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Extracellular vesicles: pathogenic messengers and potential therapy for neonatal lung diseases

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Extracellular vesicles (EVs) are a heterogeneous group of nano-sized membranous structures increasingly recognized as mediators of intercellular and inter-organ communication. EVs contain a cargo of proteins, lipids and nucleic acids, and their cargo composition is highly dependent on the biological function of the parental cells. Their cargo is protected from the extracellular environment by the phospholipid membrane, thus allowing for safe transport and delivery of their intact cargo to nearby or distant target cells, resulting in modification of the target cell's gene expression, signaling pathways and overall function. The highly selective, sophisticated network through which EVs facilitate cell signaling and modulate cellular processes make studying EVs a major focus of interest in understanding various biological functions and mechanisms of disease. Tracheal aspirate EV-miRNA profiling has been suggested as a potential biomarker for respiratory outcome in preterm infants and there is strong preclinical evidence showing that EVs released from stem cells protect the developing lung from the deleterious effects of hyperoxia and infection. This article will review the role of EVs as pathogenic messengers, biomarkers, and potential therapies for neonatal lung diseases.

KEYWORDS

extracellular vesicle, neonatal lung disease, bronchopulmonary dysplasia, mesenchymal stem cell (MSC), biomarkers

1. Introduction

Airway cells are often exposed to microbes, environmental insults such as hyperoxia, hypoxia, and mechanical stimuli. These ecological cues induce airway injury, inflammatory responses, and repair processes in the respiratory system. Coordinated intercellular communication is required to maintain lung homeostasis. However, constant exposure to these environmental insults can damage the epithelial barrier leading to excessive inflammatory responses and lung pathology. In the last decade, extracellular vesicles (EVs) have been recognized as important mediators of lung homeostasis and disease (1).

EVs are nano-sized particles characterized based on their physical properties such as size (small EVs are <200 nm and large or medium EVs are >200 nm) or density (low, middle or high), biochemical composition (CD63$^+$/CD81$^-$ EVs, Annexin A5 EVs, etc.) and description of conditions or cells of origin (lung epithelial cell-derived EVs, podocyte-derived EVs, hypoxia-induced EVs, etc.) (2). EVs contain a cargo of cell-specific lipids, proteins,

metabolites, and nucleotides that influence the molecular and functional properties of neighboring and distant target cells (2).

EVs are also categorized based on how they are generated (2). EVs generated by directly budding of the cell plasma membrane have been termed microvesicles, and these are typically 100–1,000 nm in size (3). On the other hand, exosomes (30–100 nm in diameter) are formed from exocytosis of intraluminal vesicles (ILVs). ILVs are generated by endocytosis of cellular cargo (proteins, lipids, metabolites, nucleotides), forming endosomes and subsequently multivesicular bodies (MVBs). MVBs are transported to the plasma membrane through the cytoskeletal and microtubule network. They undergo fusion with the plasma membrane and secretion of ILVs into the extracellular space as exosomes (4). This is regulated by various signaling mechanisms and stimuli, including receptor activation by adenosine triphosphate (ATP) and lipopolysaccharide (LPS) (5, 6). The process also involves the assembly of SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) complexes, which draw opposing membranes together to create the energy required for membrane fusion (7). Microvesicles are released through the outward budding and fission of the plasma membrane; this is calcium dependent and associated with cytoskeleton remodeling (3, 8–10).

Specific combinations of proteins and lipids such as tetraspanins, adhesion molecules, glycoproteins, cholesterol, sphingomyelin, and antigen presenting molecules are present on the surface of EVs (2). The exact composition is however dependent on the EV cellular origin, pathogenic conditions, and the mechanism of biogenesis (2). These proteins and lipids influence cellular transport, target cell identification and reception, cargo sorting, and cell programming (8).

EVs are produced by almost all cell types in the respiratory tract (11). Cell types already studied include alveolar type II pneumocytes, pulmonary vascular endothelial cells (PVECs), macrophages, mast cells, and fibroblasts. Under stress such as infection, oxidative stress, and mechanical stress, EVs released by injured lung cells contribute to the development of lung pathologies (12). In addition, lung cell-derived EVs may serve as biomarkers for lung disease risk and severity (11). We will review the mechanisms by which EVs induce lung pathology, the role of EVs as biomarkers in both adult and neonatal lung diseases, and the potential of EVs as vehicles for drug delivery (**Figure 1**).

