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Review Article

Extracellular vesicles and their therapeutic applications: a review article (part1)

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Abstract: Extracellular vesicles (EVs) have emerged as a captivating field of study in molecular biology with diverse applications in therapeutics. These small membrane-bound structures, released by cells into the extracellular space, play a vital role in intercellular communication and hold immense potential for advancing medical treatments. EVs, including exosomes, microvesicles, and apoptotic bodies, are classified based on size and biogenesis pathways, with exosomes being the most extensively studied. The aim of this study was to examine the molecular secretory pathway of exosomes and to discuss the medical applications of exosomes and the methods for employing them in laboratory models. The therapeutic potential of EVs has garnered significant attention. Their unique properties, such as stability, biocompatibility, and capacity to traverse biological barriers, make them promising vehicles for targeted drug delivery. By engineering EVs to carry specific cargo molecules, such as therapeutic proteins, small interfering Ribonucleic Acid (RNAs) (siRNAs), or anti-cancer drugs, researchers can enhance drug stability and improve their targeted delivery to specific cells or tissues. This approach has the potential to minimize off-target effects and increase therapeutic efficacy, offering a more precise and effective treatment strategy. EVs represent a captivating and rapidly evolving field with significant therapeutic implications. Their role in intercellular communication, targeted drug delivery, and regenerative medicine makes them valuable tools for advancing medical treatments. As our understanding of EV biology and their therapeutic applications continues to expand, we can expect remarkable advancements that will revolutionize the field of medicine and lead to more personalized and effective therapies.

Keywords: Extracellular vehicles (EVs), intercellular communication, targeted drug delivery, regenerative medicine, therapeutic proteins, stem cells

Introduction

Cells communicate with each other through various pathways within a biological microenvironment. These mechanisms, including paracrine, juxtocrine, and autocrine methods, facilitate communication among different cells. Over the past decade, scientists have come to understand that cells utilize not only soluble factors but also nanovesicles known as exosomes to communicate with nearby and distant cells, thus adding another dimension to cellular communication [1].

Initially, it was believed that exosome production primarily served to eliminate intracellular waste. However, subsequent research has revealed the significant role of exosomes in both natural and pathological biological processes. These vesicles are part of the extracellular vesicle family, distinguished by their size and origin. Exosomes, the smallest members of this family, are generated through endocytosis. A plethora of molecules participate in their production, intracellular transport, and secretion, while various mechanisms have been elucidated regarding their formation and cargo loading [2].

Extracellular vesicles (EVs) are small membrane-bound particles released by cells into their external environment. Their main function is to facilitate intercellular communication by transferring proteins, lipids, and nucleic acids between cells. Exosomes are a subtype of EVs that are formed within the endosomal pathway and released when multivesicular bodies fuse with the cell membrane [2]. They play a significant role in mediating cell-to-cell communica-

