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Abstract

Lung and brain injury that occurs during the perinatal period leads to lifelong disability and is often driven and/or exacerbated by inflammation. Human amniotic epithelial cells (hAEC), which demonstrate immunomodulatory, anti-fibrotic, and regenerative capabilities, are being explored as a therapeutic candidate for perinatal injury. However, limitations regarding scalable manufacturing, storage, transport, and dose-related toxicity have impeded clinical translation. Isolated therapeutic extracellular vesicles (EVs) from stem and stem-like cells are thought to be key paracrine mediators of therapeutic efficacy. The unique characteristics of EVs suggest that they potentially circumvent the limitations of traditional cell-based therapies. However, given the novelty of EVs as a therapeutic, recommendations around ideal methods of production, isolation, storage, and delivery have not yet been created by regulatory agencies. In this concise review, we discuss the pertinence and limitations of cell-based therapeutics in perinatal medicine. We also review the preclinical evidence supporting the use of therapeutic EVs for perinatal therapy. Further, we summarize the arising considerations regarding adequate cell source, biodistribution, isolation and storage methods, and regulatory roadblocks for the development of therapeutic EVs.

Key words: extracellular vesicles; human amniotic epithelial cells; perinatal injury; brain; lung.
Significance statement
Perinatal brain and lung injury results in lifelong disability. While there is considerable evidence showing that human amniotic epithelial cells can treat perinatal injury, limitations involving cell source, and dose-related toxicity have hindered clinical translation. Extracellular vesicles (EVs) are messenger molecules that have shown therapeutic effects comparable to their cells of origin. This review discusses how EVs circumnavigate the limitations associated with whole-cell therapy and summarizes the preclinical data demonstrating the efficacy of EVs in reducing perinatal organ injury. Further, we discuss the manufacturing and regulatory considerations that need to be addressed to move EVs into clinical use.

Introduction
Perinatal pulmonary and cerebral injury
The perinatal period is critical for the adequate development of the pulmonary and cerebral systems. Adverse events during this time, such as preterm birth (birth before 37 weeks of gestation) and chorioamnionitis (invasion of the intrauterine membranes and amniotic fluid by bacterial pathogens), can compromise adequate development and result in lifelong diseases such as bronchopulmonary dysplasia (BPD) and cerebral palsy (CP).\(^1,\)\(^2\) Importantly, chorioamnionitis increases the risk of preterm birth. BPD is a pulmonary disorder clinically diagnosed by the need for supplemental oxygen at 36 weeks postmenstrual age and histologically observed by arrested and impaired lung structure with few and large alveoli.\(^2\) CP is a neurological disorder diagnosed following impaired or uncontrolled motor function such as spasticity, rigidity, and/or ataxia and occurs following injury to the developing white matter. White matter injury can occur during pregnancy, birth, or shortly after birth and is associated with elevated systemic and localized proinflammatory mediators (cytokines, chemokines, prostaglandins, and other immune mediators).\(^3,\)\(^4\) Although distinctly different conditions, the proinflammatory cascade involved in both prematurity and chorioamnionitis strongly contributes to the development of both BPD and CP.\(^5\) Congenital diaphragmatic hernia (CDH) is another antenatal condition known to impair pulmonary development and cause cerebral injury.\(^6\) CDH is characterized by a defective diaphragmatic development resulting in herniation of the abdominal contents into the thorax. This leads to compression of the fetal lungs and subsequent malformation, resulting in pulmonary hypoplasia and pulmonary hypertension (PH).\(^7\)

The economic impacts of prematurity and subsequent lifelong diseases is $1.4 billion in the first 18 years of life.\(^8\) Given the current inability to reduce injury and improve outcomes, novel therapies that target the perinatal inflammatory response and reduce/reverse organ injury are in dire need.
No methods currently exist to prevent preterm birth, cure chorioamnionitis, or prevent diaphragm malformation. Chorioamnionitis is generally managed by delivering maternal antibiotics, but maternal antibiotic treatment does not prevent chorioamnionitis-associated neonatal morbidities.9 Management of preterm neonates centers around providing ex-utero care that minimizes organ injury. Incomplete lung development often results in the need for pulmonary intervention. This includes corticosteroid administration to mothers at risk for preterm delivery to induce fetal lung maturation. Following delivery, neonates who are unable to maintain independent respiration are provided with exogenous surfactant and respiratory support. While lifesaving, mechanical ventilation is a known contributor to inflammatory lung injury, which arrests lung development, resulting in the pathogenic phenotype of BPD. Rates of BPD have not decreased in the past 5 decades. Additionally, recent evidence suggests that the pulmonary inflammatory response caused by mechanical ventilation may lead to the development of CP.10