2. EV isolation

The EV membrane is composed of a phospholipid bilayer containing major histocompatibility complex molecules and tetraspanins. A major challenge of EV research however is achieving high purity EVs while maintaining their integrity and biological activity. **Table 1** summarizes common methods of EV isolation.

FIGURE 1

Structure, cargo and function of extracellular vesicles. Extracellular vesicles (EVs) are composed of a lipid bilayer containing transmembrane proteins with cargo consisting of proteins, mRNA, miRNA, DNA, and lipids. EVs can be isolated from various body fluids and have diverse sizes ranging from 100 to 1,000 nm. EVs isolated from the lung fluids and peripheral blood can be used as biomarkers for neonatal lung diseases. EVs have also been linked to the pathogenesis neonatal lung diseases. Mesenchymal stromal cell (MSC)-derived EVs and bioengineered EVs are potential novel therapies for neonatal lung diseases.

TABLE 1 Isolation of EVs.

Method	Advantages	Disadvantages	References
Ultracentrifugation	• Gold-standard • Cost-effective • High yield	• Time-consuming • Costly • Easily contaminated • Poor preservation of EV integrity and bioactivity due to high G force	(13, 14)
Ultrafiltration	• Simple • Cheap	• Low-yield • Poor preservation of EV integrity	(15)
Size-exclusion chromatography	• Cheap • Biologically intact EVs • Consistent yield	• Time-consuming but quicker than ultracentrifugation	(15–17)
Polymer precipitation	• Quick, simple process • Cost-effective	• Only smaller volume samples • Extremely prone to contamination by precipitation with non-exosome particles • Inconsistent results	(18, 19)
Immunoaffinity	• Quick, simple process • High purity	• Costly • Only smaller volume samples	(11, 13)
Membrane-based separation	• Quick • High yield • High purity	• Specific sample types only (e.g. urine), unable to handle samples with heterogenous cell types–will affect purity • Membrane clogging	(16)
Microfluidic platforms	• High-throughput • High yield • High purity	• Lack of standardization of devices resulting in heterogenous data • Only smaller volume samples	(20)

Ultracentrifugation is considered the gold standard. It utilizes extremely high centrifugal forces to separate EVs from other biological particles, is affordable and requires little technical skill. However, ultracentrifugation as a purification method is time-consuming, prone to contamination by other particles of similar weight and density, and EV integrity and bioactivity may not be preserved after ultracentrifugation (13, 14, 21).

Ultrafiltration employs physical filters of varying pore sizes and properties like application of electric charge and transmembrane pressure. Ultrafiltration can also be combined with other techniques such as low-speed centrifugation, ultracentrifugation, or size-exclusion chromatography (15). The process of ultrafiltration is simple to perform. However, there are limitations to sample processing – low-yield, membranes clogging, damage to EVs, types of samples that can be ultrafiltered, and the process of ultrafiltration is time-consuming (9).

Size-exclusion chromatography (SEC) is an increasingly popular technique that involves running samples through porous beads leading to separation of molecules by size. EVs isolated by SEC are biologically intact, making this method ideal for functional research (16). When used in conjunction with ultracentrifugation, EV yield and purity are significantly increased (17). The polymer precipitation method utilizes reagents such as polyethylene glycol (PEG) to cause precipitation of EVs, allowing isolation of EVs by simple centrifugation, making it cost-effective and efficient, but prone to contamination (18).

The immunoaffinity technique is done by priming a medium with target antibodies to bind with specific surface antigens or receptors present on EVs of interest. This isolates EVs with high purity, but this method is costly and difficult to sustain. Membrane-based separation methods isolate EVs through binding of membrane hydrophilic phosphate of EVs to metal oxides or the negatively charged membranes to positively charged molecules. This method is high yield, efficient and has high purity rates (19).

