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Free and hydrogel encapsulated exosome-based therapies in regenerative medicine

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ABSTRACT

Over the last few decades, mesenchymal stem cells-derived exosomes (MSCs-Ex) have attracted a lot of attention as a therapeutic tool in regenerative medicine. Exosomes are extracellular vehicles (EVs) that play important roles in cell-cell communication through various processes such as stress response, senescence, angiogenesis, and cell differentiation. Success in the field of regenerative medicine sparked exploration of the potential use of exosomes as key therapeutic effectors of MSCs to promote tissue regeneration. Various approaches including direct injection, intravenous injection, intraperitoneal injection, oral administration, and hydrogel-based encapsulation have been exploited to deliver exosomes to target tissues in different disease models. Despite significant advances in exosome therapy, it is unclear which approach is more effective for administering exosomes. Herein, we critically review the emerging progress in the applications of exosomes in the form of free or association with hydrogels as therapeutic agents for applications in regenerative medicine.

1. Introduction

Extracellular vehicles (EVs) are phospholipid bilayer spherical structures with substantial dynamic heterogeneity, released from almost all mammalian cells, and play pivotal roles in intercellular communications [79]. These vesicles contribute to regulation of different signaling pathways in neighboring and distantly recipient cells by delivering various biomolecules, including mRNAs, miRNAs, proteins, and lipids. Exosomes are the most well-known subclass of nano-sized EVs (40–150 nm), originated from the endosomal pathway, that has attracted growing attention in nanomedicine, regenerative medicine, pharmacology, and cancer biology due to their cell-originating nature. [1,21,119]. Many stem cells like mesenchymal stem cells (MSCs) can secret exosomes to the extracellular space [51]. MSCs are multipotent mesenchymal stromal cells, capable of self-renewal and differentiation into different cell lineages such as osteocytes, chondrocytes, adipocytes, cardiomyocytes, and endothelial cells. The therapeutic application of MSCs in regenerative medicine has captivated a lot of attention [119]. MSCs can be isolated from many adult tissues and expanded under *in vitro* conditions [15]. Engraftment and trans-differentiation into different cells are two of the most studied therapeutic applications of these cells. Numerous recent studies highlight the therapeutic effect of their paracrine signaling *in vivo*, as opposed to their cellular effect. Moreover, MSCs form and secret exosomes, which are the key therapeutic effectors in promoting tissue regeneration [112]. Lai et al. studied the beneficial effect of exosomes from MSCs (MSCs-Ex) in a mouse model of

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myocardial ischemia-reperfusion injury. They intravenously injected the exosomes in mice and noted their cardioprotective function in cardiovascular diseases [51]. Later, therapeutic application of the exosomes was expanded to other disease models. For example, exosomes derived from human induced pluripotent stem cell-derived MSCs have been shown to promote the proliferation and migration of human dermal fibroblasts and human umbilical vein endothelial cells (HUVECs) as well as promote cutaneous wound healing rate through increasing collagen synthesis and angiogenesis [117]. In the context of bone regeneration, exosomes from MSCs play an important role in the bone regeneration and show osteoinductive effects [57]. These exosomes profoundly induced cell migration and homing when they were encapsulated into polylactic acid-polyglycolic acid copolymer (PLGA) scaffolds [25]. In addition, Zhang et al. showed that exosomes derived from human MSCs supported neurogenesis and angiogenesis in a model of traumatic brain injury [121]. These studies highlight the importance of exosomes and its potential in regenerative medicine. However, a major problem with this kind of application is limitations in engraftment of the exosome in the target site.

Different routes of administration are used to deliver exosomes to the target tissue. Intravenously injection is the most widely used route for the delivery of exosomes, however, the clearance rate of this route is relatively rapid [61]. To overcome this limitation, biomaterials like hydrogels have recently been utilized to deliver a large dose of exosomes to the target tissue. Due to the lengthy healing time of regenerating tissues, it is crucial to develop novel biocompatible scaffolds as a carrier for the sustained release of exosomes and maintenance of their bioactivity at the target site. This review briefly describes the biological roles of free MSCs-Ex and hydrogel encapsulated MSCs-Ex, and their therapeutic potential in regenerative medicine.

2. Extracellular vesicles

Cells release double-layer phospholipids vesicles known as extracellular vesicles into the extracellular space, which participate in intercellular communication through the transport of biological materials including nuclide acids, proteins, and lipids [67,71]. In recent years, there has been a growing interest in the biology of EVs, due to their pivotal roles in regulation of pathological and physiological conditions. Numerous efforts have been devoted by researchers to collect information on EVs. These include: (i) International Society for Extracellular Vesicles (ISEV, www.isev.org), that is in charge of globally advancing the application of EVs in medicine by offering training, updating terminology and classification, investigating EVs functions and developing new separation and characterization techniques, along with holding annual meetings and workshops; and (ii) online databases such as Vesiclepedia (www.microvesicles.org), ExoCarta (www.exocarta.org), and EVpedia (www.evpedia.info), which collect information on exosomal cargoes for the research community. Based on their origin and size, EVs are classified into three main groups of exosomes, microvesicles, and apoptotic bodies (Table 1) [50].

2.1. Biogenesis and characters of exosomes

The most well-known class of EVs is exosomes, originate from the endosomal compartment, which is situated on the cytoplasm and has a size range between 30 and 120 nm in diameter [50]. The first evidence of cell-derived membranous particles was reported in 1977; De Broe and colleagues by analyzing human duodenal fluid found that these fluid samples contain the plasma membrane fragments, indicating the common feature of viable cells. After that, Pan, Harding, and colleagues showed that mammalian reticulocytes have the ability to produce small vesicles during differentiation process [33,69]. Concurrently, in another laboratory Trams and co-worker using electron microscopy showed that neoplastic cell lines released different vesicles posing 500 to 1000 nm in diameter and often a population of vesicles about 40 nm in diameter. They concluded that exfoliated membrane vesicles may have a physiologic function; which may be known as exosomes [100]. After that, the term exosomes is considered for multi-vesicular bodies (MVBs)-derived EVs [41]. Exosomes are cup-shaped when viewed by transmission electron microscopy (TEM) but, as depicted by cryo-TEM, their cross-sectional surface is spherical, which is their observed morphology [70]. Although exosomes have a heterogeneous size distribution, but the expression markers and morphology of exosomes are independent of their size. Numerous proteins such as CD82, CD81, CD63, CD9, ALIX, as well as TSG101 have been used as markers for the detection of exosomes [96].