Potential of cell therapies
Cell-based therapies have shown great potential in mitigating complex chronic disorders. Stem cells isolated from embryonic or fetal tissue and adult bone marrow, adipose tissue, and blood have shown regenerative and anti-inflammatory action in cardiovascular, pulmonary, and neurological conditions.11 Cell therapies are increasingly gaining traction in neonatal and pediatric disease settings, with investigations being conducted in clinical trials for BPD, CP, hypoxic-ischemic encephalopathy, and hypoplastic left heart syndrome.12 Importantly, cells isolated from gestational tissues demonstrate therapeutic action, including immune tolerance and anti-inflammatory, proangiogenic, and anti-fibrotic properties.13

BPD and CP are complex multifactorial conditions; however, the increase in proinflammatory cytokine expression in both disorders is a well-identified contributor.14,15 Perinatal inflammation is known to alter pulmonary and cerebral development and function, particularly impairing adequate vascular development.16,17 While the proinflammatory cascade is a major contributor to BPD and CP, it is also a therapeutic target, urging the development of targeted therapies. However, there is a current paucity of neonatal clinical trials, with the majority of trials involving adult males.18 Historically, hesitation in conducting trials in neonates was driven by ethical or operational concerns, which likely contributed to a lack of perinatal-specific testing. Ultimately, this has caused practitioners to adapt treatment to the neonatal population from adult therapy. Costa et al found that over 96% of premature neonates were exposed to off-label drugs, and 66% were unlicensed.19 “This is concerning, given that premature birth and birth weight impact pharmacokinetics and pharmacodynamics.”20 With the increasing prevalence of regenerative therapies, adequate safety studies must be performed in this patient population to inform adoption into widespread clinical use.

Human amniotic epithelial cell-based therapies
Preclinical studies have demonstrated the potential therapeutic benefit of human amniotic epithelial cell (hAEC) therapy for perinatal lung and brain injury. In the context of lung injury, Vosdoganes et al, Murphy et al, and Moodley et al have shown in small animal models of lung injury that hAEC treatment can modulate inflammation, reduce proinflammatory cytokines (IL-6 and TGF-β) and histopathological indices of lung injury; pulmonary arterial remodeling and collagen density. Moreover, hAEC treatment in these varying models showed their ability to improve indices of lung function, pulmonary capillary bed density, tissue-to-air space ratio, and secondary septal crest density.21-23 To validate these findings, more physiologically relevant models were used, Vosdoganes et al reported similar findings of hAEcs effect on modulating fetal pulmonary inflammation in an antenatal model of lung injury in fetal sheep.24 Moreover, Hodges et al reported the therapeutic efficacy of hAEcs in mitigating ventilation-induced lung injury in fetal sheep.25 Combined, these studies highlight the anti-inflammatory and protective action of hAEcs, which are likely attributed to the ability of hAEcs to shift the dominant phenotype of macrophages from classically activated M1 to the alternatively activated and immunomodulatory M2 phenotype.26 The findings of these studies underpinned the development of hAEcs used in first-in-human trials for the treatment of BPD. To date, allogeneic hAEcs have been shown to be safe and well-tolerated by extremely premature infants with established and severe BPD.12,27

