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Circulating exosomes in ophthalmic disease: novel carriers of biological information circulating exosomes in ophthalmic disease

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Abstract. – Exosomes, small membrane vesicles with a diameter of 30-100 nm, transport lipids, proteins, DNA, and RNA. Exosomes originate from endocytic vessels and are processed and released through exocytosis. They can be taken up by target cells and mediate intercellular communication. Initially, exosomes were thought to be waste products excreted by cells. However, with more research, they have been found to play important roles in physiological and pathological processes. Therefore, they are promising biomarkers for the diagnosis and treatment of a variety of disease conditions, including fundus diseases, ocular surface diseases, retinal diseases, tumors, ocular trauma, and light damage. In this review, we discuss the history, biogenesis, release, isolation, characterization, and biological functions of exosomes, as well as their future application prospects in ophthalmic diseases.

Key Words:
Exosomes, Biological functions, Diagnosis, Target treatment, Ophthalmic disease.

Abbreviations

MSC: mesenchymal stem cell; RPE: retinal pigment epithelium; qRT-PCR: quantitative reverse transcription-PCR; miRNAs: microRNAs; AMD: age-related macular degeneration; MVBs: multivesicular bodies; HSP: heat shock proteins; LSC: limbal stem cell; NTA: nanoparticle tracking analysis; TEM: transmission electron microscopy; TM: trabecular meshwork; UM: veal melanoma; MHC: Major Histocompatibility Complex; circular RNAs: CircRNAs.

Introduction

In 1967, Wolf[1] discovered extracellular vesicles in plasma, which at the time were thought to be platelet wastes. In 1983, Pan et al[2] discovered exosomes in the supernatant of cultured red blood cells from sheep and they were believed to be the excretion channels of transferrin receptors. The concept of exosomes was first formally proposed in 1987[3]. In 1996, Raposo et al[4] discovered that exosomes could alter the extracellular microenvironment, present antigens, stimulate T cell proliferation, induce immune responses, and affect health, and thus, they were considered as immunological vaccines. In 2007, Valadi et al[5] first confirmed that mRNA and miRNA in exosomes exhibit biological activity.

Exosomes are widely found in the peripheral blood, cerebrospinal fluid, urine, milk, alveolar lavage fluid, uterine eluent, and other body fluids, suggesting that they are potential serological diagnostic markers. Exosomes are produced and released by different cells, playing roles in various biological processes, such as immune regulation; coagulation; cell proliferation, differentiation, and migration; and information transfer. In 2013, the Nobel Prize in Physiology or Medicine was awarded to J. E. Rothman, R. W. Schekman, and T. C. Sudhof, three scientists who elucidated the molecular mechanism underlying the transport and transmission of cell vesicles. In 2015, Huan et al[6] demonstrated that exosomes from acute myeloid leukemia inhibit hematopoietic stem cells in the leukemic bone marrow microenvironment, leading to extensive studies on the function of exosomes in stem cells and cancer cells.

In recent years, the development of the high-throughput second-generation sequencing technology has led to the identification of many candidate biomarkers, which serve as markers for the occurrence and development of diseases in the circulatory, respiratory, digestive, nervous, urinary, and reproductive systems[7]. However, studies on the role of exosomes in ophthalmolog-

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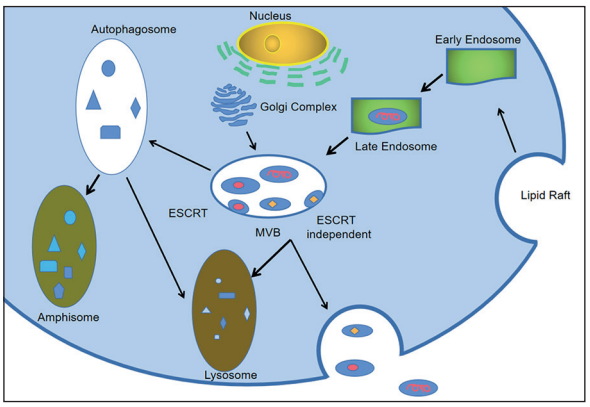

Figure 1. Biogenesis of exosomes.

ical diseases are lacking. This review discusses advances in the study of the biology and potential role of exosomes in ophthalmologic diseases.