Microfluidic platforms are sophisticated networks utilizing various methods of purification organized in a miniature device. Purification methods include immunoaffinity, membrane-based filtration, nanowire trapping, acoustic nanofiltration, deterministic lateral displacement, and viscoelastic flow sorting. Microfluidic devices can achieve high throughput, high yield and high purity EVs, but there is a lack of standardization of devices contributing to heterogeneity of results reported by multiple investigators utilizing various devices (20).

3. EV characterization

Analyzing the particle size, morphology and biocomposition of EVs by multiple, complementary techniques is critical in evaluating the likelihood that biomarkers or functions are associated with EVs and not other co-isolated materials (2).

The International Society for Extracellular Vesicles has proposed the Minimal Information for Studies of Extracellular Vesicles-2018 (MISEV2018) guidelines, which recommends that the source and preparation of the EV must be described quantitatively (2). MISEV2018 also recommends using techniques that provide images of single EVs at high resolution

such as electron microscopy, using single particle analysis techniques that estimate biophysical features of EVs, and assessing the topology of EV-associated components (2). The commonly used EV characterization techniques are listed in **Table 2**.

Dynamic light scattering can be used to measure particle size, but analysis is limited when EVs of various sizes are present, and this cannot be used for functional analysis (22, 23). High resolution flow cytometry is a reliable and popular technique that enables structural analysis, quantification, and functional EV characterization (26). Nanoparticle tracking is a method by which the concentration, size distribution and particle velocity of EVs are measured. In nanoparticle tracking, specific antigens can also be identified with fluorescent tagged antibodies, providing more functional information (22). Atomic force microscopy is a technique that provides outputs of EV quantity, morphology, structural and functional analysis at a molecular level. This technique also preserves the integrity and bioactivity of EVs (24). Electron microscopy (EM) can also be used for structural characterization of EVs. EVs can be visualized with transmission EM, with a characteristic cup-shaped appearance of EVs due to dehydration during sample processing (25). Cryo-EM, on the other hand, allows for visualization of intact EVs without dehydration, enabling ultrastructural analysis of EV membranes and contents (31). EV membrane and cargo components can be analyzed with techniques according to molecule type, such as Western Blot and mass spectroscopy for proteins, and microarray and next generation sequencing for DNA or RNA (27–30).

Given the wide range of techniques available for both isolation and characterization of EVs with varying qualities, and with numerous research studies focusing on EVs that have been reported and are ongoing, there is a need for standardization of research protocols and techniques to maximize knowledge-sharing and productivity of the scientific community. Efforts are being made through the International Society for Extracellular Vesicles (ISEV) to create task forces and research guidelines to overcome these challenges (32, 33).

4. EVs in the pathogenesis of lung diseases

Increasing evidence indicates that EVs play essential roles in the pathogenesis of various adult lung diseases, including acute lung injury (ALI), acute respiratory distress syndrome (ARDS), asthma, chronic obstructive pulmonary disease (COPD), and pulmonary hypertension. The involvement of EVs in neonatal lung diseases has also been reported in bronchopulmonary dysplasia (BPD), but much less is known.

4.1. EVs and adult lung diseases

ALI and ARDS are devastating and rapidly progressive respiratory disorders that are characterized by disruption of the integrity of alveolar and vascular endothelial barriers (34–36). In

TABLE 2 Characterization of EVs.