So far, the potential of hAEC-based therapy for BPD is promising. Building on this body of evidence Zhu et al investigated key necessary variables that could help inform future clinical translation. These included the assessment of optimal cell doses, delivery routes, and timing of cell administration and their impact on BPD in a rodent model. Findings from these studies suggest that hAEcs provided prophylactically were more efficacious and no differences between intravenous and intratracheal delivery were observed.28 Currently, a dose-escalation trial is underway to assess the feasibility and safety of prophylactic treatment using allogeneic hAEcs in infants at >80% risk of developing moderate to severe BPD.29 More recently, the anti-inflammatory potential of hAEcs has been suggested to be limited by the severity of the inflammatory insult. In neonatal lambs exposed to antenatal inflammation and mechanical ventilation, Papagianis et al showed that hAEC treatment increased alveolar septation and subsequent alveolarization, but did not alter postnatal respiratory physiology.30

Importantly, hAEcs has also demonstrated the potential to treat perinatal cerebral injury. hAEcs treatment in perinatal mice exposed to inflammatory injury (antenatal endotoxin exposure and postnatal hyperoxia) protects against white matter damage, decreases microglial apoptosis, and prevents microglial activation.31 In fetal sheep exposed to antenatal inflammation, Yawno et al showed that hAEcs reduced fetal brain inflammation, reduced gray and white matter injury, and suppressed activated microglial cell upregulation. This correlated with decreased cell death in brain tissue.32 Further, hAEcs protected endogenous oligodendrocytes.33 These findings suggest that the neuroprotective actions of hAEcs in the fetal brain were likely driven by attenuating the fetal inflammatory response via anti-inflammatory effects. Investigating hAEcs as a potential therapeutic for preterm brain injury could be the next step to understanding its clinical safety and efficacy.

Limitations of cell therapy
The perinatal events that contribute to neonatal diseases are complex, and prophylactic treatments and/or repetitive
dosing are likely required to both prevent and rescue injury. Indeed, Cindy et al reported that multiple doses of mesenchymal stromal cells (MSCs) more efficiently improved sensorimotor outcomes and recovered MAP2 and MBP area loss greater than a single dose. However, the need for multiple doses is a limitation unique to cell therapies, including the need for an increased cell yield and evaluation of dose-related toxicity, which has been implicated in mesenchymal stem-cell therapy.

While autologous cell therapies avoid the risk of immune rejection, neonatal patients who are likely to require such interventions are also likely to be affected by pregnancy complications such as preeclampsia, preterm delivery, or fetal growth restriction. There is limited information on how pregnancy complications affect the therapeutic quality of perinatal stem and stem-like cells. Additionally, mesenchymal stem cells isolated from term umbilical cords displayed greater colony-forming efficiency compared to preterm, suggesting potential differences in MSCs across gestational ages. While allogenic therapies circumvent this limitation, inherent variability among donors can impact the reproducibility of therapeutic efficacy. Elaborated later in this review, therapeutic extracellular vesicle (EV) products isolated from allogeneic donors have currently been shown to be safe with low immunogenicity risk preclinically. Thus, the need for autologous sources would no longer be necessary. This bypasses a key roadblock in providing readily available therapies for pulmonary and cerebral neonatal conditions.

The method of cell administration is also a limitation to consider, as Lim et al showed that rapid infusion of hAECs increased the risk of hypoxia and bradycardia, secondary to pulmonary microemboli likely due to hemodynamic immaturity. This suggests the potential for dose-related adverse events. While changes in the infusion protocols were resolved with larger infusion volumes and slow delivery, repeated doses in future studies may not be tolerated well with infants with low blood circulating volumes. Additionally, slow and lengthy cell infusion protocols may reduce cell viability within the intravenous delivery lines, potentially increasing the risk of adverse events caused by flushing non-viable cells at the end of the procedure. Coupled with the additional safety concerns of tumorigenesis, the long-term efficacy and safety outcomes following the treatment of perinatal organ injuries need to be investigated further.

**Extracellular vesicles**

While cell therapies have shown therapeutic potential, clinical translation into the perinatal setting remains challenging as host rejection, tumorigenicity, and scalability issues present substantial barriers. Recent evidence suggests that the therapeutic effect of cells is not mediated by engraftment but by the release of paracrine factors, including EVs. EVs are naturally occurring membranous particles that encapsulate proteins, lipids, and nucleic acids and deliver their cargo to recipient cells. Therapeutic EVs have been shown to have effects comparable to parent cells. EV-based therapeutics may allow the field to overcome challenges faced by cell therapy as they:

- Have reduced risk for immunogenicity and tumorigenicity as EVs lack the cellular components responsible for a pro-inflammatory response, cell division, and tumor formation;
- Can be engineered for targeted delivery to specific tissues and organs, minimizing any off-target effects;
- Can be loaded with therapeutic cargo, such as proteins, nucleic acids, or drugs; and, simplified cold chain logistics.