Exosome biogenesis is a complex process involving many biological compounds in the formation, trafficking, and secretion steps of the exosomal pathway. Exosomes originate from the multivesicular body (MVB), an endosomal compartment, and they are formed by inward budding of MVB's membrane which results in intraluminal vesicles (ILVs) (Fig. 1). ILVs are resident inside MVB until the fate of the MVBs is determined. Indeed, a regulatory system composed of different proteins called ESCRT (The Endosomal Sorting Complexes Required for Transport) machinery along with ESCRT-independent molecules and accessory protein constructively and/or stimulatingly participate in generation and loading exosomes ([50,89]. ESCRT-dependent machinery is composed of four complexes including ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III which located on MVBs membrane and contribute to cargo sorting and exosome formation [35]. In the context of ESCRT-independent machinery, Ceramide, a waxy lipid molecule, has a pivotal role in budding of exosomes into MVBs lumen [99]. However, unconventional secretion of exosomes from MVBs has been reported in tumor cells, which mediates aggressive behavior of tumor cells. For instance, Hoshino et al. demonstrated a synergistic interplay between invadopodia formation and exosome secretion. Indeed, inhibition of invadopodia formation in tumor cells considerably suppressed exosome release and invasiveness [36]. Intracellular trafficking of MVBs/exosome is preferentially mediated by different Rap-GTPases like Rab7, Rab11, Rab27, and Rab35 [75] (Fig. 1). According to previous observations, MVBs endure three subcellular fates [75]. MVB may fuse with the plasma membrane (PM) and secrete ILVs as exosomes to the

Table 1
Characteristics of extracellular vesicles (exosomes, microvesicles, and apoptotic bodies).

Extracellular vesicles	Exosomes	Microvesicles	Apoptotic bodies
Origin	Multivesicular bodies/Late endosome	Plasma membrane	Cells undergone apoptosis
Size	30–120 nm	100–1000 nm	1000–6000 nm
Mechanism of generation	Inward budding of multivesicular body	Outward budding of the plasma membrane	Cell shrinkage and segmentation
Pathway	ESCRT-dependent and ESCRT-independent Constitutive dependent Stimuli-dependent	Ca^{2+}-dependent Constitutive-dependent Stimuli-dependent	Apoptosis-dependent
Content	mRNA, miRNA, Proteins, lipids	mRNA, miRNA, proteins, lipids	proteins, nuclear segments, DNA, RNAs, cell organelles, lipids
Shape	Spheroid/cup-shaped	Irregular	Irregular

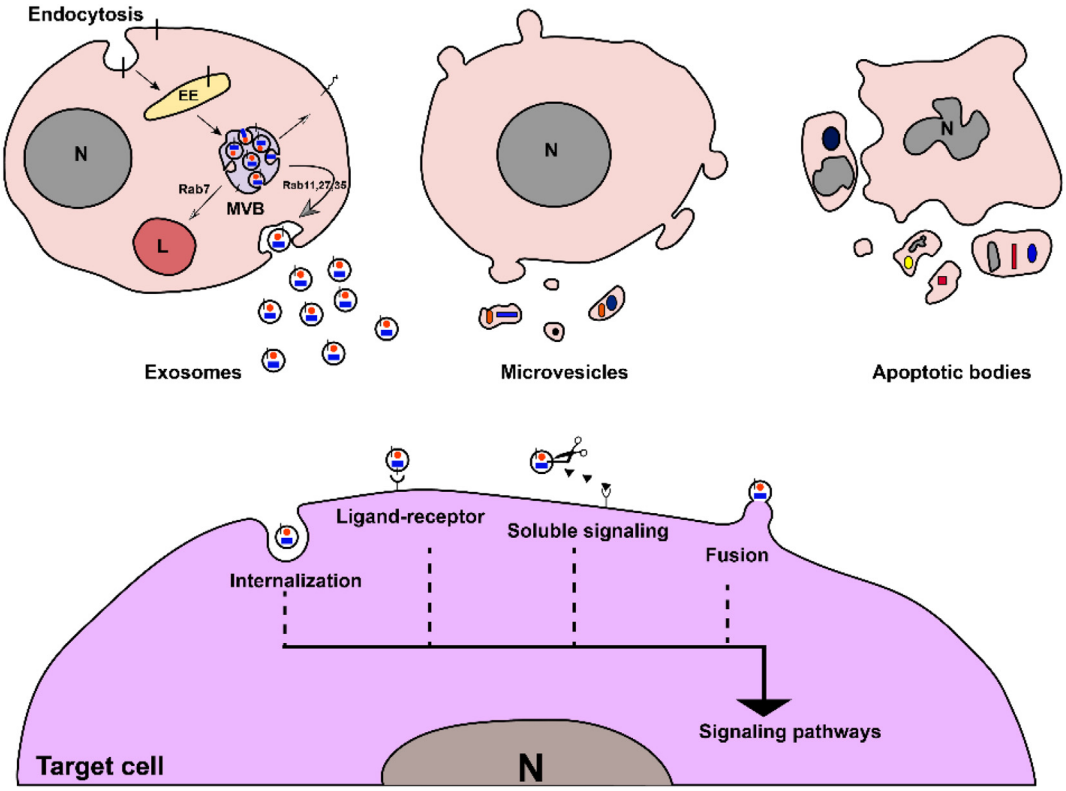

Fig. 1. Origin and intracellular communication of extracellular vesicles (EVs). Three types of EVs have been categorized according to their size and origin; exosomes, microvesicles, apoptotic body. There are four possible ways that EVs interact with target cells and affect signaling pathways. EE: Early endosome; L: Lysosome; M: multivesicular body; N: Nucleus.

extracellular space. In this scenario, signaling molecules direct MVB to the lysosomal degradation pathway, where MVB's cargo like excess receptors and unwanted materials are digested. Alternatively in immune cells, MVB can back-fuse to the PM and express immunological antigens on the PM [67]. Once secreted, exosomes distribute through biological fluids like blood, urine, breast milk, CSF, amnion, ascites, saliva, bile, and bronchoalveolar lavage and reach the target cells [50]. In a paracrine situation, exosome can deliver cargoes to the distant or neighboring cells to affect the function of recipient cells function by three proposed mechanisms including internalization, direct-fusion, and ligand-receptor interaction [27] (Fig. 1).

Alternatively, it has been proposed that protease enzymes in the ECM may activate exosomal proteins, which in turn induces target cell signaling [64]. Through those routes, exosomes deliver biomolecules and participate in inhibition and/or induction of signaling pathways of the target cells. Another EV subtype is microvesicles (MVs), released from different cells including platelets and endothelial cells. MVs are heterogeneous in size and morphology (100–1000 nm) [13] (Table 1). MVs are generated from the PM with irregular shapes through a mechanism known as the PM shedding. Such shedding is a critical process for RBCs hemostasis and leads to a loss of approximately 20% of the hemoglobin. Since, MVs are produced through the process of PM shedding in which the components of cytoplasm are engulfed, the cytoplasmic components of cell of origin contribute to regulating the function of nearby and distant cells [13]. The largest class of EVs is apoptotic bodies (ABs), generated during the final step of apoptosis with a size range between 1000 and 6000 nm [29] (Table 1). Based on Surveys like that conducted by Sebbagh et al., Rho-associated kinase 1 (ROCK1) participates in the development of various ABs. In this scenario, caspase-3 activates ROCK1, which in turn phosphorylates myosin light chain leading to membrane segmentation [82]. Phosphorylation of myosin light chain and its ATPase activity initiate the actin-myosin cytoskeleton interaction that breaks down nuclear integrity, consequently leading to the encapsulation of genome materials and DNA fragments into irregular-shaped ABs particles. Similar to other EVs, ABs potentially participate in the cell-cell communication through engulfment of biological molecules such as nuclear-related fragments, microRNAs, proteins, and even organelles, and facilitate initiation of the pathological conditions [119] (Table 1).