Biological Characteristics of Exosomes

Exosomes originate from the invaginations of cell membranes, form intracellular vesicles; get encapsulated by endocytosis to form early endocytosomes, and selectively absorb proteins, lipids, and RNA in the cytoplasm to finally form late endosomes (multivesicular bodies, MVBs). Some of the MVBs are phagocytized and degraded by lysosomes, while the others fuse with the cell membrane and are released into the extracellular space (Figure 1). These vesicles are of different types and are classified based on their diameter: exosomes (30-150 nm), apoptotic bodies (800-5,000 nm), and microvesicles (100-1,000 nm) (Figure 2). Exosomes are nanosized, double-layered phospholipid membrane vesicles, which are uniform in shape (cup-shaped or oblate) and size and are present in body fluids. The formation of exosomes can be divided into either endosomal sorting complex required for transport (ESCRT)-dependent or non-dependent[7].

Sorting of Cargo in Exosomes

Exosomes are released from many kinds of cells, such as epithelial cells, endothelial cells, neurons, dendritic cells, mast cells, lymphocytes, and platelets[8]. They widely exist in blood, urine, saliva, and ascites[9]. In addition, exosomes can be extracted from cell culture media. Exosomes are essentially different from other types of vesicles because their composition is more complex, and exosomes originating from different cells differ in their contents. The three main contents of exosomes are proteins, lipids, and nucleic acids, with proteins being the main cargo (up to 41,860), followed by RNAs (7,540) and lipids (1,116)[10]. The RNAs enclosed in exosomes include both coding and non-coding RNAs, such as miRNA, long non-coding RNA (lncRNAs), and circular RNA. Exosomes transport these contents to target cells, facilitating cell-to-cell communication.

The proteins found in exosome include both non-specific and specific proteins, with non-specific proteins forming the majority[11], including membrane transport and fusion proteins (such as GTPases, annexin, and flotillin), transmembrane proteins (such as CD9, CD63, and CD81), skeletal proteins (such as tubulin and myokines), heat shock proteins (HSPs) (such as HSP70, HSP84, and Hsp90), and metabolic enzymes (such as phospholipase and pyruvate esterase). These non-specific proteins are involved in the formation of exosomes, determine their structure, and maintain basic cell functions. On the other hand, the specific proteins[12] are closely related to the specific functions of cells. For example, exosomes from B cells carry major histocompatibility complex (MHC) class I, MHC class II, and co-stimulatory factors, which directly stimulate the anti-tumor response of $CD4^+$ cells, and exosomes from dendritic cells include more contents that have antigen presentation ability, which enhances the cytotoxic T cell-killing ability.

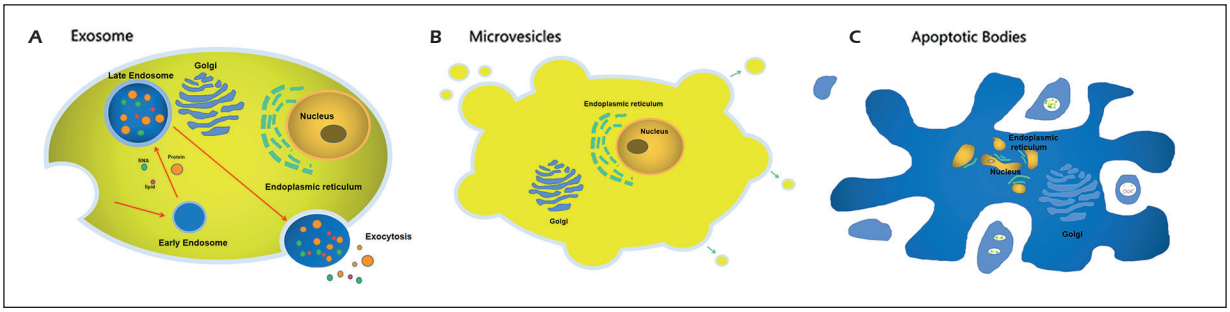

Figure 2. Classifications of extracellular vesicles. **A,** Exosomes. **B,** Microvesicles. **C,** Apoptotic bodies.