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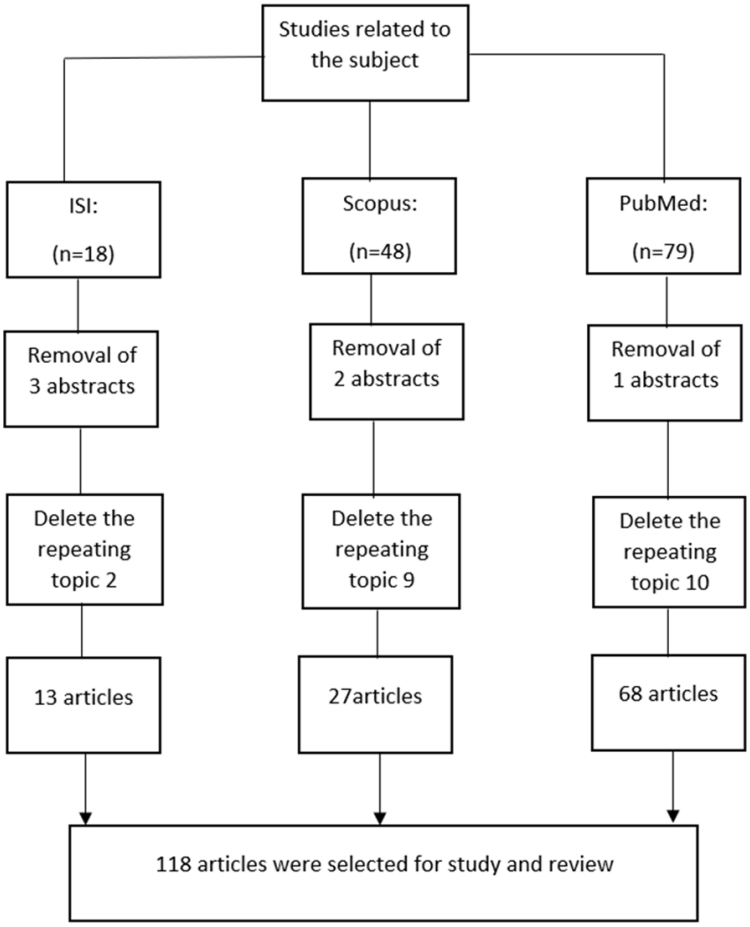

Figure 1. Search strategy in selected databases between 2000-2018 and identification of final articles.

tion by delivering bioactive molecules to target cells, thereby influencing various physiological and pathological processes [3].

These vesicles contain a variety of biomolecules, such as proteins, nucleic acids, and lipids. Once released into the extracellular environment, they reach target cells, causing changes in their fate and function due to the presence of these biological molecules. Researchers believe that exosomes have potential applications in medical fields, including biomarkers, biocarriers, and gene therapy. The use of exosomes in clinical trials is on the rise, and researchers in this field are actively studying their behavior and biology in pathological conditions among patients. Overall, the focus of these experiments is the utilization of exosomes as biomarkers and drug carriers for various diseases, particularly cancer [3].

This review article aims to examine the molecular secretory pathway of exosomes. Additionally, it will discuss the medical applications of exosomes and methods for employing them in laboratory models.

Methods

The method of the current study is a simple review. In this study, articles related to descriptive and analytical studies indexed in Scopus, Institution for Scientific Information (ISI) and Pubmed databases have been used. Searching for articles in the Scientific Information Database, the keywords used included extracellular vesicles, exosome, endocytic-lysosomal (ESCRT), Multivesicular bodies (MVB).

The primary criterion for the selection of articles was its relationship with exosomes and the presence of one of the keywords. In general, in this review, the collection of studied articles included 145 articles, and finally 118 articles were considered suitable for this purpose.

The inclusion or exclusion criteria of the studies included the following: 1. The articles should be between 2000 and 2018. 2. The articles should be research and review type. 3. Articles should have complete and accessible text (**Figure 1**).

Extracellular vesicles

Extracellular vesicles constitute a heterogeneous population of small bilayer phospholipid vesicles that are secreted by various cells in the body, including immune cells, red blood cells, platelets, cancer cells, stem cells, and other cell types.

These vesicles serve as biocarriers, transporting a wide range of biomolecules such as proteins, Deoxyribonucleic acid (DNA), RNA, and