3. Free exosome therapy

Stem cells are capable of differentiation into various cell types and contribute to maintenance of tissue homeostasis by replacement of damaged cells [116]. According to previous reports, stem cells can be categorized into embryonic and adult stem cells, based on their origin and developmental stage [116]. In the field of regenerative medicine, stem cells appear to be very promising for repair and regeneration of injured tissues. Despite their availability and differentiation potential, stem cell based therapies suffer from several major drawbacks [112] including: (*i*) the necessity for a consistent source of cells with phenotypic stability; (*ii*) the risk of rejection due to immunological incompatibility; (*iii*) the higher cost of generation and difficulty of handling; and (*iv*) the potential risks of tumor or ectopic tissue formation. Growing evidence suggests that paracrine signaling rather than trans-differentiation of the transplanted stem cells participate in repair tissue [80]. In this regard, paracrine factors released from the transplanted stem cells play a pivotal role in the cell-based healings of tissues such as nerve, kidney and heart. Interestingly, the key role that exosomes play in the biology of stem cells is well established. For instance, it has been shown that exosomes participate in self-renewal and proliferation of pluripotent/multipotent stem cells and play a key role in defining the fate of neighboring cells, rendering them to produce EVs [74].

As mentioned above in the context of intercellular communication, the secreted exosomes act as messengers and transfer various

Table 2
MSCs-Ex based clinical trials up to September 2019.

Study title	Conditions	Status	Identifier
microRNAs Role in Pre-eclampsia Diagnosis	Preeclampsia	completed	NCT03562715
Allogenic Mesenchymal Stem Cell Derived Exosome in Patients with Acute Ischemic Stroke	Cerebrovascular Disorders	Not yet recruiting	NCT03384433
MSCs-Ex Promote Healing of MHs	Macular Holes	Recruiting	NCT03437759
Effect of Microvesicles and Exosomes Therapy on β-cell Mass in Type I Diabetes Mellitus (T1DM)	Diabetes Mellitus Type 1	Unknown	NCT02138331
A Safety Study of IV Stem Cell-derived Extracellular Vesicles (UNEX-42) in Preterm Neonates at High Risk for BPD	Bronchopulmonary Dysplasia	Recruiting	NCT03857841

biomolecules to regulate the function of neighboring cells. Recent developments have shifted scientific interest from conventional stem cell therapy to exosomes therapy. This is because the exosomes from stem cell therapy to exosome therapy because exosomes from diverse cellular origins contain sets of biomolecules from the corresponding cells of origin, which is needed for regeneration of multicellular tissues [83]. EVs/exosomes have many advantages that make them superior to stem cells in regenerative medicine [93]. Exosomes act as a natural depot for encapsulation bioactive molecules like RNAs and proteins to target them to the desired cells and to protect them against enzymatic degradation. The nanosized exosomes are capable of penetrating through biological barriers like the brain-blood barrier. In addition, exosome-based therapies eliminate the safety concerns and practical restrictions of stem cell expansion, cell differentiation to undesired lineages, and tumorigenesis. Notably, the flexible nature of these biological vesicles allows scientists to manipulate their cargoes to produce desired exosomes carrying specific drugs, RNAs, and proteins. Moreover, various surface receptors present on the membrane of exosomes may be specifically engineered to target the sites of injury. Although exosomes show advantages in regenerative medicine, however, there exist challenges about the design and use ideal ways for delivering desirable exosomes to target tissues. In this regard, exosomes and their cargo should not be changed/deformed, and a large number of exosomes would be delivered to target area. Based on the type of diseases, different administration routes can be used to deliver exosomes. The administration routes should be suitably chosen and optimized to obtain therapeutic advantages, specifically in the case of newly formed tumors in sensitive tissues.

3.1. Exosomes from mesenchymal stem cells

MSCs have attracted much attention in the field of cellular therapy due to their distinctive characteristics. MSCs can be harvested from various tissues such as bone marrow aspirate, umbilical cord, and fat tissue. MSCs have a high proliferation rate for *ex vivo* expansion. Furthermore, these cells exhibit immunosuppressive properties, which make them useful for allogeneic transplantation. Of note, frequent administration of allogenic exosomes isolated from MSCs has demonstrated that exosomes possess a limited amount of antigenic components and are non-immunogenic therapeutic agents [6]. MSCs-Ex is similar to exosomes from other sources in shape, structure, and exosomal markers [62,77]. These exosomes represent density range of 1.10–1.18 g/mL in sucrose, and are enriched in exosomal markers like CD81, CD9, CD63, and Alix, and membrane lipids including sphingomyelin, cholesterol, and phosphatidylcholine [51,98]. However, several reports showed that MSCs-Ex transfer distinct biological cargo, which mirrors the parental cells genomic pool. For instance, these exosomes contain more than distinctive 150 miRNA [11] and 850 proteins [52] that are involved in both pathological and physiological events. Exosomes derived from MSCs bear several cytokines and growth factors, including interleukin-6 (IL-6), IL-10, transforming growth factor β (TGFβ1), and hepatocyte growth factor (HGF), which have been confirmed to mediate immunoregulation [10]. Beside these, high levels of extracellular matrix metalloproteinase inducer (EMMPRIN), VEGF, and MMP-9 have been found in MSCs-Ex, these proteins contribute to angiogenesis, which could be essential for tissue regeneration [103].

Over the past decade, exosomes from MSCs and their function have been extensively studied in different diseases models [111,117]. Several groups have explored the clinical applications of MSCs-Ex as listed in the clinical trial database (https://clinicaltrials.gov/). When "mesenchymal stem cells exosomes" was used as keyword, five clinical studies related to MSCs-Ex was found up to September 2019. In one clinical study (NCT03562715) that has been completed, the expression of miRNAs 136, 494 and 495 in exosomes isolated from the MSCs of peripheral blood was compared with those from umbilical cord in patients with preeclampsia. The characteristics of these clinical trials are presented in the Table 2. The present section aims to discuss therapeutic effects of MSCs-Ex in cardiovascular, neurological, and kidney diseases and wound healing.

