Review Article

Stem Cell INVESTIGATION

Exosomal therapy—a new frontier in regenerative medicine

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Abstract: The recent advances in translational and nanomedicine have paved the way for developing the targeted drug delivery system at a greater pace among global researchers. On par with these technologies, exosomes act as a potential portal for cell-free drug delivery systems as these are bestowed with the native characteristics of the parent cell of origin. Exosomes, called extracellular vesicles (EcVs), are present in almost all cells, tissues, and body fluids. They help in intercellular signaling and maintains tissue homeostasis in the disease pathobiology. Researchers have characterized 9,769 proteins, 2,838 miRNAs, 3,408 mRNAs, and 1,116 lipids being present in exosomal cargo. The separation of exosomes from cells, tissues, and body fluids follow different patterned kinetics. Exosomes interact with the recipient cells through their surface receptor molecules and ligands and internalize within recipient cells through micropinocytosis and phagocytosis. Advancing technologies in regenerative medicine have facilitated the researchers to isolate exosomes from mesenchymal stem cells (MSCs) as these cells are blessed with supreme regenerative potentiality in targeting a disease. Exosomal cargo is a key player in establishing the diagnosis and executing therapeutic role whilst regulating a disease process. Various *in vitro* studies have exhibited the safety, efficacy, and therapeutic potentiality of exosomes in various cancers, neurodegenerative, cardiovascular, and orthopedic diseases. This article throws light on the composition, therapeutic role, and regulatory potentials of exosomes with the widening of the horizon in the field of regenerative medicine.

Keywords: Exosomes; extracellular vesicles (EcVs); cellular therapy; biological medicine

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Introduction

The contemporary developments in the field of translational medicine include the development of targeted drug delivery systems for harnessing the full potentiality and utilizing the therapeutic products effectively by overcoming the limitations of the existing methods to address the pathogenesis of a disease. In that context, various polymer-based nano delivery systems have been developed (1). Utilizing extracellular vesicles (EcVs) as a drug delivery

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tool has been given special attention due to their naïve characteristics derived from the parent or host cells (2-4). Despite the normal cellular homeostasis, EcVs play a major role in intervening in the pathobiology of disease processes through the intercellular signaling cascade (5-9).

Based on the size, exosomes are subcategorized under EcVs, which are endosome derived lipid bi-layered spherical vesicles of 40–150 nm in size (2,10-12). Almost all cells, tissues, and body fluids [plasma, urine, saliva, tears, gastrointestinal (GI) secretions, semen, and breast milk] secrete exosomes (13-16). Exosomal cargo carries an array of micro-biomolecules that consists of proteins, lipids, RNA, and DNA from the secreting parent cells (2,17-19). Moreover, the characteristics and behavior of the exosomes closely relate to the parent cell of origin (20-22). On account of their appropriate size and property along with their evident role in numerous pathobiological processes, the potential of exosomal therapy in the management of various neurodegenerative disorders, infectious diseases, musculoskeletal disorders, and cardiovascular disorders is overwhelming. This has enabled the researchers to target the disease process at a cellular level by using natural engineered defense mechanisms.

Characterization of exosomes

The composition of an exosome vesicle (EV) includes proteins, RNA, DNA, and other substances. Currently, 9,769 proteins, 2,838 miRNAs, 3,408 mRNAs, and 1,116 lipids have been described in their composition (23). Of these components, the exosomal proteins differ based on the nature of the primitive cells or tissues (24). Proteins like membrane transport and fusion proteins, chaperones, adhesion molecules, MHCs, cytoskeletal proteins, and lipid-related proteins are major exosomal proteins (24-28). With a pre-existing knowledge of the functioning of these proteins, ALG-2-interacting protein X (ALIX), heat shock protein 70 (HSP 70), tumor susceptibility gene 101 (TSG101), and tetraspanins (CD9, CD 63, CD 81, and CD 82) have been identified with a higher concentration in exosomes (29,30). Moreover, they serve as a biomarker for exosomal identification as shown in *Figure 1* (31). Exosomes are also rich in lipid layering molecules like glycosylphosphatidylinositol-anchored protein (LBPA) and flotillin (32,33). Apart from the metabolic enzymes and signal transduction molecules such as G protein and protein kinases, exosomes contain mRNAs, miRNAs, non-coding RNAs (ncRNAs), and mitochondrial DNA in their composition (34,35). Noteworthy, the first types of nucleic acids identified in the exosomes were mRNA and miRNA (36,37).