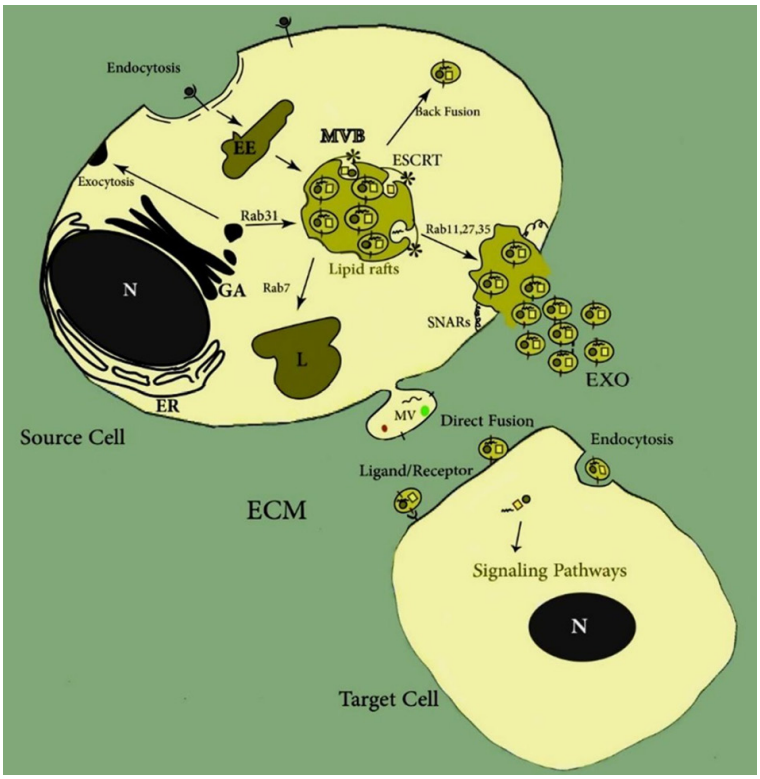

Figure 2. How to form, secrete and absorb exosome. Exosomes are formed by budding towards the inside of the MVB. Exosomes are produced through both ESCRT-dependent and ESCRT-independent pathways. The contents of exosomes are provided by endocytosis, Golgi apparatus and products inside the cytoplasm. Rab proteins mediate the intracellular transfer of MVB and its consequences. Based on its membrane composition or a series of unknown mechanisms, MVB can choose secretory, lysosomal, and direct integration routes. After secretion, exosomes can reach the target cell through 3 ways: 1) endocytosis, 2) ligand-receptor, and 3) direct integration. After exosomes reach the target cell, they cause changes in function, growth, fate, and morphology. MVs are released by budding and shedding from the cell membrane.

lipids [4]. Scientists have classified extracellular vesicles into three types based on their origin, formation process, and size: 1) exosomes, 2) microvesicles, and 3) apoptotic bodies. Exosomes originate from endosomes, forming vesicles within the secondary endosome's lumen, and are eventually released through the fusion of the secondary endosome with the plasma membrane. These vesicles contain DNA, mRNA, microRNA, and other non-coding RNAs, as well as cytoplasmic and membrane proteins. They are composed of lipids and have a size ranging from 30 to 120 nm [5] (**Figure 2**).

Microvesicles (MVs), also known as shedding vesicles, comprise a heterogeneous population of extracellular vesicles with a size ranging from 100 to 1000 nm. These vesicles are released into the extracellular environment through a process of budding and tearing from the cell membrane surface. This formation mechanism is akin to the cytokinesis cutting step [6]. Furthermore, the formation of MVs resembles the release of viruses from cells. Various cell types, including endothelial cells, platelets, and erythrocytes, release MVs. It is believed that MVs are released in response to stimuli, whereas exosomes are also released in a basal state. Observations indicate that these vesicles exhibit active binding to annexin V, and their membrane is abundant in phosphatidylserine. Larson and colleagues demonstrated that some of these MVs do not exhibit binding to annexin V, while others are rich in phospholipids. Studies indicate that both MVs and exosomes play a role in various cellular signaling pathways, facilitating the transport of numerous biomolecules through a series of well-defined mechanisms [7].

The content of MVs undergoes changes in response to stimuli. For instance, in pre-thrombosis conditions, platelets release larger microvesicles that contain factors capable of activating endothelial cells. Following thrombus expansion, platelets release microvesicles containing factors aimed at thrombus removal. These findings demonstrate that microvesicles exhibit heterogeneity in terms of size and content. The identification and isolation of specific populations of microvesicles can serve as a significant objective in studying the function and biology of extracellular vesicles. Apoptotic bodies (ABs) are the largest extracellular vesicles in terms of size. These bodies are released as cell fragments from apoptotic cells. The ROCK-1 protein plays a crucial role in the formation of these 1-5 micrometer bodies [8].

