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RESEARCH ARTICLE

Mesenchymal stromal cell extracellular vesicles as therapy for acute and chronic respiratory diseases: A meta-analysis

Alvin Tieu[1,2,3] | **Kevin Hu**[2] | **Catherine Gnyra**[2] | **Joshua Montroy**[2] | **Dean A. Fergusson**[2,4] | **David S. Allan**[2,3,4] | **Duncan J. Stewart**[1,3,4] | **Bernard Thébaud**[1,3,5] | **Manoj M. Lalu**[1,2,3,6]

[1] Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada

[2] Clinical Epidemiology Program, BLUEPRINT Translational Research Group, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada

[3] Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada

[4] Department of Medicine, The Ottawa Hospital, Ottawa, Ontario, Canada

[5] Division of Neonatology, Department of Pediatrics, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada

[6] Departments of Anesthesiology and Pain Medicine, The Ottawa Hospital, Ottawa, Ontario, Canada

Correspondence
Manoj M. Lalu, Department of Anesthesiology and Pain Medicine, University of Ottawa, and Clinical Epidemiology Program and Regenerative Medicine Program, Ottawa Hospital Research Institute, 501 Smyth Road, PO Box 201B, Ottawa, Ontario, K1H 8L6, Canada.
Email: mlalu@toh.ca

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Abstract

Preclinical studies suggest mesenchymal stromal cell extracellular vesicles (MSC-EVs) reduce inflammation and improve organ function in lung diseases; however, an objective analysis of all available data is needed prior to translation. Using rigorous meta-research methods, we determined the effectiveness of MSC-EVs for preclinical respiratory diseases and identified experimental conditions that may further refine this therapy. A systematic search of MEDLINE/Embase identified 1167 records. After screening, 52 articles were included for data extraction and evaluated for risk of bias and quality of reporting in study design. A random effects meta-analysis was conducted for acute lung injury (ALI; N = 23), bronchopulmonary dysplasia (BPD; N = 8) and pulmonary arterial hypertension (PAH; N = 7). Subgroup analyses identified EV methods/characteristics that may be associated with improved efficacy. Data is presented as standardized mean differences (SMD) or risk ratios (RR) with 95% confidence intervals (CI). For ALI, MSC-EVs markedly reduced lung injury (SMD -4.33, CI -5.73 to -2.92), vascular permeability (SMD -2.43, CI -3.05 to -1.82), and mortality (RR 0.39, CI 0.22 to 0.68). Small EVs were more consistently effective than large EVs whereas no differences were observed between tissue sources, immunocompatibility or isolation techniques. For BPD, alveolarization was improved by MSC-EVs (SMD -1.45, CI -2.08 to -0.82) with small EVs more consistently beneficial then small/large EVs. In PAH, right ventricular systolic pressure (SMD -4.16, CI -5.68 to -2.64) and hypertrophy (SMD -2.80, CI -3.68 to -1.91) were significantly attenuated by EVs. In BPD and PAH, EVs isolated by ultracentrifugation demonstrated therapeutic benefit whereas tangential flow filtration (N = 2) displayed minimal efficacy. Lastly, risk of bias and quality of reporting for experimental design were consistently unclear across all studies. Our findings demonstrate clear potential of MSC-EVs to be developed as therapy for acute and chronic lung diseases. However, greater transparency in research design and direct comparisons of isolation technique and EV subtypes are needed to generate robust evidence to guide clinical translation.

Protocol Registration: PROSPERO CRD42020145334

KEYWORDS

exosomes, lung, mesenchymal stem cells, mesenchymal stromal cells extracellular vesicles, meta-analysis, respiratory, systematic review