Being a nanoscale vesicular component in complex body fluids, it is challenging to procure exosomes of high-yield and fineness (38). However, their isolation and characterization is an essential prerequisite to establish their therapeutic potential with a better understanding of their physiology. Exosomes are derived from the cell culture supernatants or plasma with their identification based on the physical and morphological characteristics (39,40). Although separation can be made by several methods like ultracentrifugation, ultrafiltration, gradient ultracentrifugation, precipitation, size-exclusion chromatography, immune-affinity capture, mass spectrometric immunoassay, magnetic-activated cell sorting, and microfluidics-based techniques with each having their advantages and disadvantages (41-45) Ultracentrifugation is the most commonly used method for isolating exosomes. The complex proteins in the exosomes are analyzed by processes like western blot, flow cytometry, and mass spectrometry (46). Additionally, sample analysis with high precision fluorescence is a possibility in the nanoparticle tracking method (47). Homogeneity without exosome isolation is possible with the resistance pulse sensing method (48). Profiling the miRNA content of the exosomes was made with next-generation sequencing, microarray processing, and RT-PCR. Confirmation of the isolated samples can be done by electron microscopy (49). Disparate use of various methods of isolation, confirmation, quantification, and analysis used by the researchers with their own set of advantages and disadvantages, it brings added heterogeneity in their analysis which needs standardization.

Cellular physiology of exosomes

The formation of exosomes involves the invagination of the plasma membrane and the formation of intracellular multivesicular bodies with intraluminal vesicles. This endocytic pathway of the donor cell is followed by the transport of the transmembrane and intra-vesicular proteins from the Golgi complex resulting in the formation of early endosomes. After maturation and differentiation, they get transformed into late endosomes (50-53). They are degraded by fusing with lysosomes or plasma membrane or autophagosomes to release the intraluminal vesicles as exosomes (measuring 40–150 nm diameter) into the extracellular milieu as shown in *Figure 2* (5,32,54).

