and the organism as a whole. Although, the main cause of HD patients' death is medium spiny neurons, many specific events occur at presymptomatic and symptomatic HD phases. Currently, Huntington's chorea is in the focus of pharmaceutical companies, producing drugs able to combat this HD symptom. However, these drugs are always not possible to delay the disease and present moderate to severe side effects.

In contrast to conventional drugs, MSC is safe, and they did not present any side effects as shown in multiple clinical trials. MSC showed therapeutic potential distinct from, for example, small molecules and biologics. Cells are deposited multiple drugs, they can sense diverse signals, migrate to specific sites in the body, make decisions, and carry out complex responses inside one specific tissue environment.

Our knowledge about the biology and therapeutic potential of these cells is still minimal; however, as demonstrated by scientific literature, these cells and their derivatives as exosomes and mitochondria have tremendous therapeutic potential. Pre-clinical studies provided evidence about the paracrine effect of these cells' such as regenerative, anti-apoptotic, anti-fibrotic anti-inflammatory, immunosuppressive, immunomodulatory, and angiogenic.

More recently, the potential effect of MSC against different pathophysiological aspects of HD, such as mitochondrial dysfunction; transcriptional dysregulation; altered axonal transport of critical factors; disrupted calcium signaling; abnormal protein interactions, and impaired autophagy, has been demonstrated.

This review tries to provide insight into cellular and cell-free technologies from the exact cellular origin. These cell and cell-free products may share similar features and present specific characteristics, as demonstrated for MSC, exosomes, and mitochondria. We tried to clarify that these products aim at different cellular targets or molecular pathways involved in Huntington's disease. Therefore, we should study how to use these new therapeutics, which can delay or even stop neurodegenerative devasting diseases.

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Author details

Irina Kerkis[1*], Rodrigo Pinheiro Araldi[2], Cristiane Valverde Wenceslau[3] and Thais Biude Mendes[1]

1 Genetics Laboratory, Butantan Institute, São Paulo, Brazil

2 Federal University of São Paulo, Cellavita Pesquisas Científicas, São Paulo, Brazil

3 Cellavita Pesquisas Científicas, Valinhos, SP, Brazil

*Address all correspondence to: irina.kerkis@butantan.gov.br

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