Technique	Main features	Drawbacks	References
Nanoparticle tracking analysis (NTA)	• One of the most used methods • Provides parameters of concentration and particle size (10–2,000 nm)	• Critical parameters for success of NTA are sample preparation and the correct dilution factor	(2, 22)
Dynamic light scattering (DLS)	• Used for size measurements in the range of 1–6,000 nm • Possible recovering samples after analysis	• Detection of smaller particles becomes challenging in the mixture of small and large particles	(22, 23)
Atomic force microscopy (AFM)	• Detects the morphology of the sample in three-dimensional space • Generates topographic images of the samples with a resolution limit around 1 nm	• Measures samples in their native condition, which can turn into a limitation of the method as native state of different samples can be varied	(24)
Transmission electron microscopy (TEM)	• Images of high-resolution particles • Using immunogold-labeling to further reveal EV proteins	• Loss of material during extensive sample preparation • Lack of multiparametric phenotyping and low throughput capacity	(25)
Flow cytometry	• Records both the scattering and fluorescence signals • Analyzes multiple labels on individual particles • Identifies various types and subsets	• Low sensitivity to discriminate small size EVs • Low fluorescence being emitted by labeled EVs • Limited feasibility of post-stain washing to reduce background fluorescence	(26)
Protein content of EVs	• Proteomics technology allows the creation of large-scale profiling of proteins secreted through EVs • Immunoblotting can be used to detect EV markers and target proteins	• EVs must be broken prior to analysis • Some of the makers are not present in every/each EV • No single protein or combination of proteins can be recommended as universal EV markers	(27–30)
RNA content of EVs	• High-throughput RNA-seq • Validates by RT-qPCR	• Low yield of materials often below the detection limit of the most common quantification techniques	(27–30)

response to inflammatory stimuli, EVs and microparticles (MPs) are released from circulating inflammatory cells, damaged PVECs, and epithelial cells (37). In preclinical models, PVEC-derived EVs induce significant lung injury, as demonstrated by alveolar-capillary barrier failure, lung edema, and neutrophil infiltration in mice (38). These pathological effects are linked to and presumably at least in part mediated by the detrimental effects of PVEC-derived EVs on endothelial function. In ALI models, PVEC-derived EVs induce a reduction in endothelial nitric oxide (NO) production and an increased release of lung inflammatory cytokines (39).

Alveolar macrophage derived EVs are also abundant in the bronchoalveolar lavage fluid (BALF) in animal models of ALI. They are capable of inducing inflammatory responses both in vivo and in vitro (37, 40–42). Alveolar macrophage derived EVs trigger EV release by epithelial cells and neutrophils and deliver high concentrations of TNF-α to alveolar epithelial cells, leading to increased production of keratinocyte-derived chemokine and intercellular adhesion molecule-1 (37, 40–42), inducing a vicious cycle of inflammatory injury.

Alveolar epithelial cell derived EVs are also important mediators of ALI. In hyperoxia-induced ALI, alveolar epithelial cell-derived EVs are increased in BALF and serum (43) and they activate proinflammatory responses in systemic and pulmonary macrophages leading to disease progression (44).

COPD is characterized by severe airway inflammation and subsequent lung parenchymal damage. Mononuclear/macrophage-derived EVs rich in inflammatory mediators such as cytokines, chemokines, adhesion molecules, and proteases have been linked to alveolar wall destruction and emphysema, the hallmarks of COPD (11, 45). Endothelial-derived microparticles can promote the progression of COPD by inducing apoptosis of neighboring health endothelial cells upon delivery of inflammatory cargo (46). Epithelial-derived EVs have also been linked to the pathogenesis of COPD. Cigarette smoke stimulates human bronchial epithelial cells to release EVs enriched in full-length CYR61/CTGF/NOV family 1 (CCN1) protein that not only mediates IL-18 induced inflammation but also helps maintain lung homeostasis by increasing the levels of vascular endothelial growth factor (VEGF) (47). Cigarette smoke extract-induced human bronchial epithelial cell-derived EVs promote myofibroblast differentiation of lung fibroblasts, leading to the development of fibrosis (48). Cigarette smoke-exposed lung epithelial cells also release EVs that contain pro-inflammatory cytokines and Wnt-5a into the circulation, and these EVs can reach distant cells and organs (49).

EVs are also implicated in the pathogenesis of pulmonary hypertension. Patients with pulmonary arterial hypertension (PAH) have increased endothelial-derived CD62e microparticles in their pulmonary arterial blood (50). PAH patients also have increased microparticles positive for endothelial PECAM and VE-cadherin in their plasma samples (51). In monocrotaline-induced PAH, lung- and plasma-derived small-sized EVs isolated from monocrotaline-exposed mice induce PAH in healthy mice (52). EVs from PAH mice and patients contain elevated levels of miR-19b, miR-20a, miR-20b, and miR-145, known to target bone

morphogenesis protein receptor signaling, apoptosis, and cell proliferation. EVs from the lungs of PAH mice reduce apoptosis of PVECs (53). Furthermore, EVs released by PVECs from PAH mice convert healthy bone marrow-derived endothelial progenitor cells into a pathological progenitor phenotype. These cells induce pulmonary vascular remodeling when injected into the lungs of healthy mice (54).