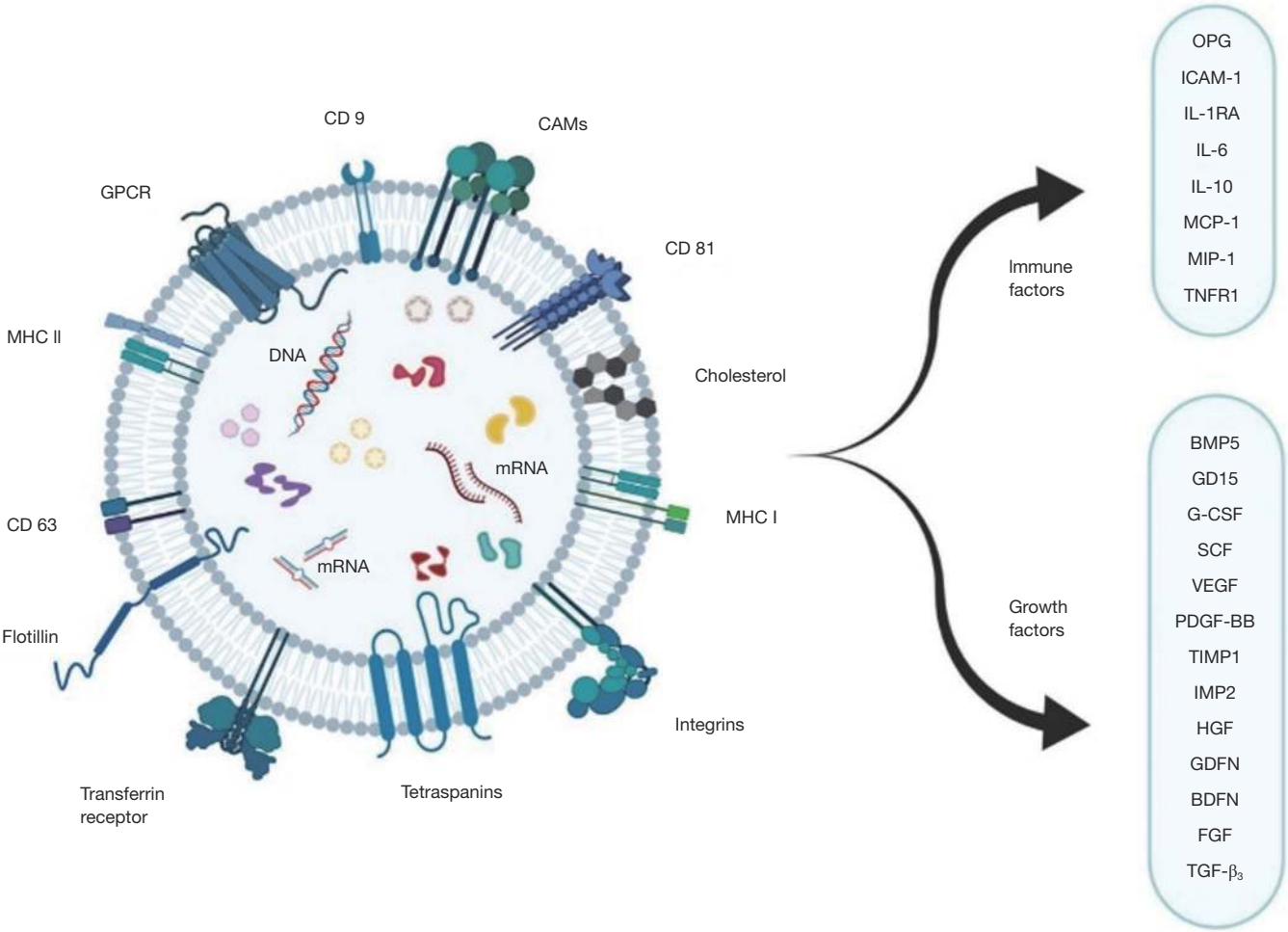

Figure 1 Basic structure of mesenchymal stem cell exosome depicting the surface and core proteins along with the key immune and growth factors.

Exosomes interact with the recipient cells through their surface receptor molecules and ligands. Some of the exosomes persist on the cell membranes of the donor cells after secretion while the rest of them interacts with the recipient cells (54-59). Internalization of exosomes occurs via membrane integration process mediated by raft or caveolae or clathrin dependent endocytosis. Micropinocytosis and phagocytosis have also been described as a method of internalization of the exosomes by recipient cells. This physiological integration process on the targeted recipient cells has been considered to have therapeutic potential as a targeted delivery system for effectively executing biological functions (60-64). Yet, the exosomal components in specific accounting for cell-type or organ specificity remain unclear (65).

Diagnostic role of exosomes

Exosomes are the key players of the intercellular communication portal and thereby owe the capability of determining the progression of a disease. Studies are emerging in the diagnostic and therapeutic facets of exosomes for various systemic pathologies. The detection of substances (intracellular and extracellular) carried by these nanoparticles and promoting their immune capture with surface proteins aid in the diagnosis of pathological processes. The spectrum of diseases in which exosomes play a key role in diagnosis includes cerebrovascular disease (66,67), diseases involving the central nervous system and neoplasm (8,68-70) along with disease involving kidney, liver, and lungs (70-72). Let us consider the scenario of cancer. Exosomes can protect the rapid degradation of