1 | INTRODUCTION

Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells with multipotent differentiation potential and diverse secretory, immunomodulatory, and therapeutic functions (Le Blanc & Mougiakakos, 2012; Viswanathan et al., 2019). They can be derived from readily available tissues, such as bone marrow, umbilical cord, and adipose tissue (Jung et al., 2012; Romanov, 2003). Many in vitro and in vivo studies show that MSCs can modulate inflammatory pathways, improve organ function and prolong survival (Harrell et al., 2019; Jung et al., 2012), and there are currently over 1000 clinical trials registered on ClinicalTrials.gov investigating MSCs as an intervention across a variety of diseases. Despite this widespread interest, MSCs have received approval for only two conditions, graft versus host disease and perianal fistulas in Crohn's disease (Galipeau & Sensébé, 2018). The lack of successful clinical translation is multifactorial but may include technical challenges associated with generating a consistent, viable and effective cell therapy (Caplan et al., 2019; Lukomska et al., 2019; Rizk et al., 2016). For example, post-hoc analysis from a clinical trial of MSCs for moderate to severe acute respiratory distress syndrome demonstrated an extensive range in cell viability from 36 to 85% (Matthay et al., 2019). Interestingly, it has now been suggested that MSCs exert their therapeutic effects at least in part by secretion of extracellular vesicles (EVs), which may represent a more robust therapeutic product than delivery of intact cells (Akyurekli et al., 2015; Allan et al., 2019; Börger et al., 2017; Colombo et al., 2014; Gnecchi et al., 2005).

Respiratory disorders, whether acute or chronic, remain a leading cause of death worldwide (WHO, 2020). Greater than 500 million people are affected by chronic lung diseases with minimal curative options (Soriano et al., 2020). Moreover, COVID-19 related acute respiratory distress syndrome has shed light on the lack of available specific therapies to combat severe respiratory infections/injury. Given the anti-inflammatory and regenerative properties of MSC-derived EVs (MSC-EVs), pulmonary diseases have become a prominent focus for preclinical development of EV therapeutics (Guo et al., 2020). Administration of MSC-EVs improved outcomes in both acute (e.g. acute lung injury, the preclinical correlate of acute respiratory distress syndrome) and chronic (e.g. pulmonary arterial hypertension) conditions, as well as neonatal diseases (e.g. bronchopulmonary dysplasia) (Harrell et al., 2019; Lee et al., 2019; Monsel et al., 2016; Worthington & Hagood, 2020). MSC-EVs carry a variety of key mRNAs, microRNAs and proteins known to mediate cell processes including inflammation, angiogenesis, apoptosis and fibrosis (Tieu et al., 2020), all of which are critical to lung recovery. Hence, EVs can potentially harness the benefits of whole cells while overcoming many of the common issues that accompany live cell therapies. These advantages include EVs potentially having lower immunogenicity, enhanced ability to cross important biological barriers (e.g. lung endothelium and epithelium), and favourable manufacturing/storage procedures (Chen et al., 2011).

As EV therapies have garnered interest over the past decade, there remain challenges and unanswered questions associated with developing this cell-free therapy. In our previous systematic review, we analysed the methodology and outcomes of more than 200 in vivo studies investigating MSC-EVs as a therapy (Tieu et al., 2020). There was high heterogeneity in EV nomenclature, experimental approach, interventional traits, dosage regimen and study design. The Minimal Information for Studies of Extracellular Vesicles (MISEV 2018) attempts to address many of these issues (Théry et al., 2018). However, given the diversity of EV methodology available, there is no consensus as to which isolation technique, interventional traits (e.g. MSC source or EV subtype) or administration protocols (e.g. delivery route, timing of treatment) will result in greatest therapeutic benefit. A systematic and comprehensive understanding of these experimental details and the overall impact of MSC-EVs on lung repair are needed prior to considering clinical translation.

In the clinical world, scientifically rigorous systematic reviews and meta-analyses are the gold standard approach for evaluating the efficacy of interventions objectively and comprehensively (Crowther et al., 2010; Gurevitch et al., 2018; Murad et al., 2016; Sena et al., 2014). They objectively assess the quality of research being conducted, elucidate specific therapy or patient population characteristics that affect treatment outcomes, and identify knowledge gaps. By applying meta-research methods in preclinical EV research, our study aims to determine the efficacy of MSC-EVs as treatment for various acute and chronic respiratory conditions. A meta-analysis of acute lung injury, bronchopulmonary dysplasia, and pulmonary arterial hypertension was completed. We also conducted subgroup analyses to explore the specific EV characteristics and methodology that may be associated with greater benefits to help refine MSC-EV therapy. Lastly, we assessed the quality of reporting experimental parameters and risk of bias in study design that currently exists in MSC-EV research.

2 | MATERIALS AND METHODS

2.1 | Protocol and registration

Our protocol was registered in the International Prospective Registry of Systematic Reviews (PROSPERO CRD42020145334). Reporting of this systematic review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines (Supplemental File 1).