Table 1. Comparison of the characteristics of extracellular vesicles

Vesicle type	Source	Size	Marker	Content	Morphology	Extraction method
Exosome	By budding to the inside of the MVB membrane/secondary endosome	30-100 nm	CD9, CD63, CD81, Tsg101, Alix, Hsp60, Hsp70, Hsp90	Protein, lipid, mRNA, miRNA, double-stranded DNA	Cup shape	Sequential centrifugation with sucrose gradient and ultracentrifugation with a speed of 100,000-200,000 g
Microvesicle	By shedding from the plasma membrane of the cell	100-1000 nm	Lipids and TF and flotillin molecules	Protein, lipid, miRNA, mRNA	Irregular shape	Centrifuge with a speed of 20000-18000 g
Apoptotic bodies	From apoptotic cells	1-5 micrometers	PS statement			

Table 2. ESCRT machine components

Function	Subsets	Complex
Categorizing goods according to ubiquitin protein	HRS STAM1	ESCRT-0
Binding to ubiquitin protein and ESCRT-0 machine and stimulation of bud formation	Tsg101 Vps28 Vps37 Mvb12	ESCRT-I
Binding to ubiquitin protein and ESCRT-I machinery and stimulation of bud formation	Vps36 Vps22 2Vps25	ESCRT-II
Stimulation of vesicle division	Vps20 Vps32 Vps24	ESCRT-III
ESCRT machine analysis and recycling	Vps2 Vps20 Vps32	Subproteins

Studies on the formation of apoptotic bodies (ABs) indicate that the caspase 3 protein activates the ROCK-1 protein, which, in turn, phosphorylates the myosin light chain and triggers detachment of cell fragments. Phosphorylation of the myosin light chain, coupled with the ATPase activity of this chain, stimulates the actin-myosin cytoskeleton reaction, eventually leading to the disintegration of its cohesive core [9]. As a result, this process leads to chromosomal breakage and DNA fragmentation, which is then encapsulated within apoptotic bodies. These bodies can contain organelles, DNA, and histone fragments. Due to their diverse composition of proteins, DNA, and miRNA, ABs can contribute to cell communication and disease dissemination. Endothelial cell-derived apoptotic bodies are notably abundant in the cytokine IL-α, which can enhance chemokine release and induce inflammation. Additionally, it has been discovered that ABs play a crucial role in phagocytosis by providing a set of signals, thereby preventing the initiation of necrosis. The comparison of these vesicles is presented in **Table 1** [10].

Exosomes

For the first time, Pan, Harding, and their colleagues reported that mammalian reticulocytes secrete nanovesicles during their differentiation. Initially, it was believed that the secretion of these vesicles served as a compensatory cellular response to expel incoming waste materials, including transport receptors. Since then, the term “exosome” has commonly been used to refer to a group of vesicles that are released as extracellular vesicles. In vitro experiments demonstrate that various types of mammalian cells secrete exosomes. Exosomes are present in the intracellular space as well as biological fluids such as plasma, amniotic fluid, joint fluid, cerebrospinal fluid, urine, saliva, breast milk, alveolar fluid, and even bile. Under normal or abnormal conditions, the biological activity and kinetics of exosomes may vary [11] (**Tables 2**-**4**).