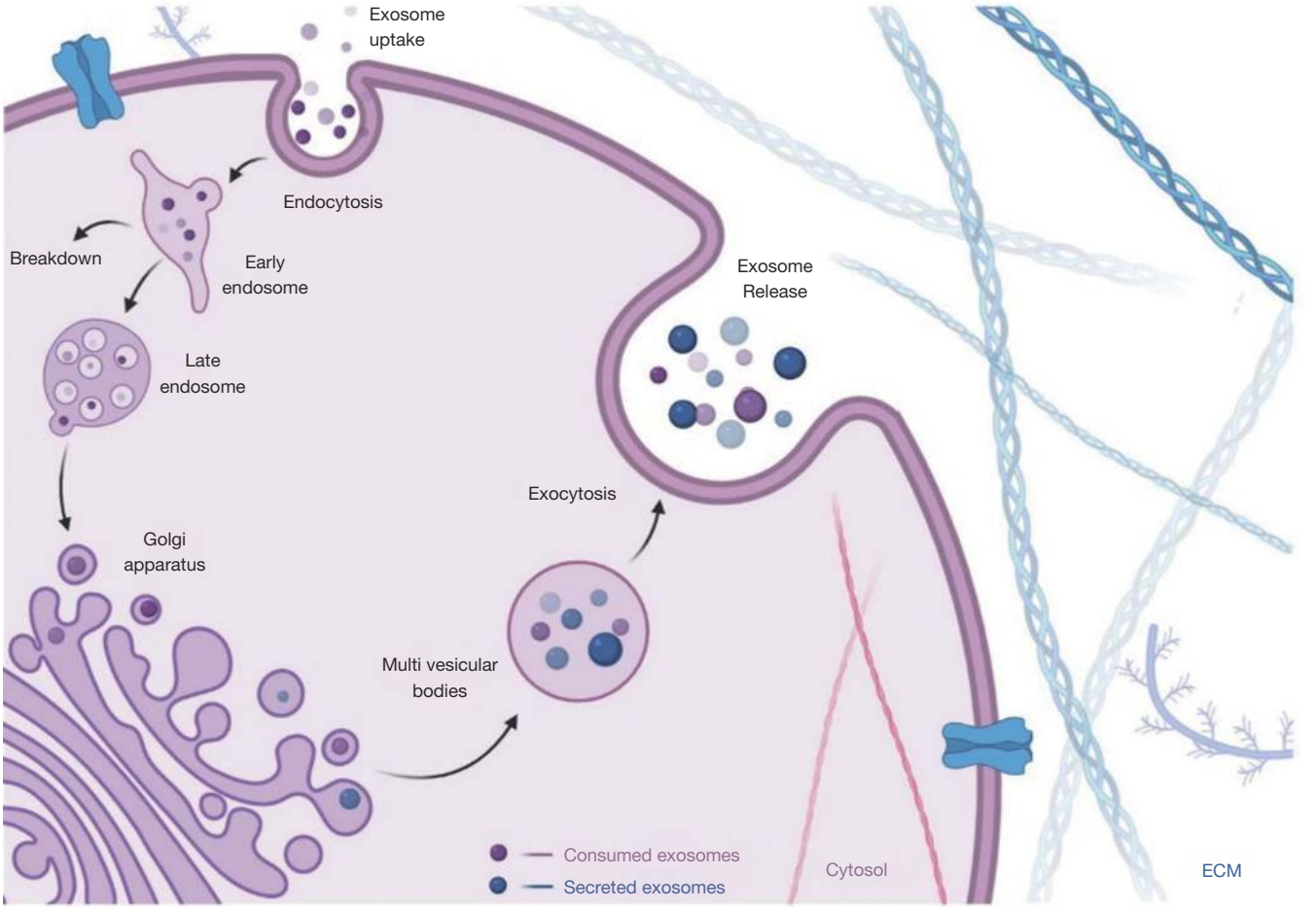

Figure 2 Physiology of formation and degradation of exosomes. ECM, extracellular matrix.

nucleic acids and remain highly stable in plasma (73). With an exosome-based liquid biopsy, direct detection of the circulating tumor cells and their DNA or cell-free RNA from body fluids like blood, saliva can be made. This DNA or RNA (including mRNA, miRNA, ncRNA) from serum exosomes can be used for the detection of cancer-related mutations making them a promising biomarker in cancer diagnosis (74-77). Various studies have identified proteins and RNAs rich in cancer-derived exosomes which act as biomarkers that can be used in the detection and prognosticating treatment response (78-85).

Therapeutic role of exosomes

Exosomes possess a higher therapeutic potential for various disease spectra due to their ability for intracellular shuttling. Nanomedicine technologies have given rise to explore the usage of pathogenic importance of exosomal particles in various diseases. The targeted drug delivery system in nanomedicine focus on the sustained release of exosomes for exerting the biological activity in the targeted site. Exosomes are used as vectors or carrier molecules to elicit a biological response.

Under given physiological circumstances, exosomes demonstrate very low immunogenicity and the potential to circumvent the physiological blood-brain-barrier (86). With the help of a stable lipid bilayer, the cargoes loaded in the exosomal vesicles are guarded against the action of native immune cells and digestive enzymes. The engineered exosomal vesicles deliver the cargoes loaded to them to the site of action through various mechanisms of endocytosis or membrane fusion as shown in *Figures 3* and *4* (87,88). EVs are derived from various cell types and tissues. On delivery to specific diseased tissue, under specific conditions, EV elicits