**Extracellular vesicles for perinatal disease**

As previously discussed, the mechanisms that underlie the development of perinatal disease are complex and multifactorial. Interestingly, Zhong et al demonstrated that altered miRNA signaling arrests pulmonary vascular development and may contribute toward an increased risk of BPD development. The cargo carried by EVs can include a complex mixture of proteins, mRNAs, microRNAs, and nucleic acids. The delivery of cargo from therapeutic EVs, such as microRNA, that promotes pulmonary vascular growth showcases potential therapeutic modality. Recipient cells internalize EVs through mechanisms such as endocytosis or direct fusion with the cell membrane to deliver cargo to recipient cells. Delivered cargo can then alter gene expression, signaling pathways, and cellular processes in the recipient cell, thereby modulating critical cellular processes that are implicated in diseases. In the context of neonatal conditions, the transfer of bioactive cargo can heavily influence processes such as proliferation, differentiation, migration, and angiogenesis. The excessive immune responses that facilitate sustained tissue damage are implicated in many neonatal conditions. EV therapy is postulated to work by interacting with immune cells to modulate the immune system and create an anti-inflammatory environment to repair and regenerate damaged tissues.

Several studies demonstrate the potential of isolated EVs to ameliorate injury in various preclinical perinatal injury models such as hypoxemic-ischemic encephalopathy, necrotizing enterocolitis retinopathy or prematurity, and spina bifida. Currently, MSCs are the most investigated EV source. Tieu et al summarized the findings of 52 articles demonstrating the therapeutic efficacy of MSC-EVs for BPD, acute lung injury, and pulmonary arterial hypertension (PAH). Interestingly, while all EVs were protective against acute lung injury, EVs isolated by ultracentrifugation demonstrated greater therapeutic efficacy than tangential flow filtration for BPD and PAH. This may suggest that key therapeutic components may be removed depending on the isolation technique. While mechanisms are unknown, these findings demonstrate the clear potential of MSC-EVs to be developed as a therapy for acute and chronic lung diseases. It is also important to note that the therapeutic effects of other EV sources are also being investigated. These include astrocytes, umbilical cord blood (UCB), and MSCs derived from Wharton’s jelly (WJ), umbilical cord (UC), and bone marrow (BM), as described in Figure 1. EVs isolated from these sources demonstrate therapeutic potential in perinatal brain and lung injury. In this review, we explore hAECs as a promising source of therapeutic EVs.

A key benefit of hAEC-EVs over other cell types is the source material. hAECs are isolated from the amniotic membrane which is discarded after birth. They, therefore, represent an abundant EV source with minimal ethical concerns, unlike embryonic stem cells, and can be collected without invasive techniques, like BM-MSCs. Additionally, hAECs
are relatively easy to obtain and can be collected in large numbers. While UC-MSCs are also obtained from discarded perinatal tissue, Zhang et al have shown that only ~1 650 000 UCB-MSCs can be obtained from one cord blood unit.100 In contrast, ~120 000 000 hAECs can be isolated from a single amnion.101 A key limitation is the low proliferative capacity of hAECs, which limits the EVs that can be isolated to a single passage. Whereas ~1 650 000 UCB-MSCs can be passaged to reach over a billion cells. The therapeutic efficacy of EVs isolated from different sources is a current area of interest.