4.2. EVs and bronchopulmonary dysplasia (BPD)

BPD is the most common adverse outcome of extreme prematurity (55). It is the result of antenatal injury to the developing lung combined with repetitive and multiple post-natal insults, including oxygen therapy and ventilation, leading to alveolar simplification and vascular rarefaction (55). Not much is, however, known about the role of EVs in BPD pathogenesis. Genschmer and collaborators compared the function of EVs derived from BALF from BPD and non-BPD infants in a murine model (56). Intriguingly, mice that received intranasal BPD-derived EVs had significant alveolar hypoplasia and right ventricular hypertrophy, suggesting a role for EVs in BPD pathogenesis (56).

Recently, Lal et al. also demonstrated that the tracheal aspirate of infants with severe BPD had higher EV particle concentrations as compared to control infants, and the majority of these EVs were derived from epithelial cells (57). EVs shed from hyperoxia and LPS-exposed epithelial cells had reduced miR-876-3p. Gain of miR-876-3p in murine models attenuated hyperoxia and LPS-induced alveolar simplification, highlighting a potential critical role of lung epithelial cell-derived EV-miRNAs in the pathogenesis of BPD (57). miRNAs are non-coding RNAs that bind to sequences in the 3′ untranslated region (3′UTR) of target mRNA, resulting in the destruction of target mRNA or its repression (58).

Recently, our laboratory investigated the critical role of circulating EVs from hyperoxia-exposed and mechanical ventilated newborn rats in inducing brain injury in healthy newborn rats (59, 60). In the hyperoxia model, newborn rats were exposed to room air or 85% oxygen for two weeks, and circulating EVs were isolated from the plasma of these rats. Fluorescence activated cell sorting (FACS) and Western blot analyses demonstrated that the EVs from hyperoxia-exposed rats contain increased levels of both surfactant C (SPC) and gasdermin D (GSDMD), a key executor of inflammasome-induced cell pyroptosis. When these EVs were adoptively transferred into healthy newborn rats by intra-tail vein injection, they were taken up by the lung and brain. In the lung, the EVs from the hyperoxia-exposed rats induced inflammation, indicated by increased inflammatory cell infiltration in the alveolar airspaces and expression of inflammatory cytokines and chemokines. Furthermore, alveolarization and vascular density were drastically reduced in the lungs that received EVs from hyperoxia-exposed rats. In vitro experiments with PVECs demonstrated reduced cell

proliferation and increased cell death when cultured with EVs from hyperoxia-exposed rats (59). Upon examining the brain, EVs from hyperoxia-exposed rats induced brain inflammation by activating microglia and increasing expression of pro-inflammatory cytokines. These changes were associated with increased cell death in the cortex, subventricular zone, and subgranular zone. Additionally, in vitro experiments showed that neural stem cells (NSC) had decreased proliferation and increased cell death when cultured with EVs from hyperoxia-exposed rats (59). EVs from cultured hyperoxia-exposed lung epithelial cells induced pyroptosis in NSC (59). This data revealed a novel lung-brain crosstalk mediated by lung epithelial-derived EVs in both lung and brain injury.

This EV-mediated lung-brain crosstalk was further investigated in mechanical ventilation-associated brain injury in newborn rat models (60). We demonstrated that injurious mechanical ventilation induced similar markers of inflammation and pyroptosis, such as IL-1β and activated caspase-1/GSDMD in both lung and brain, in addition to inducing microglial activation and cell death in the brain (60). EVs isolated from neonatal rats with ventilator-induced lung injury had increased caspase-1. Adoptive transfer of these EVs into healthy newborn rats led to neuroinflammation with microglial activation and activation of caspase-1 and GSDMD in the brain, similar to that observed in neonatal rats that were mechanically ventilated (60). Thus, circulating EVs can contribute to brain injury and possibly poor neurodevelopmental outcomes in preterm infants exposed to hyperoxia and mechanical ventilation (60).