Exosomes are also rich in a variety of lipids, including cholesterol, sphingomyelin, phosphoglycerate, ceramide, and saturated fatty acids. The intrinsic rigidity of saturated phospholipids plays an important role in maintaining the morphology and the high stability of exosomes. In addition, there are a variety of mRNAs, miRNAs, lncRNAs, circular RNAs, and other non-coding RNAs (ncRNAs) in exosomes[13,14]. Therefore, exosomes act as effective carriers that transport RNAs to target cells and regulate protein synthesis and cellular function. MiRNAs from exosomes are associated with many diseases. In cancer patients, exosomal miRNAs from the systemic circulation are similar to those in the exosomes from the original cancer cells, suggesting that exosomal miRNAs are potential diagnostic biomarkers for cancer[15-17]. Exosomes can be effectively separated and detected from almost all body fluids. Exosomal miRNAs extracted from blood, urine, and other body fluids[18] can be used as diagnostic markers, providing a wide possibility for the non-invasive diagnosis of diseases.

Circular RNAs (circRNAs) are endogenous ncRNAs with a closed loop structure; they are highly stable in the extracellular matrix and blood and they also act as a molecular sponge that competitively binds to miRNA and reverses miRNA-mediated inhibition of gene expression, thereby increasing target gene expression. CircRNAs are a positive regulator of polymerase II, resulting in a positive regulation of parental genes[19,20]. The amount of circRNAs in exosomes is at least twice that in cells. Exosomes act as transport tools and protect circRNA stability during transport. However, the mechanism underlying exosomal circRNA transport and clearance is unclear.

Physiological Functions of Exosomes

Exosomes released by cells are taken up by receptor cells wherein they release their contents and mediate intercellular information exchange[21]. Exosomes from different cell types have different functions. Exosomes derived from immune cells (such as B cells and dendritic cells) can mediate specific immune responses in tumor or target cells. Exosomes from tumor cells play an important role in tumor metastasis[22,23]. Prions, bacteria, and other pathogens release exosomes, which transport the pathogenic source between host cells and participate in immune escape[24]. Exosomes and their contents promote information exchange between cells and receptor cells, resulting in a variety of pathophysiological reactions. However, the detailed mechanisms that regulate exosome release and uptake remain unclear. ISGylation promotes the fusion of MVBs with lysosomes and reduces the release of exosomes from cells[25]. Rab5a and Rab9a are two proteins that promote the degradation of MVBs. Rab27a, the actin-binding protein cortactin, GTPases, such as RhoGTPases, and soluble N-ethylmaleimide-sensitive factor attachment protein receptor E proteins can promote exosome fusion with membranes and release from cells[13]. The mechanisms of the uptake of exosomes by receptor cells also remain unclear. There are three methods of exosome uptake by receptor cells: direct fusion, receptor-ligand interactions, and endocytosis[26]. Endocytosis is believed to be the major method of uptake, which includes clathrin, caveolin, and lipid raft-mediated endocytosis[27]. In addition, some special surface proteins in exosomes also mediate exosome uptake by target cells.

Isolation of Exosomes

Current methods for the isolation of exosomes are time-consuming and laborious and include ultracentrifugation, microfluidics techniques, ultrafiltration, and commercial exoQuick kits[28]. The different isolation methods have their own advantages and disadvantages (Table I).

Ultracentrifugation

Ultracentrifugation, a classical method for exosome isolation, has been widely used in many laboratories. The detailed steps of ultracentrifugation are as follows: centrifugation of samples at 300× g for 10 min, 2000×g for 10 min, 10000× g for 30 min, followed by 100000× *g* for 80 minutes. It is simple, convenient, and cost-effective, which is especially suitable for large sample volumes[29]. However, this method also has some disadvantages. The ultracentrifuge (Beckman Coulter, CA, USA) is expensive, and the process of isolation is time-consuming. In addition, small amounts of samples or rare clinical samples are not suitable for this method[30].

Density-Gradient Centrifugation

Density-gradient centrifugation increases the separation efficiency and exosome purity. The theory of density-gradient centrifugation such as Three-Phase Partitioning method using Ammonium sulfate powder (Fisher Scientific, Princeton, NJ, USA) is the use of a gradient medium to enrich the exosomes at an appointment position and

Table I. Methods for isolation of exosomes.

Method	Advantages	Disadvantages
Ultracentrifugation	1. Large samples can be analyzed 2. Simple to operate 3. Less destructive	1. Time-consuming 2. Instrumentation is expensive
Density-gradient centrifugation	1. Highly pure exosomes 2. Good integrity of the structure and function	1. Time-consuming 2. Instrumentation is expensive 3. Includes multiple overnight centrifugation steps
Ultrafiltration	1. Rapid and simple 2. High purity of exosomes	1. Protein contaminants 2. Expensive
Immuno-Affinity Purification	1. High specificity 2. Large quantities can be analyzed 3. High quality 4. High sensitivity	1. Expensive 2. Separating exosomes from magnetic beads is complex 3. Biological activity of exosomes is uncertain
ExoQuick techniques	1. Simple operation 2. High extraction efficiency 3. Saves time	1. Expensive 2. Unsuitable for large-scale analysis

then separate the exosomes from the medium[31]. This method is more complex than ultracentrifugation because it involves the addition of sucrose solution before the final step of ultracentrifugation at 100000× g. However, exosomes with higher purity and structural integrity structure can be obtained using the sucrose buffer[32].