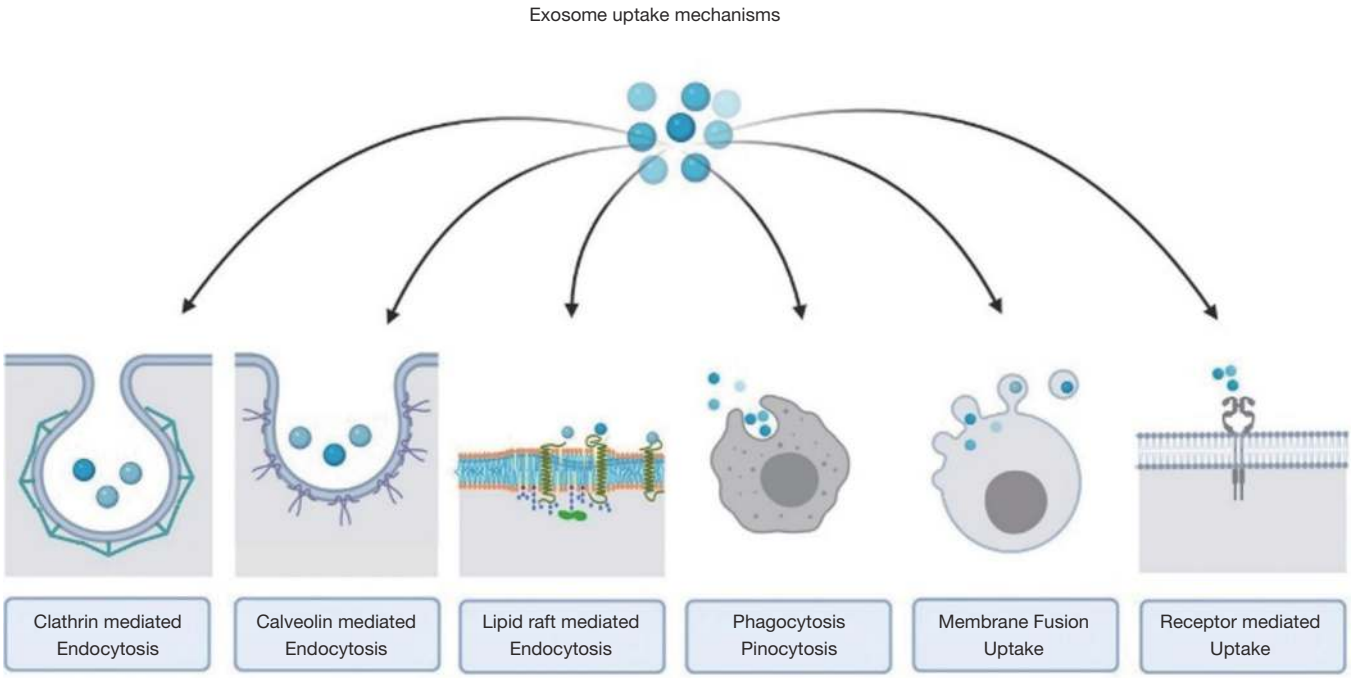

Figure 3 Methods of uptake of exosomes by the target cells.