The use of EVs isolated from hAECs (hAEC-EVs) as a therapeutic agent for neonatal conditions is relatively novel and treatment efficacy is a current focus of study. Similar to hAECs, Zhu et al showed that hAEC-EVs reduced indices of lung injury through improved tissue-to-airspace ratios and secondary septal crest density in a murine BPD model.84 Further, other preclinical studies have shown hAEC-EV therapeutic efficacy in models of idiopathic pulmonary fibrosis,102 corneal injury repair,103 kidney damage,104 and myocardial infarction.105 Furthermore, several studies reported the potential of amniotic fluid stem cells (AFSC)-EVs at reducing or repairing CDH-derived lung injury in different preclinical models. Antounians et al CDH rodents treated with AFSC-EVs had restored lung tissue homeostasis and improved epithelial cell and fibroblast differentiation.73,75 Khalaj et al reported the rescue branching morphogenesis, differentiation of alveolar type 1 and 2 cell expression, and ciliated epithelial and pulmonary neuroendocrine cells following AFSC-EV treatment. Similar to the findings of Zhong et al for BPD, these studies attribute the observed effects to the presence of microRNA identified as EV cargo. Interestingly, Blundell et al also showed that AFSC-EVs could protect against cerebral inflammation caused by CDH.74 Taken together, these studies further highlight the robust potential of amnion-derived EVs to treat perinatal organ injury. We suggest that EVs isolated directly from amniotic cells will have increased therapeutic benefit to AFSC-EVs, secondary to increased purity and yield.

**Extracellular vesicles; the road to clinical utility**

Despite the therapeutic potential of EVs, several challenges limit clinical translation. These include the absence of reliable technologies for understanding their biodistribution, a lack of consensus around best practices for preservation, large-scale production, and regulatory guidance.

Understanding the upstream considerations of extracellular vesicle therapy

Establishing a cell source

EVs exert comparable therapeutic effects as their parent cells,106 and therefore, an integral aspect of EV production is identifying the appropriate cell source that demonstrates promising preclinical results. The development of a master bank is needed to enable the establishment of a sizeable, well-characterized collection of cells that serve as an EV source. To achieve this, cell characterization is required to assess cellular properties, such as cell purity, viability, and functionality. These can be validated by varying laboratory techniques such as flow cytometry, gene expression analysis, and functional potency assay assessments.107-109 In the case of EV-based therapeutics, the expansion and cultivation of cells need to be monitored carefully in controlled laboratory conditions with optimized culture protocols while monitoring cell behavior, as these can inherently affect EV composition and yield.

A deep understanding of the selected cell type will inform how a cell bank is established. There are 2 obvious options for creating a master cell bank, namely the pooling of allogenic donors and the use of genetically engineered cell lines, as stated in Table 1, each possessing its advantages and disadvantages. The simplest way to establish a cell bank involved using unmodified allogenic cells which is a faster and more cost-effective process. However, this method has significant drawbacks, including contamination risks, lack of clonal stability, and the potential for source variability to
be greater than what can be addressed by pooling. An alternative approach is creating immortalized master cell clone/s. This approach offers benefits such as controlled variability due to the homogeneity of the cell source, reduced contamination risk, and the ability to expand cells that were previously non-proliferative. Nevertheless, using genetically modified cell lines comes with disadvantages, such as increased regulatory oversight and approvals and safety concerns, which can be time-consuming and costly to address. Additionally, ongoing measures, validations and characterizations as stated in FDA guidance document Q5D 2.1.2, 2.3.3, are necessary to monitor genetic drift and cell stability and ensure that the cells are maintaining their desired characteristics whilst not eliciting any off-target effects like tumorigenesis or immunogenicity. Such measures include recording of sub-cultivations at defined dilution ratios and characterization of recombinant DNA expression constructs, and the consistency of the coding sequence of the expression construct in cells. Regardless of the chosen method for creating master and working cell banks, regulatory information can be found in ICH Topic Q 5 D Quality of Biotechnological Products: Derivation and Characterization of Cell substrates Used for Production of Biotechnological/Biological Products 1998 (107, 108).