3.2. MSCs-Ex in wound healing

The wound healing process consists of different steps and involves many biomolecules that contribute to induction of angiogenesis, cell growth and proliferation, migration, tissue reorganization, and ECM remodeling [85]. In this scenario, exosomes potentially can reach cells that are involved in skin regeneration such as keratinocytes, endothelial cells, and fibroblast to regulate signaling pathways in the target cells [81]. Exosomes are capable of promoting one/all steps of the wound healing process as they can carry multiple biological cargoes such as miRNAs, mRNAs, and proteins that can regulate signaling pathways in the target cells [95]. For instance, it was demonstrated that exosomes from human umbilical cord MSCs induce β-catenin activation by transferring Wnt-4,thus prompting angiogenesis in a rat model of skin burn [120]. As once exosomes are delivered to target cells, different signaling pathways were activated to promote the expression of various growth factors involved in the wound healing including STAT3, Interleukin-6 (IL-6), stromal cell-derived factor-1 (SDF-1), insulin-like growth factor-1 (IGF-1), and HGF [73]. In this regard, MSCs from the umbilical cord have been utilized in numerous studies to (*i*) promote wound healing through its anti-scarring effect as well as through inhibiting TGF-SMAD signaling and decreasing collagen deposition [20] (ii) the induction of angiogenesis [110] and suppression of immune response [102] (*iii*) foster proliferation and migration of cells [113]; (*iv*) decrease inflammation [58]; and (*v*) induce tissue remodeling in rodent models of cutaneous regeneration [20]. Similarly, in nother *in vivo* studies, the beneficial effects of exosomes from human adipose MSCs [59], bone marrow MSCs [83], and healing of wound MSCs [117] have been reported in skin regeneration and wound healing. In general, MSCs-Ex activate several signaling pathways and biomolecules can be activated by MSCs-Exs to improve the wound healing process. These findings suggest that MSCs-Ex can support wound healing by activation of different signaling pathways and molecular events within the target cells in the wound.

3.3. MSCs-Ex in cardiovascular diseases

Preliminary work on MSC derivatives was undertaken by Gnecchi et al. demonstrating that the conditioned medium collected from MSCs overexpressing Akt-1 inhibited apoptosis of rat cardiomyocytes under *in*

vitro settings and reduced infarct size in a rat model of ischemic heart [26]. Likewise, Arslan and colleagues showed that exosomes from MSCs exert a therapeutic effect in a mouse model of myocardial infarction by reducing the infarct size. They found that the intravenous administration of exosomes five minutes prior to reperfusion led to nearly 50% reduction in infarct size. Interestingly, exosomes restored energy supply and decreased oxidative stress *via* the elevation of ATP and NADH levels in mice heart, 30 min after myocardial ischemia/reperfusion injury. Further observations showed that MSCs-Ex contain CD73 and other essential enzymes that are involved in ATP generation. Therefore, these vesicles may be a viable candidate in treating patients with acute myocardial infarction [3]. In support, it was confirmed that conditioned media from MSCs had potential to cause 60% reduction in infarct size and showed improvement of systolic and diastolic cardiac performance [97]. Similarly, Bian et al. showed that cardiac indices as blood flow, cardiac systolic and diastolic performances recovered and infarct size significantly reduced following the intramyocardial injection of MSCs-Ex in a rat model of myocardial infarction [5].

Exosomes released from mouse MSCs decreased the vascular remodeling and hypoxic pulmonary hypertension in the mice model of hypoxic pulmonary hypertension. At the subcellular level, these neovesicles inhibited signal transducer and activator of transcription-3 (STAT3) activation and augmented levels of miR-204 in the lung tissue, indicating the therapeutic effect of exosomes. The same results were observed when exosomes from human MSCs were co-cultured with human pulmonary artery endothelial cells *in vitro* [53]. Interestingly, exosome from genetically lentiviral CXCR4-modified MSCs (Exo^{CR4}) was found to increase IGF-1α and pAkt levels, inhibit active caspase 3, and amplify VEGF expression and tubulogenesis in cardiomyocytes *in vitro*. Moreover, the implantation of MSC-sheet treated by Exo^{CR4} exhibited beneficial effects such as decreasing infarct size, inducing angiogenesis, and improving cardiac function in the rat model of myocardial infarction [43]. Yu et al. reported that exosomes from MSC^{GATA-4} (Exo^{GATA-4}) have the potential to inhibit apoptosis, promote survival, and preserved mitochondrial ATP synthesis in cardiomyocytes subjected to a hypoxic condition. Intramyocardial administration of exosomes in the border of an ischemic region extensively improved heart contractile function and decreased infarct size in a rat model of myocardial ischemia/infarction [117].

3.4. MSCs-Ex in neurological diseases

MSCs-Ex has been shown to play a key role in the treatment of neurodegenerative diseases (Fig. 2). For example, Doeppner et al. demonstrated that the bone marrow-derived MSCs-Ex have a therapeutic effect in restoring post-ischemic neurological injuries, increasing angioneurogenesis, modulating post-ischemic immune responses, and providing long-term neuroprotection in a mice model stork [16]. Intravenous administration of the MSCs-Ex in a rat stroke model also improved neurovascular plasticity, enhanced axonal density and synaptophysin-positive regions in the ischemic margin zone of the striatum and cortex in the brain of treated rats [107]. Along the same line, other studies indicate the MSCs-Ex contain miR-133b, which can be transferred to astrocytes and neuron cells, and consequently contribute to neurite remodeling and promote efficient recovery in the rat stroke model [108]. Furthermore, it has been reported that MSCs-Exs contain the miR-17-92 cluster, which can enhance oligodendrogenesis, neurogenesis, and neural remodeling in the ischemic boundary region [106]. Moreover, it has been shown that the miR-17-92 cluster inhibits phosphatase and tensin homolog, a potential target of miR-17-92 cluster, and subsequently activates the protein kinase B/mechanistic target of rapamycin/glycogen synthase kinase 3β signaling pathway [106]. In a rat model of preterm brain injury, Drommelschmidt and coworkers reported that the MSCs-Ex inhibits neuron degeneration, reactive astrogliosis, and microgliosis [17]. Similarly, following the MSCs-Ex administration, brain function was improved by reducing the neurological sequelae [68]. Researchers have also examined the therapeutic effect of the MSCs-Ex in the traumatic brain injury (TBI) models [120]. In this regard, Zhang et al. demonstrated that the MSCs-Ex elevates angiogenesis and growth rate of neurons, whereas it reduces inflammation of the lesion boundary zone and dentate gyrus of brains, indicating an improvement in the functional of neurons after TBI [120]. Interestingly, MSCs-Ex was shown to mitigate the adverse symptoms of the Alzheimer's disease when loaded with neprilysin, an enzyme that degrades β-amyloid peptide in the central nervous system (CNS) at intracellular as well as extracellular levels. It was revealed in a 3D culture system that exosomes from dental pulp-derived MSCs suppress apoptosis in dopaminergic neurons, indicating their potential in treatment of patients with Parkinson's disease. These findings demonstrate the therapeutic potential of MSCs-Ex in CNS diseases.