5. EVs as biomarkers for lung diseases

The stability of EVs is a potential advantage over traditional biomarkers. Traditional biomarkers such as proteins and RNA molecules are often unstable and susceptible to degradation over time, making them less reliable for diagnostic purposes. In contrast, EVs are surrounded by a protective lipid membrane that helps to stabilize their contents, including proteins, nucleic acids, and other molecular components (61). Proteomic and phosphoproteomic studies conducted on EVs from different cell types have suggested that they transport a diverse range of biologically relevant molecules, such as lipids, carbohydrates, RNAs, and some are believed to exhibit heterogeneity in composition, which is dependent on their cellular origin (62). EVs can carry specific proteins or RNA molecules that are unique to lung diseases. For example, sputum of patients with severe asthma has elevated levels of miR-142-3p, miR-629-3p, and miR-223-3p (63), and sputum-derived EVs from idiopathic pulmonary fibrosis (IPF) patients show an aberrant expression of miR-142-3p, miR-33a-5p, and let-7d-5p compared to healthy subjects (64).

There are few reports that EV-miRNAs can be used as biomarkers for BPD (65). In the study by Lal et al., EV miR876-3p was a potential biomarker for severe BPD in preterm infants. Decreased expression of EV miR-876-3p at birth predicted the future development of severe BPD in ELBW infants (57). This

study established the predictive potential and causative role of microbiota-regulated miR-876-3p in severe BPD (57).

More recently, Ransom et al. characterized tracheal aspirate EVs in preterm infants between 22- and 35-week gestational age. Across all gestational ages, the majority of tracheal aspirate EVs expressed epithelial and immune cell markers. Moreover, infants who developed BPD had increased CD14+ EVs in their first tracheal aspirate obtained within 24 h of birth (66).

6. EVs as therapies for neonatal lung diseases

Mesenchymal stromal cells (MSCs) have regenerative properties and it is increasingly known that MSC-derived EVs replicate many of the beneficial effects of MSCs. EVs may also be bioengineered for drug delivery and genetically modified to carry specific target molecules. Although these therapeutic strategies are in the early stage of development, the prospect of using them in newborn infants is encouraging.

6.1. Stem cell derived EVs for newborn lung diseases

MSCs are efficacious in neonatal lung injury models (67–69). The pleiotropic properties of these cells make them particularly attractive and given their paracrine-mediated mechanism of action, MSC-derived EVs have been investigated as potential therapies.

In an experimental model of chorioamnionitis, antenatal administration of MSC-EVs reduced placental inflammation, and preserved lung structure, suggesting that antenatal MSC-EVs are efficacious in alleviating the deleterious effects of intrauterine inflammation. In experimental pre-eclampsia, MSC-EVs restore placental vascularity and preserve neonatal lung structure (70). In experimental BPD models, MSC-derived EVs restore alveolar structure, prevent lung vascular rarefaction, and alleviate PH by altering macrophage polarization, reprogramming bone marrow myeloid cells and increasing pro-angiogenic signaling pathways (70–77).

We recently compared the therapeutic efficacy of intra-tracheal (IT) and intravenously (IV) delivered MSC-EVs in a preclinical model of BPD. We demonstrated that systemically and IT delivered MSC-EVs have similar beneficial effects in experimental BPD (78). This finding is promising as IV MSC-EVs may also have beneficial effects on the developing brain (79). Another important question which we recently sought to address is the duration of MSC-EV therapeutic effects in experimental BPD. We administered MSC-EVs to neonatal pups with hyperoxia-induced BPD on postnatal day 3 and followed the pups into young adulthood (78). We found that one dose of MSC-EVs at postnatal day 3 had persistent beneficial effects at three month follow up (78). Importantly, late administration of MSC-EVs in an established BPD model was also found to partially reverse lung injury (79, 80). Clinical trials are now on the horizon but identifying the ideal patient will be critical.