2.2 | Eligibility criteria

Eligibility criteria for study inclusion was created a priori and defined according to five parameters: population, intervention, comparator, outcomes and study design (PICOS). Predefining PICOS parameters is standard practice in meta-research methodology that enables unbiased search, inclusion, and assessment of pertinent articles.

2.2.1 | Population

We included any in vivo animal models of respiratory disease or injury. Invertebrate animals, in vitro or ex vivo preclinical studies were excluded.

2.2.2 | Intervention

Studies must have administered MSC-EVs for inclusion. All animal or tissue source of MSCs were included. MSCs or EVs may have been pre-treated, co-treated or modified (e.g. gene transfection). All routes, doses, timing and frequency of administration were included. Studies may have administered EVs as xenogeneic, allogeneic or autologous products. Studies in which EVs were not derived from MSCs, or were not administered to animals directly, were excluded.

2.2.3 | Comparator

All comparators including (but not limited to) placebo, vehicle control, MSCs and/or fibroblasts were considered.

2.2.4 | Outcome

Primary, secondary and tertiary outcomes were selected for acute lung injury (ALI), bronchopulmonary dysplasia (BPD), and pulmonary arterial hypertension (PAH), the three most studied lung conditions for MSC-EV therapy. For lung conditions outside of ALI, BPD, and PAH, the primary and secondary outcomes reported in the articles were extracted for narrative synthesis. Studies in which outcomes did not assess EV efficacy (e.g. only the biodistribution of EVs was assessed) were excluded.

2.2.5 | Study Design

We included controlled interventional studies (randomized, pseudo-randomized, or non-randomized), while unpublished grey literature, abstracts, review articles, editorials, commentaries, and letters were excluded. Studies were not excluded based on language or publication date.

2.3 | Search strategy

A systematic search of Ovid MEDLINE, Ovid MEDLINE In-Process & Other Non-Indexed Citations, and Embase Classic + Embase was conducted until March 6, 2019 using a broad search strategy for any in vivo study investigating MSC-EVs. To update our study, an additional search was conducted on 4 August, 2020 with lung disease filters. Both search strategies (Supplemental File 2) were designed with the help of an information specialist and assessed by a second information specialist through the Peer Review of Electronic Search Strategies (PRESS) (McGowan et al., 2016). The search strategies were modified according to database and used controlled vocabulary, MeSH terms (e.g. mesenchymal stem cells, mesenchymal stromal cells, extracellular vesicles, exosomes, microvesicles), abbreviations (e.g. MSCs, EVs, MVs), and filters for preclinical animal models. No restrictions were applied for publication date.