3.5. MSCs-Ex in kidney diseases

The renoprotective function of the MSCs-Ex in kidney injury models has been described. (Fig. 2). Human bone marrow-derived MSCs-Ex have been shown to promote proliferation of tubular cells in a glycerol-induced acute kidney injury (AKI) in the SCID mice, and subsequently recover renal function through horizontal transferring of miRNAs [7].

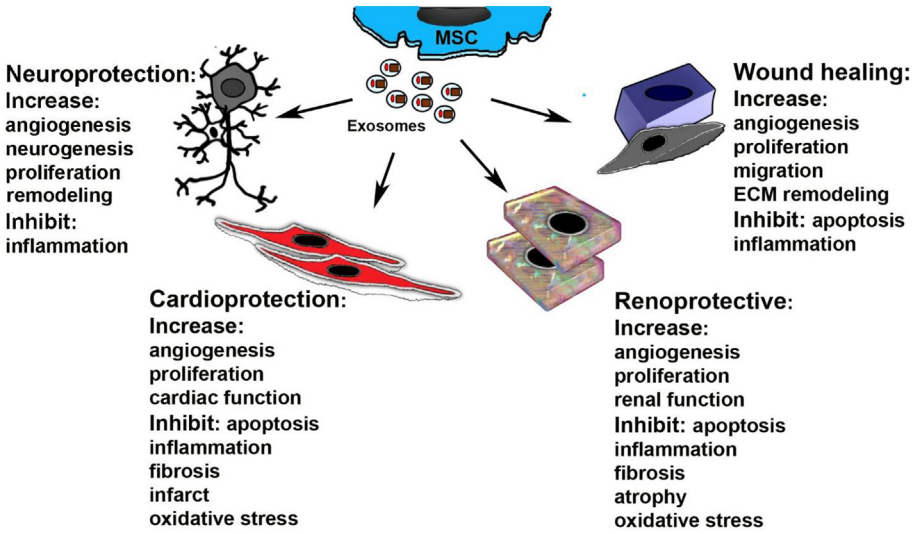

Fig. 2. Schematic diagram of therapeutic effects of mesenchymal stem cell (MSCs) derived exosome in different diseases.

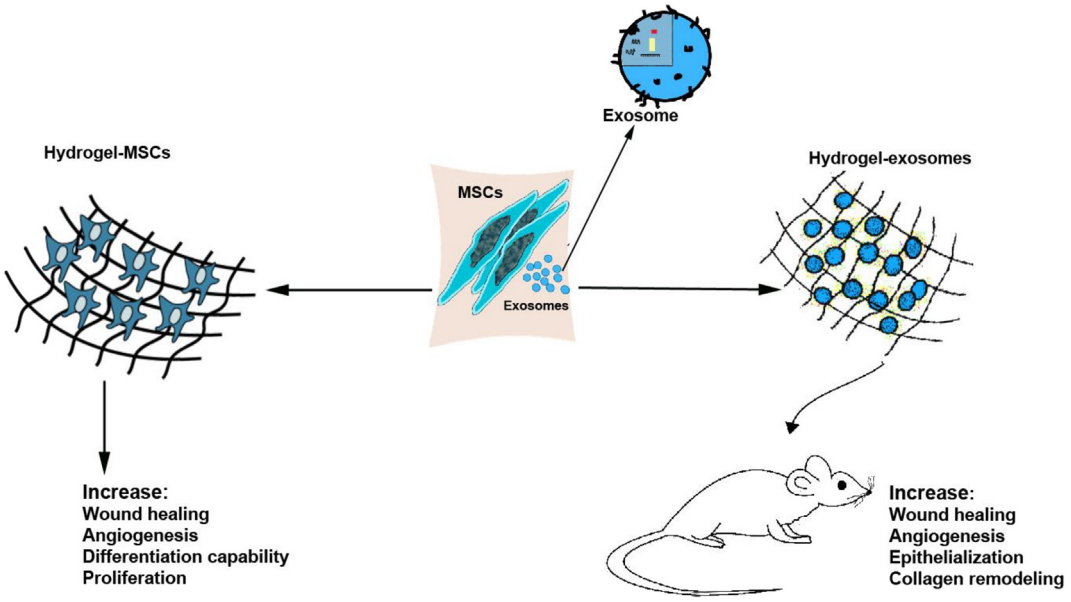

Fig. 3. The encapsulation of mesenchymal derived exosomes into chitosan/silk hydrogel for the treatment of diabetic diseases.

In the same model, and using human bone marrow-derived MSCs, Bruno et al. reported that mRNAs from MSCs-Ex such as CDC6, CDK8, and CCNB1 along with specific growth factors like hepatocyte growth factor and insulin-like growth factor-1 are involved in the cell cycle of tubular epithelial cells to enhance proliferation and inhibit apoptosis [8]. It was also shown that the MSCs-Ex can suppress fibrosis and tubular atrophy in a mouse model of nephrectomy [34]. In a mouse AKI model induced by cisplatin, a single administration of the MSCs-Ex was found to improve renal function and cell survival, whereas multiple injections of MSCs-Ex significantly decreased the rate of mortality [6].

It is reported that MSCs-Ex inhibit the apoptosis by suppression of the pro-apoptotic genes including lymphotoxin alpha, caspase8, and caspase1, and amplify the expression of anti-apoptotic geneslike Bcl2, Bcl-xL, and BIRC8 *in vitro* [6]. Therapeutic effects of the MSCs-Ex in the kidney were investigated in an ischemia-reperfusion injury (IRI) model. In this regard, Gatti et al. reported that the administration of the MSCs-Ex improved the adverse condition of AKI in a rat model by promoting proliferation and survival of tubular epithelial cells [24]. These beneficial effects can be attributed to the specific miRNAs and mRNAs profile of exosomes that mediate renoprotective signaling in tubular cells [72]. It has also been proposed that the MSCs-Ex may induce angiogenesis in the kidney injury model. For example, kidney-derived MSCs-EX was recently shown to promote angiogenesis in the renal tissue. Transcript profiling revealed that the exosomes contain pro-angiogenesis mRNAs such as bFGF, IGF-1, and VEGF, which are known to improve kidney function [12]. A growing body of research indicates the pro-angiogenic features of exosomes derived from human umbilical cord MSCs improve kidney function in a rat model of kidney IRI [42]. Anti-inflammatory effects of MSCs-Ex were recently reported. In a rat model of kidney IRI, Zou et al. observed that exosomes from human umbilical cord-derived MSCs recovered renal injury in the acute and chronic phases, which was attributed to suppression of macrophages and inhibition of CX3CL1 expression [126]. Lin and co-workers also observed the same results with exosomes from adipose-derived MSCs, that is, improvement in kidney function through decreasing mRNAs levels of inflammatory cytokines including IL-1β and TNFα [58]. The administration of MSCs-Ex in a model of gentamycin induced AKI in rats significantly increased IL-10 (an anti-inflammatory marker) expression, yet reduced expression of TNF-α and IL-6 (pro-inflammatory cytokines) [76].