6.2. Engineered EVs

EVs are also being investigated as "drug vehicles" (81). The ability of EVs to target a particular tissue or cell could be used to deliver drugs to intended targets while avoiding off-targets selectively (81). The "drug cargo" is selectively loaded into the EVs and the EVs are engineered to have specific properties to enhance their targeting and biomimetic features (82, 83). The lower number of transmembrane proteins, such as MHC complexes on their surface, make EVs less immunogenic than their parental source (84, 85). In addition, EVs do not replicate after injection. Thus, EVs are less likely to transfer latent viral pathogens or enable tumor generation (86). Compared to synthetic drug carriers, the intrinsic ability of EVs to cross cell barriers and penetrate tissues gives them an advantage (87). Synthetic drug carriers such as polymeric micelles and lipid nanoparticles cause high toxicity and immunogenicity compared to EVs (88). As therapeutic EVs are derived from benign biological or autologous sources, they are less likely to induce adverse effects.

Harnessing these unique properties of EVs to develop smart drug delivery systems with enhanced targeting, safety and pharmacokinetics has however been challenging (89). One study showed that that after intravenous injection, EVs are rapidly distributed and retained in the liver, spleen, gastrointestinal tract and lungs (90). Another study however showed rapid clearance of plasma-derived EVs following intravenous administration, with a half-life of approximately 7 min (91). Moving forward, more studies will be needed to understand EV circulation kinetics, biodistribution, cell tropism, and intracellular trafficking routes as the cellular origin, dose and route of administration may affect EV biodistribution pattern (92).

Other obstacles such as low isolation yield, the lack of purification protocols, large-scale clinical grade production, parental cell-dependent composition, and inefficient drug payload of the EVs continue to hamper the therapeutic ability of EVs (93). To improve *de novo* EV yield and therapeutic efficacy, re-engineering of the parental cell has been done through genome modification, stimulation with exogenous biomolecules and specific environmental factors (93). Bioreactors are also being extensively used to scale up the production of cell-based therapy and EVs. Bioreactors provide well-controlled nutrients, uniform culture conditions and biomimetic stimuli to regulate cell growth, differentiation and tissue development (94). While bioengineering of the parental cell predictably loads only a small proportion of the modified content into EVs, direct modification of isolated EVs may be another strategy to enrich EVs (95). For example, hydrophobically modified small interfering RNAs efficiently load into EVs upon coincubation, without altering EV size or integrity (96). Active EV loading can also be done by electroporation, sonication, extrusion, freeze-thawing and by surfactant-assisted loading, where surfactant saponin disrupts the membrane and increases its permeability (97).

Another option currently being investigated is the development of artificial EVs, namely the top-down and bottom-up approaches.

The top-down approach is based on the disruption of the cultured cells to produce membrane fragments that will be used to form vesicles, while retaining the same membrane features of the initial cell (98). The bottom-up approach starts from small components of molecular building blocks to create complex structures, namely synthetic EVs (99).

7. Conclusion

We presented the evidence for lung-derived EVs as novel biomarkers and mediators for neonatal lung diseases and the potential for MSC-derived EVs as novel therapeutic modalities for neonatal lung diseases. Many of the studies discussed in this review are preclinical investigations that require successful translation from the bench to the bedside. Given that lung diseases are among the most common complications in preterm infants, with few effective therapies, it is crucial to continue discovering and understanding how EVs contribute to neonatal lung diseases and how to harness EVs to prevent and treat neonatal lung diseases. The incredible features of EVs in terms of their biocompatibility, cargo loading, cellular uptake, and escaping the immune system make them an appealing therapeutic strategy, but determining the ideal patient, route, dosing and timing will be essential to move forward. Procurement of EVs from physiologically relevant environments, the ability to scale up their manufacturing, optimize their biodistribution, and *in vivo* kinetics will also be crucial (93). This will contribute immensely to increasing the potential of EVs as acellular nanoscale therapeutics for neonatal lung diseases.

Author contributions

SW, MB, JD, KW, AT, AS, KY: conceived, drafted and reviewed the final submitted version of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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