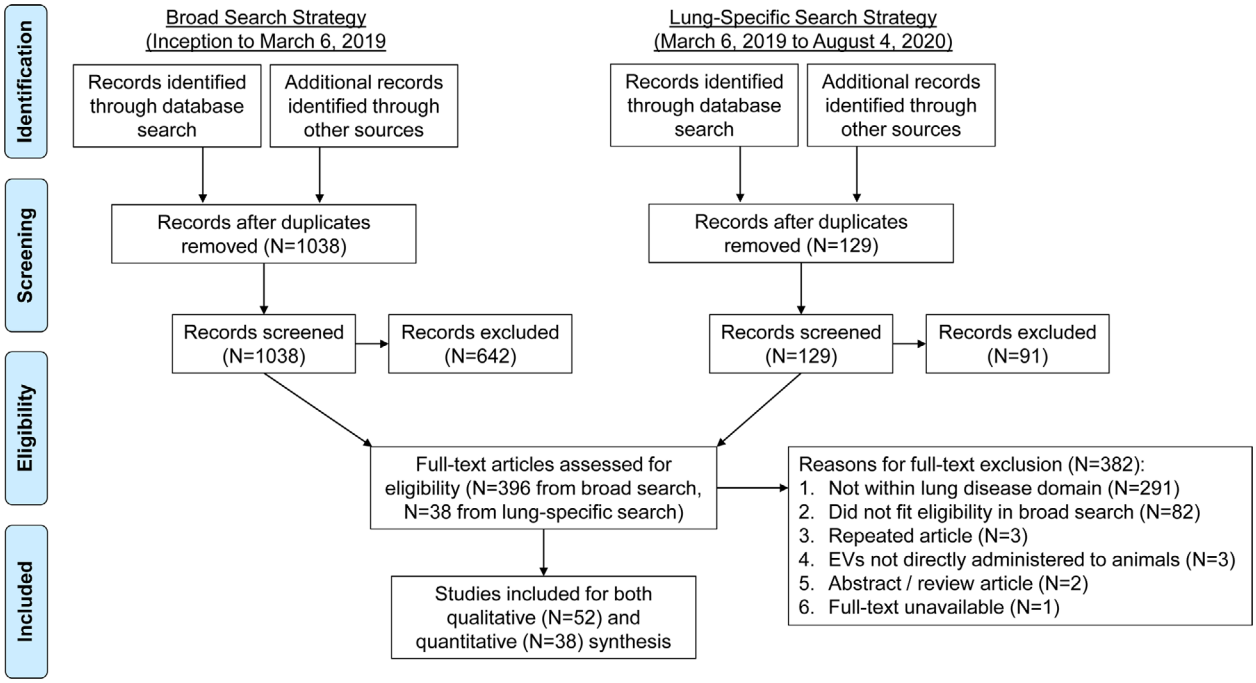

FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram detailing study screening and selection

2.4 | Study selection and data extraction

Titles and abstracts were independently screened in duplicate (AT, MS, CG and/or KH) using the Distiller Systematic Review Software (DistillerSR, Evidence Partners, Ottawa, Canada). A calibration test using 10 studies was performed before beginning the formal screening process to ensure high inter-rater correlation (kappa 0.8). Titles and abstracts that met criteria or that could not be excluded with certainty moved on to full-text screening. Following a calibration test involving 10 studies, two reviewers (AT, MS, CG and/or KH) independently reviewed the full-text articles. Reasons for exclusion were recorded at this stage. Disagreements between reviewers were resolved through discussion. The selection process is presented in a PRISMA flow diagram (Figure 1).

Standardized data extraction forms were created in DistillerSR. Data pertaining to study characteristics, risk of bias, and quality assessment were extracted independently and in duplicate (AT, CG and/or KH). A calibration exercise for data extraction was conducted on the first three studies. Data was collected on study characteristics, including: general study characteristics, animal model characteristics (e.g. species, sex, model of disease or injury), MSC and EV isolation and characterization techniques, MSC or EV modifications, and dosage. Details for all data collected can be found in our registered PROSPERO protocol. We used the generally accepted size-based definitions of EV subtypes, *i.e.* exosomes or small EVs (30-150 nm in diameter) and microvesicles or large EVs (150-1000 nm in diameter) (Théry et al., 2018; Tieu et al., 2020), to determine whether authors use of these terms was consistent with MISEV recommendations.

Since MSCs are a heterogeneous population of cells widely accessible from a variety of tissue sources, the International Society for Cell Therapy (ISCT) published guidelines to standardize MSC characterization. Studies were assessed for their adherence to the ISCT criteria including: (1) adherence to plastic in standard culture conditions, (2) positive and negative expression of specific surface antigens, and (3) multipotent differentiation potential. Furthermore, international guidelines for investigating EVs were published in 2018 entitled "Minimal Information for Studies of EVs" (MISEV 2018) (Théry et al., 2018). Researchers are encouraged to characterize EVs by amount, two measures of single vesicle analysis, and at least three positive and one negative protein marker. These criteria were used to assess study adherence to EV characterization.

2.5 | Primary and secondary outcomes

Outcomes were predetermined by consulting experts within the field of regenerative medicine and lung disease. Primary outcomes included histological lung injury scores for ALI, mean linear intercept (a measure of alveolarization) for BPD, and right ventricular systolic pressure for PAH. Secondary outcomes for ALI and BPD included protein concentration and neutrophil count in bronchoalveolar lavage fluid (measures of alveolar-capillary membrane permeability and inflammation, respectively).

For BPD and PAH, Fulton's index (a measure of right ventricular hypertrophy and indicator of pulmonary hypertension) was extracted as a secondary outcome. Tertiary outcomes, if reported, included measures of survival and adverse events of EV therapy. The corresponding authors of the individual studies included in our review were contacted to obtain relevant missing data.