Several researchers have investigated the renoprotective effects of MSCs-Ex by monitoring antioxidant pathways in animal kidney models. In this regard, studies indicate that MSCs-Ex suppress NADPH oxidase and reactive oxygen species [115] and elevate the activity of Nrf2/antioxidant response element [114]. These responses improved renal function while inhibiting apoptosis. Interestingly, it has also been proposed that the MSCs-Ex activates autophagy signaling pathway through increasing the expression of ATG5 and ATG7, and LC3B (genes related to autophagy pathway in a rat model of AKI induced with cisplatin), indicating the suppression of apoptosis and inflammatory response [104]. These findings demonstrate the contribution of MSCs-Ex to renal function recovery restoration from injury through different signaling pathways in animal models of kidney disease.

4. Hydrogel-encapsulated exosome therapy

4.1. Hydrogels as soft materials

Hydrogels are three-dimensional hydrophilic polymeric networks that are physically or chemically cross-linked, and can adsorb an enormous amount of water in aqueous solutions without undergoing dissolution process [44,54,88]. They have recently captivated a lot of attention in various fields such as bio-separation, water treatment, and agriculture, as well as regenerative and biomedicine, due to their biocompatibility and tissue-like behaviors. In regenerative medicine, hydrogels can serve as scaffolds, barriers, drug delivery systems, and cell encapsulation matrices. It has been shown that the incorporated cells or bioactive molecules retain their structure and function for a longer period, compared to their hydrogel-free administration [78] (Fig. 3). This indicates the potential benefit of cell encapsulation and emphasizes their importance in regenerative medicine. However, due to the living nature of the cells, designing an appropriate hydrogel for cell encapsulation is challenging and requires considering many parameters and criteria into consideration. Therefore, over the past two decades, a wide range of biopolymeric hydrogels with different chemical structures was utilized to encapsulate various types of cells [39].

The biochemical properties of hydrogels used in regenerative medicine have been described in many review articles [38,87,109]. However, to the best of our knowledge, there are very few reviews, if any, about the application of hydrogels for exosomes encapsulation. In the following section, we will review both hydrogel-encapsulated MSCs and hydrogel-encapsulated exosomes therapies.

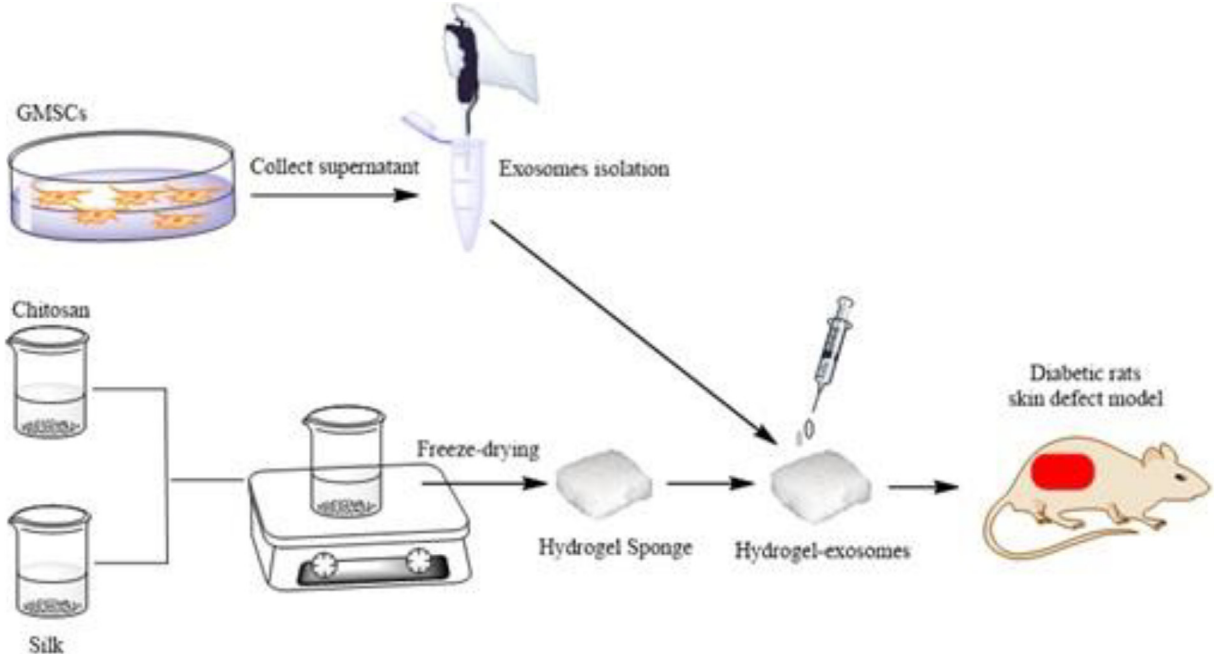

Fig. 4. Cartoon depicting the freeze-drying strategy to load MSC-derived exosomes.

4.2. Hydrogel-encapsulated MSC therapies

Recent developments in MSC therapy have heightened the need for an ideal system to deliver the cells to the target tissue while preserving their characteristics. There is evidence indicating that the cellular response of MSCs to two-dimensional (2D) culture is different compared to the three-dimensional (3D) microenvironment [14]. In the 3D cell culture environment, MSCs differentiate more efficiently into osteocytes, hepatocytes, and adipose cells. It was also shown that the differentiation capability of MSCs improved in 3D co-culture with other cell types such as osteoblasts [37], hematopoietic stem cells (HSPCs) [22], and HUVECs [47]. This highlights the importance of the 3D microenvironment to cellular response, cell-cell communications and suggests that the 3D microenvironment better mimics the *in vivo* conditions.

In the case of cell encapsulation, MSCs were embedded in a 3D permeable hydrogel matrix by solidification of a cell-liquid material mixture. Previous studies have reported that encapsulated MSCs are capable of differentiation to a defined phenotype such as bone [94], cartilage [46] and even tendon [66]. Using a microfluidic system and fibrin hydrogel, Trkov and colleagues showed that MSCs and HUVECs form stable capillary-like networks similar to blood vessels [101]. Liu's group has shown that the mouse bone marrow-derived MSCs distributed into an alginate hydrogel *via* a microfiber technology can form small-diameter vascular grafts. Further studies confirmed that such cellular system can enhance proliferation and angiogenic potential of MSCs under *in vitro* conditions. Moreover, the transplantation of these vascular grafts in the mice abdomens led to endothelialization, with a mild host reaction [60]. Laser bioprinted human bone marrow-derived MSCs have been shown to increase the rate of cardiac regeneration when encapsulated in a polyester urethane urea hydrogel [23].