ExoQuick Techniques

In recent years, many commercial exoQuick kits have been developed for quick isolation of exosomes[33]. One of the famous kits is the Invitrogen exoQuick kit, which can extract exosomes from culture supernatants. This commercial exoQuick kit isolates exosomes in a shorter time compared to the traditional methods, because ultracentrifugation is not required during the extraction process[34]. In brief, samples are centrifuged at 300× *g* for 10 min, 2000× *g* for 30 min, and 10000× g for 60 min. The existence of commercial exoQuick kits enables (Invitrogen Thermo Fisher Scientific, Waltham, MA, USA) easy extraction of exosomes with high extraction efficiency. However, commercial ExoQuick kits are too expensive to be widely used. In addition, they are not suitable for large-scale sample processing[35].

Characterization of Exosomes

Nanoparticle tracking analysis (NTA), Western blotting, and transmission electron microscopy (TEM) are the main methods used to evaluate the morphology and size of exosomes (Table II).

TEM

TEM (HD-2700, Hitachi High-Technologies Corporation, Japan) is one of the most commonly used methods to observe the characteristics of exosomes[36]. This method involves many preparation steps, making it slightly complex. In brief, samples are fixed in glutaraldehyde and dehydrated before detection. Then, a vacuum environment is created. A beam of electrons passes through the samples, resulting in a beam of secondary electrons, which are collected and magnified. However, the morphology of exosomes might change during dehydration. Therefore, cryo-electron microscopy is considered the perfect method for observing exosomes[37].

NTA

NTA performed with a NanoSight LM20 (NanoSight, Amesbury, United Kingdom) is a method for detecting exosomes based on Brownian motion. NTA obtains the particle velocity data and finally analyzes the size and concentration[38]. The advantage of NTA is its high efficiency because it requires only a few minutes to detect the characteristics of exosomes. Sample preparation for NTA is easy, and it does not destroy the exosomes in the liquid. The samples can be restored to their original form and utilized for repeated applications[39].

Exosomes in Ophthalmology

Exosomes are widely distributed in body fluids and are secreted or taken up by almost all cells.

Table II. Identification of exosomes.

Method	Advantages	Disadvantages
Nanoparticle Tracking Analysis	1. Simple and fast 2. Suitable for up to 30 nm diameter 3. Samples can be recycled	1. Expensive 2. The correct dilution of sample remains ambiguous
Resistive Pulse Sensing	1. Concentration 2. Size distribution 3. More suitable for 50 nm diameter	1. Poor stability 2. Background noise
Atomic Force Microscopy	1. Sample saving 2. Non-destructive mode of operation	1. Excessive analyses
Transmission Electron Microscopy	1. Simple and fast 2. Widely used	1. Preparation is complex 2. Destructive methodology
Flow Cytometry	1. Provides physical and chemical characteristics 2. Measurement of size is possible	1. Not suitable for detection of exosomes 2. Flow cytometer with high sensitivity is expensive

Exosomes are involved in several biological functions and have been used in translational medicine (Figure 3). Currently, an increasing number of studies have found that exosomes regulate tumor metastasis, cardiovascular disease progression, and nervous system diseases. However, the number of studies on exosomes in ophthalmology remains limited.