2.6 | Risk of bias and quality of reporting

Risk of bias was evaluated by two independent reviewers (AT, CG) using the SYRCLE (Systematic Review Centre for Laboratory Animal Experimentation) risk of bias tool (Hooijmans et al., 2014). The SYRCLE tool features 10 different parameters including randomization, blinding and outcome reporting, each of which was scored as having a low, high, or unclear risk of bias for each study. Quality of reporting was evaluated by two independent reviewers (AT, CG) using the NIH standards for transparency in reporting preclinical research. Each element in the NIH standards for transparency was scored as having been reported or not reported. Disagreements between reviewers were resolved through discussion (Fergusson et al., 2019a; NIH 2014).

2.7 | Data analysis

Extracted data on study characteristics, methodology and intervention characteristics are presented as frequencies, proportions and/or percentages. Studies may have used multiple techniques and interventions, either as separate experiments or in combination. Hence, the cumulative percentage of certain categories may be greater than 100%.

Study outcomes were pooled using Comprehensive Meta-Analyst (version 3; BioStat Inc., USA) and represented by forest plots. Continuous outcome measures were expressed as standardized mean differences (SMDs) with 95% confidence intervals (CIs), which were calculated using a random effects inverse variance meta-analysis. Standardized mean difference was chosen due to the expected heterogeneity in the measurement techniques of outcomes. Dichotomous outcome measures were expressed as risk ratios (RRs) with 95% confidence intervals (CIs), which were calculated using a random effects meta-analysis based on the DerSimonian Laird model. We corrected for the multiple use of a single control group using the method described by Vesterinen *et al* (Vesterinen et al., 2014). Statistical heterogeneity was assessed using the Cochrane I^2 test. Planned subgroup analyses included subgroups based on animal characteristics (e.g. sex, age, species), intervention characteristics (e.g. tissue source of MSCs, subtype of EVs, method of EV isolation), timing of treatment and respiratory disease modelled (e.g. ALI, BPD, PAH).

3 | RESULTS

3.1 | Study characteristics

Our systematic searches yielded a total of 1167 records (Figure 1). Following level 1 screening of titles and abstracts, 434 articles underwent level 2 full-text screening which resulted in a total of 52 studies included for our systematic review (Ahn et al., 2018; Aliotta et al., 2016, 2017; Bandeira et al., 2018; Braun et al., 2018; Chaubey et al., 2018; Chen et al., 2014, 2019, 2020; Choi et al., 2014; Cruz et al., 2015; de Castro et al., 2017; Deng et al., 2020; Dinh et al., 2020; Fang et al., 2020a, 2020b; Gao et al., 2020; Hao et al., 2019; Harrell et al., 2020; Hogan et al., 2019; Huang et al., 2019; Khatri et al., 2018; Kim et al., 2020; Klinger et al., 2020; Lee et al., 2012; Lei et al., 2020; Li et al., 2015, 2019a, 2019b, 2020; Liu et al., 2018, 2019a, 2019b; Mansouri et al., 2019; Maremanda et al., 2019; Monroe et al., 2020; Monsel et al., 2015; Porzionato et al., 2019; Potter et al., 2018; Silva et al., 2019; Stone et al., 2017; Tang et al., 2017; Varkouhi et al., 2019; Wang et al., 2020; Willis et al., 2018, 2020; Xu et al., 2019, 2020; Yi et al., 2019; Zhang et al., 2020; Zhaorigetu et al., 2020; Zhu et al., 2014). Our meta-analysis focussed on the three most frequently investigated diseases (ALI, BPD and PAH).

Baseline characteristics of the included studies are reported in Table 1. A majority of studies were conducted in the United States (N = 22; 42%) or China (N = 21; 42%) (Figure S3.1). The first paper examining MSC-EVs in a model of lung disease was published in 2012. Since then, there has been a marked growth in the number of preclinical animal studies investigating MSC-EVs with over 60% of studies (N = 32) published in 2019 and 2020 (Figure S3.1). A variety of lung conditions have been investigated including ALI (N = 23; 44%), PAH (N = 8; 15%), BPD (N = 7; 13%), asthma / allergic inflammation (N = 5; 10%), pulmonary fibrosis (N = 5; 10%), congenital diaphragmatic hernia (N = 2; 4%) and chronic obstructive pulmonary disease (N = 2; 4%). Most studies utilized either a mouse (N = 31; 60%) and/or rat (N = 21; 40%) model, with only one study applying a pig model (2%). Nearly half of the studies used only male animals (N = 24; 46%), with 8 studies using females only (15%), 2 studies using both sexes (4%), and 18 studies not reporting of the sex of their animals (35%).