Different cell encapsulation approaches have been applied to differentiation of MSCs for bone regeneration. For example, rat bone marrow-derived MSCs encapsulated into a gelatin methacryloyl (GelMA) hydrogel using a microfluidics droplet approach, have shown to promote mineralization and osteogenesis under *in vitro* and *in vivo* (rabbit model) conditions [122]. It was reported that hydrogel encapsulation by a microfluidics droplet approach [40] or bioprinting [18,19] is an efficient delivery system for MSCs to enhance osteogenesis in the bone tissue engineering. The cartilage differentiation ability of encapsulated MSCs has been described [66]. Recently, Li et al. used a microfluidics encapsulation method and demonstrated that human bone marrow-derived MSCs show a significantly high level of chondrogenesis, indicating the therapeutic importance of MSCs encapsulation in articular cartilage regeneration [55]. Bioprinting has been used to study the effect of MSC encapsulation in hydrogels (PEG-GelMA scaffold, nanofibrillated cellulose, and alginate) on chondrogenesis [2]. Skardal et al. have shown that bioprinted amniotic fluid-derived MSCs can promote the wound healing process in nu/nu mice [86]. Further, bioprinted human adipose-derived MSCs enhanced neovascularization, epithelization, and blood flow *in vivo* [45]. It has been reported that the cell encapsulation method as well as shape and size of the delivery vehicle influence proliferation and differentiation of MSCs [30]. Therefore, the size and shape of the 3D delivery system should be tailored to the desired tissue target to achieve ideal tissue regeneration [30].

4.3. Hydrogel-encapsulated exosome delivery system

Different approaches have been used to deliver exosomes to the injured sites [8,51]. The delivery of large number of exosomes without difficulty of administering the dose is critical to determining the success of exosome therapy. Therefore, the method of administration should be optimized to achieve a high therapeutic efficacy and specificity for a given disease. In this section, we review the application of hydrogels in exosomes delivery to target tissues alongside non-hydrogel approaches to exosomes delivery.

In recent years, increasing attention has been given to hydrogels as exosome carriers in regenerative medicine. It is essential to design a simple, active and non-invasive system for clinically translatable exosomes delivery. In this regard, biocompatible hydrogels offer a practical choice to deliver large amount of exosomes to the target site [123]. In the past decade, many researchers have sought to investigate the application of hydrogel-based exosomes delivery systems for exosomes delivery in regenerative medicine. For example, Shi et al. prepared a chitosan/silk hydrogel sponge, a biodegradable and biocompatible carrier, by the method of freeze drying for the delivery of human gingival MSC-derived exosomes [84].

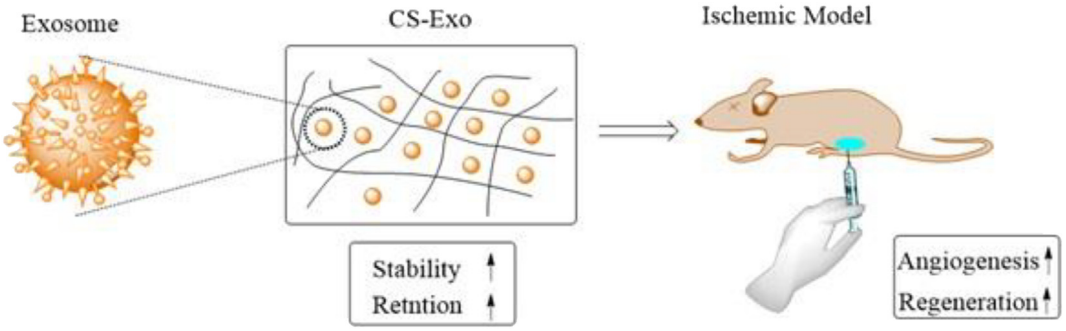

Fig. 5. Schematic illustration of hP-MSC-derived exosome encapsulated in thermosensitive chitosan hydrogel for muscle regeneration.

Using STZ-induced diabetic rats, they reported enhanced rate of re-epithelialization, collagen deposition, angiogenesis, and neurite growth in the skin wound bed of hydrogel-exosome treated group 1 and 2 weeks post-surgery [84]. Moreover, their studies indicated that the immobilization of gingival MSC-derived exosomes in the hydrogel significantly accelerated healing of diabetic skin wounds (Fig. 4).

In another work, Zhang et al. isolated exosomes from human placenta-derived MSCs (hP-MSCs) using differential centrifugation and showed that encapsulation in a thermosensitive chitosan hydrogel enhanced the *in vivo* stability and retention of the isolated-exosomes (Fig. 5). The results implied that chitosan hydrogel remarkably augmented the stability of exosomal cargoes like microRNAs and proteins and increased the half-life of exosomes *in vivo*. It was also shown that exosomes delivered in hydrogels induced angiogenesis under both *in vitro* and *in vivo* (a mouse model of hindlimb ischemia) conditions as detected by firefly luciferase imaging of angiogenesis [118].

In addition to natural exosomes, engineered exosomes have also been encapsulated in hydrogels. Recently, synovium derived MSCs that have been genetically engineered to overexpress miRNA-126-3p were mixed with a chitosan biopolymer to form a hydrogel-exosome composite. This composite contained large number of miRNA-126-3p and released exosomes in a controlled manner. Using a rat model of diabetic wound, the investigators revealed that the composite delivery system strongly induced epithelialization, collagen remodeling, and angiogenesis [92]. In another similar work, the synovium-derived MSCs that overexpressed miRNA-126-3p were encapsulated in chitosan in the presence of hydroxyapatite (HAp) nanoparticles (NPs) [56]. Interestingly, the presence of Hap NPs delayed exosome release from 2 days to 6 days. The chitosan-based slow release system is suitable for long-term utilization to improve chronic diabetic wound healing. More recently, exosomes containing miRNA-675, isolated from the supernatant of human umbilical cord MSCs, were mixed with a solution of ultrasound-treated silk fibroin to form miRNA-675 exosome-fibroin hydrogel [31]. The silk fibroin hydrogel released the exosomes in a sustained manner while increasing exosome retention and the half-life of miRNA-675 under both *in vitro* and *in vivo* settings. Han et al. used a mouse model of ischemic hind limb to test the therapeutic potential of miRNA-675 exosome-fibroin hydrogel and observed increased blood perfusion in the dysfunctional vascular system [31]. The application of exosomes incorporated in a self-healing and antibacterial polypeptide-based hydrogel, composed of Pluronic F127 (F127), oxidative hyaluronic acid (OHA), and EPL, donated by FHE hydrogel, has been recently explored for wound healing [105]. The FHE hydrogel was formed by a reversible Schiff base reaction between OHA and EPL in combination with the thermo-responsive property of F127 [105]. The investigators showed that exosomes from adipose-derived MSCs can effectively be incorporated in FHE hydrogel with a pH-responsive and long-lasting constant release phase. Interestingly, it was reported that FHE hydrogel-exosomes significantly increased the proliferation, migration, and the rate of tubulogenesis of HUVECs compared to the control free-exosomes group *in vitro*. Furthermore, the authors reported that the FHE hydrogel-exosome promoted wound healing and collagen deposition as well as hair growth in the wound site in the mouse model of diabetic wounds in mice at day 21of post-surgery [105].