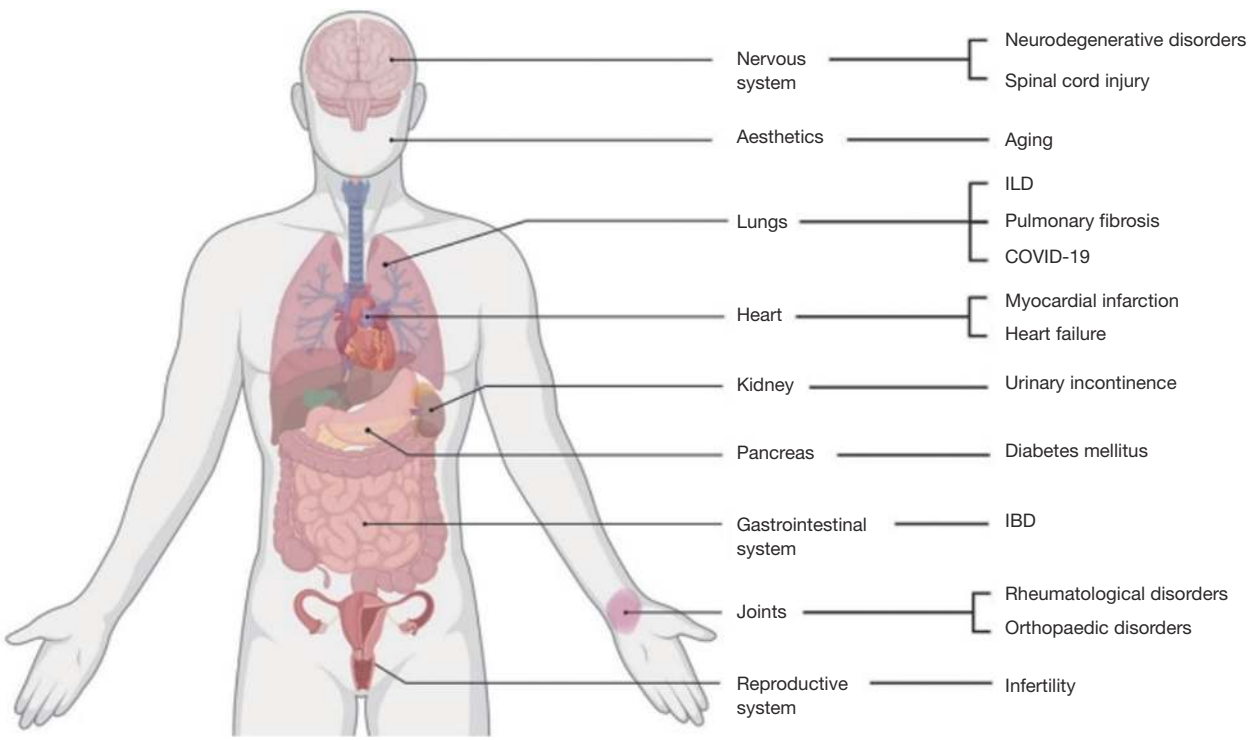

Figure 4 Potential therapeutic applications of exosomes. ILD, interstitial lung disease; IBD, inflammatory bowel disease.

tissue regeneration and homeostasis (89). Mesenchymal stromal cell-derived EV show cellular viability, trophicity, anti-inflammatory, immunomodulatory and therapeutic effects (90,91). They support neoangiogenesis and cellular proliferation (2,92-95). Exosomes demonstrate the homing effect of the parental cell type (2,96).

The superiority of the exosomal cargos compared to the stem cells on clinical and therapeutic potential is noted by

(I) Lack of inherent risk associated with any cell-based therapies including stem cells;
(II) Lack of replicating potential and the risk of malignant transformation;
(III) Lack of immunogenic response towards infections and cancers;
(IV) Targeted action at the site of interest (2,97).

The above-mentioned versatility of EVs enhances their intracellular signaling capabilities and their movement across cellular membranes to restore micromolecular homeostasis. Apart from these advantages, exosomes also offer neuroprotection and neuroplasticity by shuttling across the blood-brain barrier in neurodegenerative diseases (98). A total of 77 clinical trials have been registered among the global researchers in various pathologies. Out of 77 trials, 11 clinical trials have been completed and have proved the positive diagnostic and therapeutic effects of exosome-based treatment in various cancers, neurodegenerative and hematological diseases (99).

Sources of isolation of exosomes

The exosomes are also called intraluminal vesicles and are found in various tissues and body fluids (5-7). The exosomes derived from mesenchymal stem cells (MSCs) are of prime importance due to the greater therapeutic and regenerative potential as shown in *Figure 4*. Due to the challenges faced in isolating exosomes from various body fluids, regenerative and translational medicine experts used MSC-derived exosomes for treating various disorders (2). MSCs derived exosomal cargo exhibit intracellular signaling and communications to targeted tissues. The key sources of exosomes derived from the MSCs include bone marrow, adipose tissue, placental cells, umbilical cells, endometrial fluid, and amniotic fluid (2,100).

Exosomes of MSC origin have cell surface markers such as CD 29, CD 44, and CD 73 embedded in them (100). They play a vital role in the biomechanisms involved in the repair and regeneration, bioenergetics, immunoregulation, intracellular communication, and tissue metabolism (2,101). In a proteomic analysis, a total of 730 protein molecules in bone marrow MSC derived exosomes were isolated (102). Some researchers found the existence of transcription signaling factors in exosomal cargoes (103). Amniotic fluid exosomes are the most preferred exosomes for clinical applications than bone marrow-derived exosomes (104).

Routes of delivery of exosomes

The different state of the art approaches to deliver the exosomes to their site of action and their shortcomings were analyzed and presented with their challenges encountered in them. The most common route is intravenous (IV) despite its clearance in the liver and the kidney is rapid in this method (105). This method was widely used for conditions involving orthopedic, neoplastic, and cardiovascular pathologies (106). Intramuscular (IM) has been primarily used in neuromuscular and musculoskeletal conditions while subcutaneous (SC) route in aesthetic and cosmetic indications. The IM or SC route is chosen due to the ease of injectable area and the dosing volume (97). Intrathecal is the preferred route in neurodegenerative conditions such as Alzheimer's, Parkinson's, and Creutz-Feld Jakob's disease (98,99). Local aerosol sprays are used in the management of wounds and ulcers (107,108). For hair growth and rejuvenation in age-related therapies, this is the preferred route (108,109). During the time of the coronavirus disease-2019 (COVID-19) pandemic, there have been interesting studies discussing the role of exosomal therapy as a promising therapeutic candidate (110).