Ocular Surface

Hou et al[40] used row isotope labeling and relative and absolute quantification, combined with liquid chromatography tandem mass spectrometry, to analyze pterygium tissue and conjunctival fibroblasts in patients with pterygium. This study identified 433 proteins, including 135 exosome proteins, suggesting that exosomal proteins play an important role in the pathogenesis of pterygium. Limbal stem cell defects are the pathological features of many types of keratopathy. Traditional treatment methods include limbal stem cell (LSC) transplantation, oral mucosa epithelial transplantation, and pluripotent stem cell transplantation. However, there are many technical and ethical limitations, in addition to the difficulties in obtaining donor sources during treatment. The cell microenvironment, including paracrine factors, directly affect the survival and regeneration of LSCs[41]. Therefore, evaluating the roles of exosomes in corneal regeneration and using exosomes to transport some special proteins will facilitate treatments for LSC defects. Han et al[42] confirmed the existence of exosomes in human and mouse corneal epithelial cells and found that the exosomes secreted by mouse corneal epithelial cells fuse with stromal cells and induce fibroblast transformation. Exosomes from the corneal epithelium are involved in corneal repair and neovascularization. Therefore, exosomes can be used for the treatment of corneal diseases. Matrix metalloproteinase (MMP) 14 promotes neovascularization. Corneal fibroblasts secrete exosomes that contain activated MMP14. These exosomes are taken up by vascular endothelial cells and MMP14 is transported from corneal fibroblasts to vascular endothelial cells through exosomes. The transport of MMP14 via exosomes from corneal fibroblasts offers a new target for the treatment of corneal neovascularization[43].

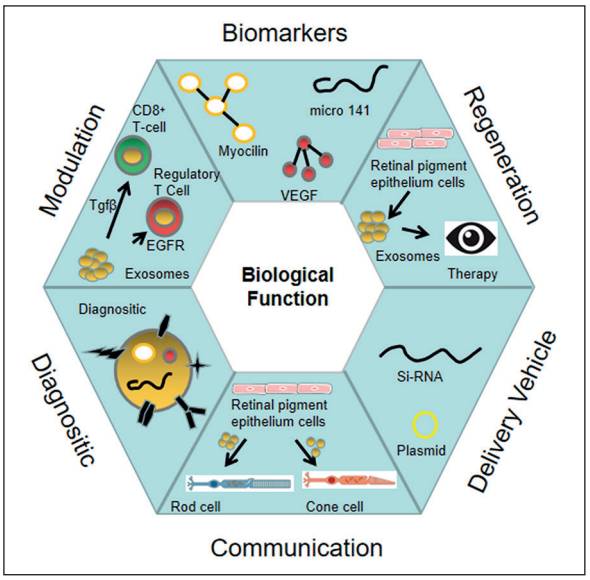

Figure 3. Functions of exosomes, including as biomarkers; diagnostic tools; communication and delivery channels; and involvement in cell/tissue regeneration as well as modulatory activities.

Glaucoma

Myocilin is a secretory protein, which when mutated elevates intraocular pressure. Approximately 3-4% of primary open-angle glaucoma cases are related to myocilin[44]. Myocilin is mainly associated with the cell membrane through its coiled structure, but the mechanism of extracellular transport remains unknown[45]. Perkumas et al[46] detected myocilin expression in the aqueous humor of cadaveric eyes, the aqueous humor of the anterior chamber obtained from patients undergoing cataract or glaucoma filtration surgery, and primary cultured trabecular meshwork (TM) cells. In addition, they also found that myocilin was abundant in exosomes. This suggests that exosomes may be the main pathway for the extracellular transport of myocilin among different cells in the eye. Perkumas et al[46] analyzed the mass spectrum of exosome proteins from primary cultured TM cells and found 108 characteristic exosomal proteins. In addition, there are a variety of TM cell-specific proteins, such as myocilin, emilin-1, and neuropilin-1, suggesting that exosomes may participate in the regulation of intraocular pressure. Liu et al[47] discovered 483 differentially expressed genes in the TM tissue from primary open-angle glaucoma patients. Among the 483 genes, 36 exosome component genes were found, suggesting that abnormal transport of exosomes might potentially be involved in the pathogenesis of primary open-angle glaucoma.

Retina

Age-related macular degeneration (AMD) is one of the main causes of central vision loss in the elderly. The pathogenesis of early AMD is related to the accumulation of lipids and proteins under the retinal pigment epithelium (RPE) layer, resulting in the thickening of the Bruch membrane and the formation of discontinuous RPE deposits (drusen). Wang et al[48] identified exosomes in drusen in AMD patients. The exosomes interact with complement H, which is related to the pathogenesis of AMD. These suggest that RPE cells are involved in the formation of drusen by transporting and releasing intracellular proteins through exosomes. Kang et al[49] isolated exosomes from the aqueous humor of patients with AMD, extracted their proteins, identified them using quantitative proteomics, and compared them with those of the control group. Six proteins with significantly increased expression, including aortic smooth muscle actin, myosin-9, HSP 70, cathepsin D, cytokeratin 8, and cytokeratin 14, were identified. These can be used as diagnostic biomarkers and therapeutic targets for AMD. Dedcrystallin D can help resist oxidative stress injury in RPE and AMD. Kannan et al[50] confirmed that this crystallin could be transported to the adjacent RPE and photoreceptor cells through exosomes and plays a protective role. Atienzar-aroca et al[51] found that more exosomes are released from RPE after induction by oxidative stress. When the exosome proteins were extracted, they exhibited high expression of vascular endothelial growth factor receptor. Further, when the isolated exosomes were co-cultured with endothelial cells, they significantly enhanced the vascularization ability of endothelial cells.