Table 3. Classification of applications of exosomes

Application of exosomes	Disease models
Biomarker	Types of cancer and non-cancer diseases
Carrier of medicinal agents	Cervical cancer, breast cancer, liver cancer, glioblastoma cancer, Alzheimer's disease, heart failure and inflammatory disease
Exosome therapy	Types of cancers, cardiovascular diseases, wound diseases, bone diseases, kidney diseases, metabolic diseases, neurological diseases

Table 4. Clinical applications of exosomes

Application of exosomes	Number	Number/percentage	Diseases
Basic studies	51	51/51	Cancer
Biomarker	38	38/38	Cancer
Treatment	7	7/07	Stroke, wound, polycystic ovary syndrome, infection, type 1 diabetes, macular retinal break disease, head and neck cancer
Carrier of medicinal agents	3	3/03	Cancer

For instance, alterations in cytosolic calcium levels in mast cells can impact the secretion of exosomes, which is relevant in severe allergic reaction conditions, as observed in the human K562 cell line. As mentioned earlier, the secretory vesicles of the cell can be classified into three types: microvesicles, apoptotic bodies, and exosomes. Microvesicles and apoptotic bodies encompass a diverse population of vacuoles, whereas exosomes exhibit a cup-shaped morphology or possess distinct sizes observable under the electron microscope [12]. Ultrasound evidence has confirmed that exosomes are generated through the breakdown of the endocytic vacuole within the MVB or new endosome containing internalized vesicles (ILVs).

ILVs are formed by budding a portion of the membrane of MVBs into their lumen. The resulting MVBs have two main fates. Firstly, they fuse with lysosomes, leading to the degradation of their cargo. Secondly, by merging with the cell membrane, ILVs are released into the extracellular environment. Additionally, another pathway has been observed within antigen-presenting cells [13]. Through this pathway, diverse antigens are processed and transferred to the outer surface of the cell membrane. For instance, in immature dendritic cells, the internal vesicles within MVBs serve as temporary storage sites for MHC type II molecules. Multiple populations of MVBs can be observed within a single type of cell. For instance, one group of MVBs, rich in tetraspanin rhizodomains and specific lipids like cholesterol, sphingomyelin, and ganglioside GM3, with a low density of lysobisphosphatidic acid and resistance to washing, follows the secretory pathway. On the other hand, another type of MVB, containing a small amount of cholesterol but a large quantity of lysobisphosphatidic acid, fuses with the lysosome. The mechanism of exosome formation within MVBs involves the incorporation of certain proteins and lipids into the endosomal membrane, the inclusion of molecules into primary ILVs, and the subsequent seclusion of ILVs [14].

The findings from various experiments reveal that proteins enter exosomes through two mechanisms. The first mechanism relies on the ESCRT machinery, situated on the cytosolic side of the MVB membrane. It recognizes transmembrane proteins, trans-Golgi network proteins, and cell surface proteins. Subsequently, these proteins undergo ubiquitination and are directed into the exosome. The second pathway operates independently of the ESCRT machinery.

Ultimately, exosomes serve as shuttles, transporting specific cargoes to target cells. As a result, exosomes play a role in both natural biological processes and pathological events [15] (**Table 5**).

ESCRT dependent mechanism

The role of the ESCRT machinery in ILV production is explained at the beginning of the text. Numerous laboratories have conducted investi-

Table 5. Exosome administration models

Exosome administration methods	Type of disease	Exosome contents	The feature of the method	Disadvantages
Intravenous injection	Cancer model	Doxorubicin	Easy	But a high number of exosomes and being trapped in other organs of the body
	Parkinson's disease model	Catalase enzyme		
	Healthy mouse model	Luciferase		
	Transgenic mouse model	siRNA		
	Healthy rat model	Alpha folate receptor		
Direct injection into the tissue	Cancer	Fluorescent sign	Efficient	Aggressive
Intraperitoneal injection	Blood infection disease model	Curcumin	Easy	Dissemination in the peritoneum
Intranasal injection	Parkinson's disease model	Curcumin	Easy	Low efficiency
Oral prescription	Model of heart failure	Fluorescent sign	Easy	Remove by the digestive system

gations on various mechanisms, such as cargo selection, vesicle budding, and vesicle emptying, using methods such as siRNA and shRNA inhibition experiments. Studies indicate that ESCRT components play a significant role in the formation of MVBs and ILVs at the molecular level. ESCRT comprises approximately 20 different proteins, which can be classified into four groups. Additionally, proteins such as VPS4, VTA1, and ALIX are associated with these protein complexes. All four groups contribute to the development of ILVs. The ESCRT machinery is a complex structure consisting of ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, and various other molecules. Each machinery subset is positioned on the cytosolic side of the endosomal membrane [16].