TABLE 1 Summary of study characteristics of all included articles

First author	Year	Country	Animal model	Human disease	MSC source	EV isolation and size / morphology analysis	Terminology consistency	Positive surface markers	Negative surface markers	Dosage regimen (dose, no. of doses, route and immuno-compatibility)
Acute Respiratory Distress Syndrome / Acute Lung Injury										
Zhu, YG	2014	United States	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Ultracentrifugation; SEM; 150- 1000 nm (large EVs)	Yes	Not described	Not described	3e6 MSC equivalent over 48h; 1 dose; Intravenous, Intratracheal; Xenogeneic
Li, L	2015	China	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Isolation kit / PEG; Not described; Not described	Unclear	CD63	Not described	1.5ug/g; 1 dose; Intravenous; Xenogeneic
Tang, XD	2017	China	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Ultracentrifugation; SEM, TEM; 150–1000 nm (large EVs)	Yes	Not described	Not described	30uL (equivalent to 3e6 MSCs over 48h); 1 dose; Intratracheal; Xenogeneic
Khatri, M	2018	United States	Pig	Acute Respiratory Distress Syndrome	Pig bone marrow	Ultracentrifugation; TEM; 30- 150 nm (small EVs)	No	CD63, CD9, CD81, CD29 (Integrin B1/B2)	Not described	80ug/kg; 1 dose; Intratracheal; Allogeneic
Potter, DR	2018	United States	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Ultracentrifugation; NTA; 30- 1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, MSC markers	Not described	30ug; 1 dose; Not Described; Xenogeneic
Yi, X	2019	China	Mouse	Acute Respiratory Distress Syndrome	Mouse bone marrow	High speed centrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63	Not described	100ug; 1 dose; Intravenous; Allogeneic
Xu, N	2019	China	Rat	Acute Respiratory Distress Syndrome	Rat bone marrow	Ultracentrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81	Not described	5e6 MSC equivalent; 1 dose; Intratracheal; Allogeneic
Varkouhi, AK	2019	Canada	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Anion-exchange chromatography; TEM; 30–150 nm (small EVs)	No	Not described	Not described	1e8 particles/kg; 1 dose; Intravenous; Xenogeneic
Silva, JD	2019	Brazil	Mouse	Acute Respiratory Distress Syndrome	Mouse bone marrow	Ultracentrifugation; NTA, DLS; 30–1000 nm (both small/large EVs)	Yes	Not described	Not described	1e5 MSC equivalent; 1 dose; Intravenous; Allogeneic
Li, JS	2019	China	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Isolation kit / PEG; DLS, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9	Not described	800 ug of RNA; 1 dose; Intravenous; Xenogeneic
Liu, J	2019	China	Rat	Acute Respiratory Distress Syndrome	Rat bone marrow	Ultracentrifugation; TEM; 30- 150 nm (small EVs)	Yes	CD63, CD81, TSG101, MSC markers	Not described	5-10ug; 1 dose; Intravenous; Allogeneic

(Continues)

TABLE 1 (Continued)