Global regulatory requirements

With the growing therapeutic spectrum of exosomal therapy, the International Society for Extracellular Vesicles (ISEV) and the European Network on Microvesicles and Exosomes in Health and Disease (ME-HaD) have formulated certain guidelines to foster their clinical usage (10). The regulations elaborate on the standard operative protocols to be followed in the process of collection, processing, testing, quality control, and manufacturing of exosomes for clinical usage. With the help of these policies, the potential of the EVs can be utilized at appropriate standards for therapeutic usage.

Any novel therapy or a drug being developed is dependent on the strategies to standardize the process to focus on validating the proposed technology. There are currently no Food and Drug Administration (FDA)-

approved exosome products for human use in the USA (2,111). According to the FDA, exosomes are classified as a 351 product that requires studies effectively showing safety and efficacy, along with the purity of the product and its potency in treating the condition (112). Therapies using the exosomes are under the Investigational New Drug (IND) developmental phase and need the approval of the regulatory agencies before initiating the clinical trial (2,113).

The regulatory framework addresses the safety standards for microbial and viral contamination and demands GxP standards (GxP = good manufacturing/good laboratory/good distribution/good clinical/good scientific practice or GMP/GLP/GDP/GCP/GSP) for the production and quality control of the corresponding therapeutics (114,115). It regulates the conduct of clinical trials (116). There remains an issue with the categorization of any emerging novel therapeutics for humans.

Pharmaceutical category of EV preparations

According to the Center for Biologics Evaluation and Research (CBER), the exosomes are regulated as biological products (117,118). Based on the individual types, the framework that was laid down for products in this category applies to the exosomes. For example, an anti-tumor vaccine that uses exosomes will be regulated under the regulations provided for therapeutic cancer vaccines (119). The functional moiety in EV-based therapy determines its medicinal type (2,120).

Hence, ISEV categorizes EV-based therapy under biological medicines with the following properties.

(I) The therapeutics acquired from unmodified cells.
(II) Therapeutics acquired from genetically manipulated cells (without trans-gene).
(III) Therapies acquired from exosomes and gene-modified cells with trans-gene classified as gene therapy products (GTP).
(IV) Native exosomal therapies; are used as drug-delivery systems, used as carriers for the biological and chemical components, and are considered as biological medicine.

As the biological medicinal products include a span of various pharmaceuticals, these were classified as Advanced Therapy Medicinal Products (ATMP's) in 2007 (2,120). It was further subgrouped to conventional biological medicinal products due to the biological properties, physicochemical and immunochemical properties (117,118). It includes somatic cell therapy, gene therapies, and tissue-engineered products.

ATMP's therapeutics involve products that have been:

(I) More than minimally manipulated such as cell expansions and cell cultures.
(II) Intended for non-homologous use. For example use of hematopoietic cells for orthopedics.
(III) Nucleated and viable cells are present in the product.
(IV) Products with therapeutically active trans-gene from genetically engineered cells are considered as ATMPs, independent of the presence of any nucleated, viable cell.
(V) EV-based therapeutics classified as ATMP's; are produced from human material by a manufacturing process comparable to the ATMP production.

Safety profile, manufacturing & standardization

The mechanism of action (MoA) is essential for the clinical translation of therapeutics based on EVs (121). The critical part of this translation is the identification of "active substances", their properties, and the essential quality controls in manufacturing a clinical-grade product (118). For phase I the hypothesis should be based on the proof of principle, reinstate the rationale based on the MoAs. Although uncertainties exist on the MoA of EV-based therapeutics on the target cells, with supportive animal models, EVs from human cells are not to be accounted as high risk in an IND (116). Commercializing a large scale manufacturing of EV based therapeutics requires a robust quality management system, technologically superior facility, and an updated technology complying with GxP (114,115). The endpoint of these investigational studies is to provide safety for the donor and the patient. The criteria for the therapeutic release of an investigational product is to determine efficacy based on the pre-IND studies for characterization (116).