In another study, Liu et al. encapsulated exosomes from pluripotent stem cell line in an acellular tissue patch (EHG) and examined the therapeutic potential of EHG–exosomes in cartilage regeneration in the rabbit model of cartilage defect. They reported that EHG hydrogel effectively retained the exosomes within the hydrogel and efficiently delivered EHG-exosomes in the site of cartilage defect. After 12 weeks post-administration, regeneration of the cartilage defect was observed, demonstrating the therapeutic effectiveness of EHG tissue patch in regenerative medicine [61]. Aside from biopolymer-based hydrogels, synthetic poly-lactic-*co*-glycolic acid (PLGA) polymers, due to their desirable mechanical and biodegradation properties, have been extensively explored, as a scaffold in regenerative medicine [63]. For example, Li, et al. extracted exosomes from human adipose-derived stem cells (hASCs) and immobilized the exosomes on polydopamine-coating PLGA (PLGA/pDA) scaffolds under ambient condition [57]. *In vitro* and *in vivo* studies revealed that PLGA/pDA scaffold provides a rigid medium for sustainable release of exosomes as a promising cell-free system for bone tissue engineering. These studies indicate the importance of matrix in the exosome-hydrogel system for applications in regenerative medicine. Therefore, designing a simple and biocompatible hydrogel that can encapsulate large number of exosomes and release them in a controllable manner in the biological system is important. In light of recent progress in the hydrogel-exosome delivery system, there are still certain questions that can be topics of future studies including: (*i*) what are the suitable techniques to load desirable amount of exosomes in hydrogels? And (*ii*) which hydrogels are suitable as a carrier for exosomes?

4.4. Other exosome administration routes

4.4.1. Direct injection

Exosomes can be directly injected in the target site of different models of disease. For example, Matsumoto et al. showed that intratumoral administration of exosomes from murine melanoma B16BL6 leads to the proliferation of tumor cells and inhibition of apoptosis in the B16BL6 cells in a mouse xenograft tumor model [65]. Direct injection of exosomes was found to inhibit the proliferation of cancer cells and shrinkage of tumor mass. It is reasonable to assume that this method has higher efficiency, but more invasive, than a systemic approach [48]. Intramyocardial injection of MSC-derived exosomes after myocardial infarction decreased infarct size and improved cardiac function [5]. While the direct injection method seems more efficient, however, it is more invasive than a systematic injection of exosomes [48].

4.4.2. Intravenous injection

Systemic administration of exosomes is the most common mode/method of delivery of the exosomes. For example, the delivery of

exosomes prepared from mouse MSCs bearing exosomal miRNA-204 to the lung tissue by intravenous injection inhibited vascular remodeling and decreased hypoxic pulmonary hypertension in the murine model of pulmonary arterial hypertension [53]. In a mouse model of kidney injury, intravenous injection

of exosomes showed a significant therapeutic effect in the renal function [8]. Intravenous administration of exosomes was shown to successfully release doxorubicin to the tumor tissue of a mouse model of breast cancer [40].

Bala et al. observed that systematic administration of exosomes bearing miRNA-155 resulted in the distribution of exosomes through various organs and tissues such as kidneys, muscle, liver, lungs, and adipose tissue [4]. Intravenous injection of exosomes from NK cells in a xenograft mouse model of glioblastoma resulted in therapeutic effects such as decreasing survival and growth of glioblastoma tumor cells [124]. In the mouse model Parkinson's disease, exosomes containing catalase were successfully delivered to the brain by intravenous injection and intranasal administration. However, the intranasal administration was found to deliver more exosomes to the brain, which resulted in a significant neuroprotective effect [32]. Despite being an easy and noninvasive method, the half-life index of systemically administrated exosomes is short and varies between a few minutes to hours. Takahashi et al. reported that exosomes derived from murine melanoma were available in circulation with a half-life approximately 2 min;, the amount of exosomes in circulation reached a minimum value after 4 h of administration [91].

4.4.3. Oral administration

It has been proposed that exosomes may provide beneficial effects on the intestinal luminal epithelial surface in the digestive tract. Kosaka et al. found that the population of intestinal stem cells in a mouse model of colitis increased when exosomes were administered orally. This indicates that the exosomes can cross through the intestinal acid barrier and lead to a functional response in the digestive tract; confirming the effectiveness of this method in an animal model. [49].

4.4.4. Intraperitoneal injection

Intraperitoneal injection of the exosomes has not been well studied, but a few studies related to their intraperitoneal administration have been documented. Bryniarski et al. reported that exosome-like nanovesicles loaded with miRNA-150 suppressed ear swelling and cutaneous contact sensitivity in mice. [9]. A recent study by Sun et al. showed the anti-inflammatory effect of curcumin-loaded exosomes injected into the peritoneal cavity. They showed that these exosomes decreased the number of CD11b + Gr-1 + cells (immunosuppressant cells) in the lung of LPS-treated mice, indicating a decrease in disease pathogenesis [90]. This approach may serve as a channel to distribute exosomes within different organs associated with the peritoneum cavity.

4.4.5. Alternative administration routes

Recent evidence suggests that, depending on the experimental design, exosomes can be administrated by alternative routes to afford high therapeutic effects. Grapp et al. examined the therapeutic effect of intraventricular injection of exosome containing folate receptor-α in mouse brain parenchyma and reported that the exosomes facilitated choroid plexus transcytosis [28]. The potential of subcutaneous vaccination by the delivery of exosomes bearing class II transactivator has been explored indicating that such a system can stimulate immune activity against the tumor and serve as a cancer vaccine. [125]. In addition, Zhang et al. reported that subcutaneous injection of MSCs-Ex increased the rate of angiogenesis in a rat wound model with a second degree skin burn [120]. Collectively, it can be stated that a proper route should be used to deliver a significant number of desirable exosomes to a given target site.

5. Conclusion

Exosomes therapy has provided a new window of hope for the treatment of several diseases. MSCs-derived exosomes show beneficial effects in different disease models. Additionally, the clinical application of exosomes derived from MSCs has recently been increased. After more than one decade of the successful exosome therapy experiments, major progress has been made in the selection of their administration route to different target tissues. Notably, it is vital to exosome therapy to choose a delivery method with high efficiency and low toxicity. In that regard, hydrogels have become a valuable tool in cell therapy as well as delivering exosomes to target tissues. Despite many hydrogel-encapsulated exosome delivery studies, further studies are needed to find answers for many questions, like are hydrogels appropriate as an exosome carrier in different disease models? Or which hydrogel is suitable for delivering a given exosomes?

Author contributions

Conception and manuscript design: J. R., A. A., N. J., and M. N. Collection of date: M. A., A. V., S. J. S., and M. M. Manuscript writing: J. R., A. A., R. A. Made important revisions and confirmed final revision: J. R., A. A., S. S., R. H., and E. J. All authors reviewed and approved the final version of manuscript.

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Declaration of competing interest

Authors declared that there is no conflict of interest.

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