First author	Year	Country	Animal model	Human disease	MSC source	EV isolation and size / morphology analysis	Terminology consistency	Positive surface markers	Negative surface markers	Dosage regimen (dose, no. of doses, route and immuno-compatibility)
Li, QC	2019	China	Rat	Acute Respiratory Distress Syndrome	Rat bone marrow	High speed centrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81	Not described	25ug; 4 doses; Intravenous; Allogeneic
Huang, R	2019	China	Mouse	Acute Respiratory Distress Syndrome	Human adipose tissue	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	Yes	CD63, CD81, MSC markers	Calnexin, GM130	100ug; 1 dose; Intravenous; Xenogeneic
Chen, W	2019	China	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Ultracentrifugation; TEM; Not described	Unclear	MSC markers	Not described	Not described; 1 dose; Intratracheal; Xenogeneic
Wang, J	2020	China	Mouse	Acute Respiratory Distress Syndrome	Human adipose tissue	Ultracentrifugation; DLS, TEM; 30–150 nm (small EVs)	Yes	CD63, CD81, CD44, CD105, CD40	Calnexin, GM130	50ug; 1 dose; Intravenous, Intratracheal; Xenogeneic
Deng, H	2020	China	Mouse	Acute Respiratory Distress Syndrome	Mouse bone marrow	Ultracentrifugation, Ultrafiltration; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD81	Not described	50ug or 100ug; 1 dose; Intratracheal; Allogeneic
Chen, WX	2020	China	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Ultracentrifugation; TEM; Not described	Unclear	Not described	Not described	1e6 particles; 1 dose; Intratracheal; Xenogeneic
Asthma / Allergic Airway Inflammation										
Cruz, FF	2015	United States	Mouse	Asthma / Allergic Airway Inflammation	Human and mouse bone marrow	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	Yes	Not described	Not described	3e6 MSC equivalent; 1 dose; Intravenous; Xenogeneic, Allogeneic
de Castro, LL	2017	Brazil	Mouse	Asthma / Allergic Airway Inflammation	Human adipose tissue	Ultracentrifugation; DLS; 30- 1000 nm (both small/large EVs)	Yes	Not described	Not described	37ug; 1 dose; Intravenous; Xenogeneic
Kim, SD	2020	South Korea	Mouse	Asthma / Allergic Airway Inflammation	Mouse adipose tissue	Ultracentrifugation, Ultrafiltration; TEM; Not described	Unclear	CD81, CD40	Not described	10ug; 4 doses; Intranasal; Allogeneic
Fang, SB	2020	China	Mouse	Asthma / Allergic Airway Inflammation	Human iPSCs	Anion-exchange chromatography; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix	Calnexin	2e10 particles; 1 dose; Intravenous; Xenogeneic

(Continues)

TABLE 1 (Continued)

First author	Year	Country	Animal model	Human disease	MSC source	EV isolation and size / morphology analysis	Terminology consistency	Positive surface markers	Negative surface markers	Dosage regimen (dose, no. of doses, route and immuno-compatibility)
Fang, SB	2020	China	Mouse	Asthma / Allergic Airway Inflammation	Human iPSCs	Anion-exchange chromatography; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix	Calnexin	1.5e10 particles; 3 doses; Intravenous; Xenogeneic
Bronchopulmonary Dysplasia										
Ahn, SY	2018	South Korea	Rat	Bronchopulmonary Dysplasia	Human umbilical cord	Ultracentrifugation; NTA, TEM; 30–150 nm (small EVs)	No	CD63, CD9	Cytochrome C, GM130, Fibrillarin	20ug; 1 dose; Intratracheal; Xenogeneic
Willis, GR	2018	United States	Mouse	Bronchopulmonary Dysplasia	Human bone marrow, human umbilical cord	Tangential flow filtration, IDX density gradient ultracentrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, Flotillin-1	Not described	0.5e6 MSC equivalent over 36 h; 1 dose; Intravenous; Xenogeneic
Chaubey, S	2018	United States	Mouse	Bronchopulmonary Dysplasia	Human umbilical cord	Ultracentrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD81, Alix	TGN48	0.7e6 MSC equivalent over 24h (2.4-2.8ug); 2 doses; intraperitoneal; Xenogeneic
Brain, RK	2018	United States	Rat	Bronchopulmonary Dysplasia	Rat bone marrow	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	No	CD63, CD9, CD81	Not described	15ug or 3.4e9 particles; 14 or 12 doses; intraperitoneal; Allogeneic
Porizonato, A	2019	Italy	Rat	Bronchopulmonary Dysplasia	Human umbilical cord	Tangential flow filtration; NTA; 30–1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, Annexin V	HLAs	8e8 particles/g for P3, 4.5e8 for P7, and 3e8 for P10; 3 doses; Intratracheal; Xenogeneic
Willis, GR	2020	United States	Mouse	Bronchopulmonary Dysplasia	Human umbilical cord	Tangential flow filtration, IDX density gradient ultracentrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix, Flotillin-1	GM130	0.5e6 MSC equivalent, or 1e6 MSC equivalent; 1 or 4 doses; Intravenous; Xenogeneic
Li, Z	2020	China	Rat	Bronchopulmonary Dysplasia	Human placenta	Ultracentrifugation; TEM; 30- 150 nm (small EVs)	Yes	CD63, CD9, CD81, HSP70/90	Not described	0.3ug; 1 dose; Intratracheal; Xenogeneic

(Continues)

TABLE 1 (Continued)