**Table 1.** Considerations of creating cell bank.

<table>
<thead>
<tr>
<th>Cell bank creation methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooling of unmodified allogeneic cells</td>
<td>• Faster production&lt;br&gt;• Cost-effective</td>
<td>• Variability&lt;br&gt;• Contamination risk&lt;br&gt;• Lack of clonal stability&lt;br&gt;• Heterogeneity</td>
</tr>
<tr>
<td>Master cell bank from single clones</td>
<td>• Homogeneity&lt;br&gt;• Controlled variability&lt;br&gt;• Reduced contamination risk&lt;br&gt;• Allows for a more targeted therapeutic approach through the manipulation of therapeutic proteins or molecules</td>
<td>• Additional regulatory compliance requirements and safety concerns that may include off-target effects, tumorigenesis and immunogenicity</td>
</tr>
</tbody>
</table>

Methods for producing and isolating EVs

The refinement of extracellular vesicles (EVs) isolation and purification is integral to their development as therapeutic agents. Diverse isolation methods can impact EV yield and purity, particularly when EVs are sourced from different cell types and biological fluids. Accordingly, the International Society of Extracellular Vesicles has developed the “minimal information for studies of extracellular vesicles 2023” (MISEV2023) guidelines to standardize EV isolation and characterization methods. This initiative advocates the use of the EV-TRACK database, recommending authors submit their experimental protocols. Despite ongoing efforts for standardization, there is currently no universal gold standard method for EV isolation, driven by considerations of cost-effectiveness to processes that are time-consuming and labor-intensive. Ultracentrifugation techniques, while common, are lengthy and subject to inter-operator variability. An alternative gaining popularity is size-based techniques, such as size exclusion chromatography, due to their lower setup costs and less time-intensive nature. Notably, each technique presents its own set of challenges. Claridge et al and Liangsupree et al highlight the impact of growing interest in EV research on driving the development of advanced isolation techniques to overcome the limitations of conventional methods. While there is no one-size-fits-all technique, efficient methods should be characterized by speed, automation, simplicity, and the ability to yield high-quality EVs reproducibly. Combining techniques like size exclusion chromatography with filtration is a common approach. Selective EV isolation is achieved through immunoadfinity and affinity-based methods, and microfluidic platforms are emerging as versatile tools for both isolation and analysis.

Another promising avenue in EV research revolves around implementing large-scale production strategies. This bridges the gap between small-scale lab production of extracellular vesicles (EVs) and practical implementation for industrialized manufacturing and clinical use. Employing innovative methods in lab-scale techniques can enhance translatability, reduce therapeutic costs, and improve the overall efficiency, affordability, and accessibility of these solutions. Here, interventions are undertaken to upscale production or modify cargo to influence EV functionality and therapeutic potential. Grangier et al and Ng et al assess these methods, such as 3D cultures, chemical stimulation (utilizing drugs and small molecules), genetic manipulation, physical stimulation (involving electrical, mechanical, ionizing, and non-ionizing radiation), and physiological modifications (such as hypoxia, oxidative stress, low pH levels, and heat shock). So far, these approaches have been shown to increase EV yield and impact cargo and functionality compared to EVs produced from 2D cultures. Therefore, careful evaluation is essential to identifying large-scale production strategies that boost yield without compromising efficacy or posing risks for clinical applications.

Understanding the downstream considerations of extracellular vesicle therapy

**Determining extracellular vesicle biodistribution in vivo**

When developing therapeutic EVs, it is critical to understand their biodistribution, which can be complicated by the limitations inherent to each method, as detailed in Table 2.

Fluorescently labeled EVs offer several advantages for the determination of biodistribution. As it is a fairly straightforward process, this method has had widespread use. However, drawbacks include the potential leaching of fluorescent dyes leading to background signals, non-specific membrane transfer, and low spatial and temporal resolution.

Radiolabeled EVs provide high labeling efficiency and sensitivity, enabling detection at nanomolar to picomolar levels with minimal impact on cellular function and tissue. They allow for quantitative imaging without the need for extensive tissue processing and possess excellent depth penetration.
However, this method involves radiation exposure, high costs, and limitations in analyzing long-time points due to radiolabel decay, and it potentially affects EV surface properties. Despite the cost, radiolabeling provides the most comprehensive information on the pharmacodynamics and pharmacokinetics of EVs.120

Bioluminescence labeling offers an efficient labeling process for EVs with high sensitivity and partial pharmacokinetic analysis capabilities. However, they require cell engineering, thus possibly altering EV composition, and lacking deep tissue penetration, low spatial, and temporal resolution.120

As there is no gold standard for analyzing EV biodistribution, researchers must carefully select the most suitable technique based on their specific requirements. It is also important to note that each method may impact EV surface properties and/or composition. This alteration could affect their therapeutic mechanisms of action, potentially limiting the relevance of the data.