At this moment the EV's do not have a standardized protocol for isolation and storage; and include homemade cocktails as protocols with no standardization for reagents, storage containers, and storage time for each desired EV-based product (2,10,122).

Direction for future research

The scope of exosomal therapy among various other clinical fields remains untouched. With the global interest of the researchers towards harnessing the potential of

Table 1 Companies targeting on exosomal research and their potential products for commercial use

Company	Exosomal source	Exosomal product	Exosomal potential
Capricor Therapeutics (128)	Cardiosphere-derived cells (CDCs)	CAP-2003 (preclinical testing)	Explore the anti-inflammatory, pro-angiogenic, antiapoptotic and antifibrotic effects associated with their parent CDCs
Kimera Labs (129)	Placental MSC-derived exosome	XoGlo	Tap into the potential for tissue repositioning, preventing healing-associated scarring
Exosomedx (130)	Plasma or serum-derived exosomes	ExoDx	CLIA certified product to conduct advanced clinical testing
NanoSomiX (131)	Brain-derived exosomes (BDEs)	ExoM and ExoC	Biomarkers for prediction of potential nervous system disorders
Evox Therapeutics (132)			For rare metabolic and lysosomal storage diseases engaging endogenous and exogenous drug loading
Codiak Biosciences (133)	Engineered therapeutic exosomes		Encompasses therapeutics for vaccines, anti-infectives, oncology, autoimmune and anti-inflammatory diseases
Aruna Biomedical (134)	Neural derived exosomes	AB126	Murine thromboembolic models of stroke and its translation to clinical therapies

MSC, mesenchymal stem cell; CLIA, Clinical Laboratory Improvement Amendments.

the exosomes, making EV based therapeutics a reality is not far from reach (10). The potential of EV based therapy is established in various orthopedic conditions, neurodegenerative disorders, auto-immune diseases, cardiovascular diseases, infectious diseases, and diagnosis of rare diseases including cancers (98,99,123). However, the current domain of research in EV based therapeutics involve developing diagnostic and therapeutic applications towards patient care.

From the level of the circulating pool of EVs, early diagnosis of a complex disease could be made by using them as biomarkers in blood (74-82). Utilising the paracrine signaling property of the MSC-derived exosomes, repair, and regeneration of organs could be achieved (87-95). For use in most orthopedic conditions exosomes are deemed anti-inflammatory concerning their immunomodulatory potential (123). Understanding the role of exosomes in the disease processes have enabled us to propel our understanding of the disease and expand the scope of the therapies evolved out of them (124). As of now therapeutic applications of the EV-based therapies from defined cell sources based on their immunomodulatory capabilities include inflammatory disorders, degenerative disorders, vehicles of drug-delivery, anti-tumor therapies, and pathogen vaccination tool (98-102). Immunomodulatin of exosomes may be exerted by either immune-activation or suppression. This novel platform with the above-mentioned diverse potentials holds promise to develop vaccines with prolonged immunogenicity against infectious diseases or cancer (125,126).

There is a paradigm shift brought down by the continual breakthroughs in research exploring the potential of the exosomes that have resulted in the development of novel therapeutic options that are reshaping the landscape of the global market from time to time. Despite being started as a subject of academic interest, exosomal therapy has now been transformed into a potential platform with immense promise for future therapeutics (127). This sowed the seeds for start-ups into exosome platforms with their proprietary technologies. Some of them are elaborated in *Table 1*. These companies are using patented technologies to tap the potential of exosomes. For example, the company Capricor Therapeutics developed an exosomal therapeutic technology from cardiosphere-derived cells (CDCs) called CAP-2003 which is in the preclinical testing phase to explore the anti-inflammatory, pro-angiogenic, antiapoptotic, and antifibrotic effects associated with their parent CDCs. Additionally, the company is evaluating the role of CDC-exosomes for the treatment of trauma-related injuries and conditions (135).

Conclusions

Exosomes play a natural role by enacting as a vehicle for the transfer of biological substances between cells and thereby renders a broader prospect to serve as a channel

for delivering drugs of therapeutic interest. However, the intricate composition and uncertain functioning are inquisitive facets warranting further exploration. In view of making the exosome-based therapy a reality, more accurate, faster, cheaper, standardized, specific, and easier methods of their separation and purification have to evolve along with concrete adducing on its safety, feasibility, pharmacokinetic and pharmacodynamic characteristics through large scale prospective research studies.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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