The interaction between the ESCRT-0 complex and 3-phosphatidyl inositol lipid, located on the endosomal membrane, triggers the activation of the ESCRT machinery and its binding to ubiquitinated proteins. Subsequently, ESCRT-0 utilizes components of ESCRT-I, leading to the recruitment of ESCRT-II monomers. The binding of ESCRT-I and ESCRT-II initiates the inward budding process from the MVB membrane. In close proximity to the site of membrane bending during ILV formation, ESCRT-II activates ESCRT-III components.

Finally, through the action of ATPase enzymes, ubiquitin proteins and subsets of the ESCRT machinery dissociate, while certain components such as TSG101, HRS, and ALIX, along with associated proteins, remain in exosomes.

Mechanisms independent of ESCRT

Several researchers have demonstrated that exosome cargoes can be loaded through an ESCRT-independent mechanism. For instance, Stauffer and his colleagues found that the depletion of four subsets of the ESCRT complex does not impact the formation of CD63 in multivesicular endosomes (MVEs). It has been discovered that oligodendroglial cells secrete proteolipid proteins that are not reliant on ESCRT function but instead depend on sphingomyelinase, an enzyme responsible for ceramide production [17].

In these cells, inhibition of sphingomyelinase disrupts ceramide synthesis and leads to a significant reduction in exosome secretion. Ceramide plays a crucial role in membrane bending of MVBs and the generation of ILVs.

In contrast, inhibition of sphingomyelinase activity in human melanoma cells leads to continuous generation of MVBs without relying on the CD63 tetraspanin-dependent mechanism.

Hence, the pathway of exosome biogenesis is referred to as the endolysosomal pathway. Ceramide is highly essential for exosome formation as it is primarily involved in the assembly, cargo loading, and release of exosomes through sphingomyelinase-mediated ceramide production. Additionally, Edgar and his colleagues have provided further evidence that CD63 plays a significant role in MVB formation in various cell types, including HeLa cells [18].

It was previously known that in melanocyte cells, the incorporation of PMEL (melanosomal protein) into ILVs does not require protein ubiquitination or ESCRT components such as TSPAN8. In exosomes secreted by rat pancreatic adenocarcinoma cells, this tetraspanin can modify both the transcript and membrane protein forms of VCAM-1 and α4 integrins.

Tetraspanin proteins, including CD9, CD63, CD81, and CD82, as well as heat shock proteins such as HSP70 and HSP90, are found in ectosomes and are sometimes used as markers for these structures. Due to their potential role in antigen presentation, many exosomes contain MHC class II and I molecules. Similarly, the upregulation of CD9 and CD82 in HEK293 cells leads to the release of exosomes carrying β-catenin in a ceramide-dependent manner. Several experts have confirmed the crucial role of ceramide in the formation of exosomes in cancer cells. It has been suggested that ceramide can induce the fusion of small microdomains into larger domains and promote budding. Cholesterol accumulation, caused by genetic or drug mutations, increases the release of vesicles carrying flotillin-2, ALIX, CD63, and cholesterol in oligodendroglial cells, confirming the key role of the flotillin-2-dependent mechanism [19].

Research has shown that phospholipase D2, which generates phosphatidic acid (PA) from phosphatidylcholine, is one of the essential molecules involved in the formation of exosomes. It appears that phosphatidic acid functions similarly to ceramide.

Depletion of proteolipid protein 2 with siRNA or CAY10594 decreases exosome secretion in the MCF-7 cell line, while induction of proteolipid protein 2 in RBL-2H3 cells shows increased exosome secretion through the PLP2-dependent pathway.