### Extracellular vesicle preservation methods and cold chain and transport considerations

A key limitation of cell therapy is the complex cold chain logistics required to maintain cell viability and quality over extended storage periods and/or during transport. The non-viable nature of EVs circumvents this limitation, and therefore, standardized methods for preserving EVs while maintaining the stability and integrity of vesicles are pivotal to clinical translation. Advancements in this field boost scalability and reduce costs in manufacturing and clinical sectors. A summary of the advantages and drawbacks of current preservation methods are summarized in Table 3.

Cryopreservation is a widely used preservation method for viable cells and tissues. The most common cryopreservation of EVs is at −20 °C and −80 °C in phosphate-buffered saline.47 This, however, is currently being optimized by the field to determine optimal storage conditions. Base buffers (eg, phosphate-buffered saline) and cryoprotectants (eg, human albumin, trehalose, and dimethylsulfoxide (DMSO)) are being evaluated for their ability to prevent osmotic damage and maintain sample stability while regulating ice crystal formation.126 An optimal concentration of cryoprotectants has to balance their potential toxicity against the chilling shock. Notably, the safety and toxicology data of cryoprotectants in the perinatal population must be elucidated prior to clinical translation, given that infusion toxicities of cryoprotectants like DMSO have been detailed.127

Alternative methods of preservation, including lyophilization, freeze drying, and spray drying,47,128,129 could potentially allow for long-term storage of EVs at ambient temperatures. These methods could be a pivotal development in EV preservation and storage, avoiding the need for cryoprotectants, increasing shelf life, and providing options for scalability. However, the impact that this process has on the stability, structure, and cargo of EVs is unknown and must be addressed.

### EV dosing and quantification approaches

Establishing reproducible quantification strategies is crucial for measuring the potential therapeutic effects of extracellular vesicles. To quantify EVs, definite parameters have been used, such as particle number, total protein, RNA, and lipid amounts. These are determined using measures such as quantitative electron microscopy, probing for specific molecular markers, or the number of source cells from which the EVs were derived.130,131 Techniques that include phenotypic characterization are preferred as they differentiate between EVs and other co-isolated particles, preventing overestimation.132

Gupta et al. assessed various techniques used to quantify EVs and found that while most studies relied on total protein amount as a measure, there were significant discrepancies in the effective doses. The observed range was from 0.001 to 100 mg of EV protein per kg of body weight. To achieve better therapeutic outcomes, the authors suggested aligning the dosing of EVs with qualitative assessments, such as EV potency assays.133

The inconsistencies in the dosing of extracellular vesicles (EVs) mainly arise from the absence of standardized quantitative measures. MISEV2023 recommends indicating the limit of detection for instruments or methods when quantifying EVs.122

### Table 2. Investigating EV biodistribution techniques.

<table>
<thead>
<tr>
<th>EV biodistribution techniques</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
</table>
| Fluorescently labeled EVs49,118 | • Most common.  
• Simple labeling process.  
• No modifications required. | • Leaching of fluorescent dyes causing background signals of released dye.  
• Non-specific dye transfer between membranes.  
• Does not provide pharmacokinetic analysis.  
• Low spatial and temporal resolution. |
| Radiolabelled EVs119 | • High labelling efficiency.  
• Highly sensitive method (nanomolar to picomolar), and signals can be detected with minimal amounts of the label, which minimizes its impact on cell function and surrounding tissues.  
• Provides pharmacokinetic analysis, allows for quantitative imaging without tissue processing.  
• Possesses the highest depth penetration of all the modalities. | • Hazardous due to radiation exposure.  
• High cost.  
• Inability to analyze long-time points due to radiolabel decay.  
• May impact the surface properties of EVs. |
| Bioluminescent labeled EVs120 | • Efficient labeling process.  
• Beneficial with its high sensitivity.  
• Allows for partial pharmacokinetic analyses. | • Requires cell engineering.  
• Lacks deep tissue penetration and has low spatial and temporal resolution.  
• Can alter EV protein composition120 |

Advantages:  
- High labelling efficiency.  
- Highly sensitive method (nanomolar to picomolar), and signals can be detected with minimal amounts of the label, which minimizes its impact on cell function and surrounding tissues.  
- Provides pharmacokinetic analysis, allows for quantitative imaging without tissue processing.  
- Possesses the highest depth penetration of all the modalities.