The immunoregulation of exosomes is involved in the pathological processes of retinal inflammation and fibrosis. Knickelbein et al[52] found that exosomes originating from the RPE stimulated by inflammatory factors could kill monocytes, mediating the local immune response. Exosomes can transport anti-inflammatory drugs to microglia, inhibit neuroinflammatory responses, and play a neuroprotective role in photoreceptor cells[53]. Exosomes also play a protective role in the pathogenesis of ischemic retinopathy. Moisseieev et al[54] injected mesenchymal stem cell-derived exosomes into the vitreous cavity of mice with induced by oxidative stress and found that the area of neovascularization was significantly reduced, suggesting that exosomes have a protective effect in ischemic retinopathy.

Tumor, Ocular Trauma, and Light Damage

Uveal melanoma (UM) is a rare malignant tumor. Ragusa et al[55] isolated and identified 179 miRNAs in exosomes originating from the vitreous humor of UM patients; 147 miRNAs were isolated from both the vitreous humor and serum. Among these 147 miRNAs, miR-146a was upregulated in the exosomes from the vitreous humor, serum, and tissue from UM patients, suggesting that miR-146a in exosomes can be a potential diagnostic marker for UM. This study also suggested that exosomes originating from body fluids or tissues provided similar diagnostic information as those originating from intraocular tissue fluid, making diagnosis simpler and more convenient.

Yu et al[56] established a mouse model of laser-induced retinal injury. Exosomes originating from mesenchymal stem cells were introduced through intravitreal injection. These exosomes

could significantly downregulate the expression of monocyte chemotactic protein 1, effectively inhibiting the inflammatory response induced by laser injury. Mead et al[57] established an animal model of optic nerve contusion and injected mesenchymal stem cell-derived exosomes into the vitreous cavity. The survival rate of retinal ganglion cells improved significantly, cell function was restored, and the axon loss rate of ganglion cells was significantly reduced. These results suggest that exosomes originating from mesenchymal stem cells play a protective role in traumatic nerve cell injury.

Conclusions

Exosomes have wide applications in tumors[58]; the advantages of exosomes in diagnosis and treatment have received increasing attention. However, research on exosomes in ophthalmic diseases is still in the initial stage[59], and the role of exosomes in ophthalmic diseases remains unclear[60]. Current evidence shows that contents in exosomes contribute to their broad biological functions including information transmission, immune regulation, and regeneration in many diseases. There are no studies on the role and applications of exosomes in crystalline lens-related diseases, uveitis, orbital inflammation, or tumor lesions. Taken together, this review advances the understanding of the role of exosomes in ocular diseases. Exosomes can transport their contents, are differentially expressed under different pathophysiological conditions, and exhibit homology in a variety of body fluids. This indicates that exosomes may become an effective tool in ophthalmic research and clinical treatment and play an important role in the diagnosis and treatment of fundus diseases. In addition, exosomes can cross biological barriers which provide better and personalized treatment to patients with eye diseases. However, further studies on the role of exosomes in ophthalmic diseases are required. There is a need for the development of non-invasive and early diagnostic tools for ophthalmic diseases. One such option is the detection of specific biomarkers in exosomes originating from the aqueous humor[61], vitreous humor[62], and other body fluids[63-65]. In addition, there is a need for research on the extraction or synthesis and injection of exosomes into the vitreous cavity or anterior chamber as drug carriers for the treatment of endophthalmic diseases. Moreover, there is no established standard for separating and purifying exosomes, let alone the mass production of exosomes, which needs urgently require research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Jiaxin You and Guanfang Su designed and supervised the study; Shounan Qi performed the analysis; Chenguang Wang contributed to the data analysis; Jiaxin You organized, designed, and wrote the paper. All authors have reviewed the final manuscript.

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