Control of MVB transport and fusion with the plasma membrane

Molecular research related to exosome transport and secretion highlights the significant role of the Rab protein family. Rab proteins have the ability to control the movement of vesicles in the endocytic and secretory pathways by interacting with specific protein effectors on the membrane surface. They can stimulate the fusion or shortening of vesicles in target membranes by interacting with the cytoskeleton or binding to the receptor domains of the membrane. More than 60 Rab proteins have been identified in humans, each of which preferentially cooperates with a specific intracellular pathway [5].

In the final stage, it appears that SNARE proteins mediate the fusion of MVBs with the plasma membrane. SNAREs are a family of proteins that regulate vesicle fusion. During membrane integration, vesicular SNAREs (v-SNAREs) located in MVBs interact with target SNAREs located on the intracellular side of the plasma membrane, forming a SNARE complex.

The presence of SNARE proteins such as SNAP-23, VAMP-7, and VAMP-8 in the fusion of secretory lysosomes with the plasma membrane has been reported in various cell types. However, the significant role of SNARE proteins in exosome release has been minimally studied. Fader and colleagues demonstrated that VAMP-7, acting as a v-SNARE, plays a role in the fusion of MVBs with the plasma membrane during the release of extracellular vesicles carrying acetylcholinesterase in the K562 cell line.

It appears that various SNARE proteins are implicated in the fusion process between the MVB membrane and the plasma membrane. However, it is still uncertain whether the same SNARE proteins are involved in this process across different cells with distinct MVBs [20].

Absorption methods of extracellular vesicles

After the release of extracellular vesicles into the extracellular environment, they utilize a series of mechanisms to reach target cells and deliver their contents. Scientists have identified three absorption methods for extracellular vesicles, considering their heterogeneity and the specific type of target cells: 1) Entry route; 2) Receptor-ligand interaction route; 3) Direct integration route.

On their path to enter target cells, extracellular vesicles are taken up through various endocytosis pathways, including clathrin-dependent endocytosis, caveolin-mediated endocytosis, macropinocytosis, phagocytosis, and lipid raft-mediated internalization. In the receptor-ligand interaction pathway, extracellular vesicles interact with surface molecules on the target cell membrane without entering the cell directly.

For instance, extracellular vesicles derived from dendritic cells carry the ICAM-1 molecule on their surface, which binds to the LFA1-R molecule present on the antigen-presenting cell membrane. This interaction initiates cell signaling or activates T lymphocytes. Finally, in the pathway of direct integration between the phospholipid layers of cell membranes and

extracellular vesicles, they merge, allowing the vesicles to release their contents into the cell cytoplasm. This process resembles membrane fusion and involves various proteins, including Rab-GTPase and SNAREs. Therefore, identifying the absorption methods of extracellular vesicles can be beneficial in designing drug delivery systems [21].

In our study, while we aimed to provide a comprehensive review of EVs and exosomes, there are opportunities for further refinement and enhancement. Firstly, a more critical evaluation of the sources and data presented could strengthen the validity and reliability of our findings. Additionally, incorporating original research findings or novel insights would enrich the discussion and contribute to advancing the field. Furthermore, future studies could explore potential challenges, controversies, or gaps in understanding EV biology and therapeutic applications, offering valuable directions for further investigation. By addressing these areas, we can enhance the depth and impact of our research.

Conclusion

Complex mechanisms and numerous biomolecules are involved in the production and loading of exosomes. Over the past three decades, significant progress has been made in understanding the biology and function of exosomes. The discovery of exosomes has opened up a promising avenue for treating various diseases. These vesicles have the ability to transport biomolecules, making them potential therapeutic agents for the treatment of diverse diseases by manipulating their biological cargoes. Additionally, exosomes can be utilized as drug carriers to specifically target cancer cells.

Disclosure of conflict of interest

None.

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