Drawbacks:  
- Hazardous due to radiation exposure.  
- High cost.  
- Inability to analyze long-time points due to radiolabel decay.  
- May impact the surface properties of EVs.

Notes:  
- Not all techniques may be applicable in all scenarios.
- The choice of technique should be based on the specific requirements of the study.
- The use of radiolabeling should be considered carefully due to potential hazards.
- Bioluminescent labeling offers high sensitivity and minimal impact on EV composition.

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when they contain functional transgenic mRNAs, while EVs under advanced therapy medicinal products, mainly in neonatal indications. For instance, the EMA classifies under pre-existing categories and little effort has been made since there is no dedicated legislation for EVs yet, they fall and are even ahead of national legislative bodies. However, developing EV products the lack of comprehensive standardization and legislation has hindered the clinical adoption of EVs. Developing EV products relies on large-scale manufacturing in GMP facilities along with simultaneous quality control. Ultracentrifugation and traditional flask-based culture methods, while easily accessible and commonly used, are time-consuming and not suitable for scale-up. It would be challenging to validate all the current data employing industrial approaches, such as bioreactor culture systems, tangential flow filtration, and chromatography-based methods. Nonetheless, to ensure accuracy and reproducibility, it would be important to characterize EVs, including particle size, concentration, the amount of surface charge, and protein markers. It is unknown how EVs pass through the blood-brain barrier bidirectionally, leading to controversy over the biodistribution mechanisms and the number of EVs within the brain. In contrast, EVs are known to accumulate and remain within the lungs due to physical entrapment in the pulmonary capillaries and cellular uptake. However, some studies suggest that while the lungs are generally the primary target destination of large EVs (>200 nm), EVs of smaller sizes (<100 nm) tend to get entrapped in the liver, kidneys, and spleen. These have a direct impact on dose optimization and considerations for neonatal patients must be made.

Various governmental regulatory agencies, including the FDA, TGA, EMA, and PMDA, have yet to form specific regulations for EVs. However, professional international societies such as ISEV and ISCT have made noteworthy contributions in this area through the creation of taskforces and are even ahead of national legislative bodies. However, since there is no dedicated legislation for EVs yet, they fall under pre-existing categories and little effort has been made in neonatal indications. For instance, the EMA classifies EVs under advanced therapy medicinal products, mainly when they contain functional transgenic mRNAs, while the TGA and FDA categorize them as biologicals. Currently, as of March 2024, no legislation has approved EV-based therapeutics for perinatal diseases.

## Conclusion

Treating perinatal conditions presents unique clinical challenges that are not present in other patient populations. Cell-based therapies have emerged as potential treatments. However, issues related to cell source and dose-related toxicity have hindered clinical translation. EVs offer a promising alternative with fewer limitations while maintaining therapeutic effectiveness. The use of EVs is a novel approach, and as the field advances in addressing hurdles facing the cell source, isolation, storage methods, and regulatory concerns will enhance the development of new therapeutic agents to mitigate perinatal pulmonary and cerebral injury.

## Author contributions

Naveen Kumar and Ishmael Miguel Inocencio: conception and design, manuscript writing, final approval of manuscript. Hamid Reza Bidkhori and Tamara Yawno manuscript writing, final approval of manuscript. Rebecca Lim conception and design, final approval of manuscript. Rebecca Lim, Ishmael Miguel Inocencio contributed equally to this work.

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## Conflicts of interest

R.L. declared leadership with CTMC Houston, Texas, United States. The other authors declared no potential conflicts of interest.

## Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.


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