First author	Year	Country	Animal model	Human disease	MSC source	EV isolation and size / morphology analysis	Terminology consistency	Positive surface markers	Negative surface markers	Dosage regimen (dose, no. of doses, route and immuno-compatibility)
Chronic Obstructive Pulmonary Disease										
Maremanda, K	2019	United States	Mouse	Chronic Obstructive Pulmonary Disease	Mouse bone marrow	Isolation kit / PEG; TRPS, TEM; 30–150 nm (small EVs)	Yes	CD63, Alix, Flotillin-1	Calnexin	15ug; 10 doses; intraperitoneal; Allogeneic
Harrell, CR	2020	Serbia	Mouse	Chronic Obstructive Pulmonary Disease	Human placenta	Ultracentrifugation, Isolation kit / PEG; Not described; Not described	Unclear	Not described	Not described	0.1 ml; 15 doses; intraperitoneal; Xenogeneic
Congenital Diaphragmatic Hernia										
Zhaorigetu, S	2020	United States	Rat	Congenital Diaphragmatic Hernia	Human bone marrow	Tangential flow filtration; TRPS, TEM; 30–150 nm (small EVs)	Yes	Not described	Not described	1e10 particles/ml; 1 dose; Intravascular; Xenogeneic
Monroe, MN	2020	United States	Rat	Congenital Diaphragmatic Hernia	Human bone marrow	Tangential flow filtration; NTA, TEM; Not described	Unclear	Not described	Not described	1e10 particles/ml; 1 dose; Direct tissue injection; Xenogeneic
Ischemia-Reperfusion Injury										
Stone, ML	2017	United States	Mouse	Ischemia-Reperfusion Injury	Human umbilical cord	Ultracentrifugation; NTA; 150-1000 nm (large EVs)	No	MSC markers	Not described	1e6 particles; 1 dose; Intratracheal; Xenogeneic
Li, JW	2019	China	Mouse	Ischemia-Reperfusion Injury	Mouse bone marrow	Ultracentrifugation, Isolation kit / PEG; NTA; Not described	Unclear	CD63, CD9, CD81	Not described	30uL (2e6 MSC equivalent); 1 dose; Intratracheal; Allogeneic
Pneumonia										
Monsel, A	2015	United States	Mouse	Pneumonia	Human bone marrow	Ultracentrifugation; SEM; 150- 1000 nm (large EVs)	Yes	CD44	Not described	EVs made by 9e6 MSCs over 48h; 1 dose; Intravenous, Intratracheal; Xenogeneic
Hao, Q	2019	United States	Mouse	Pneumonia	Human bone marrow	Ultracentrifugation; NTA, SEM; 30–1000 nm (both small/large EVs)	Yes	CD44	Not described	1e10 particles; 1 dose; Intravenous; Xenogeneic

(Continues)

TABLE 1 (Continued)

First author	Year	Country	Animal model	Human disease	MSC source	EV isolation and size / morphology analysis	Terminology consistency	Positive surface markers	Negative surface markers	Dosage regimen (dose, no. of doses, route and immuno-compatibility)
Pulmonary Arterial Hypertension										
Lee, C	2012	United States	Mouse	Pulmonary Arterial Hypertension	Mouse bone marrow	Ultracentrifugation, Size exclusion chromatography; TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix, HSP70/90, Flotillin-1	Not described	0.1ug (low dose), 10ug (high dose); 1 or 2 doses; Intravenous; Allogeneic
Chen, JY	2014	United States	Rat	Pulmonary Arterial Hypertension	Rat bone marrow	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	No	CD29 (Integrin B1/B2), Annexin V	Not described	30ug; 8 doses; Intravenous; Allogeneic
Aliotta, JM	2016	United States	Mouse	Pulmonary Arterial Hypertension	Human and mouse bone marrow	Ultracentrifugation; NTA, TEM; 30–150 nm (small EVs), 30 -1000 nm (both EVs)	No	CD63, CD9, CD81	Not described	25ug; 3 or 4 doses; Intravenous; Xenogeneic, Allogeneic
Aliotta, JM	2017	United States	Mouse	Pulmonary Arterial Hypertension	Mouse bone marrow	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	Yes	CD63	Not described	25ug; 3 doses; Intravenous; Allogeneic
Liu, Z	2018	China	Rat	Pulmonary Arterial Hypertension	Rat bone marrow	Ultracentrifugation; TEM; Not described	Unclear	Not described	Not described	30ug; 8 doses; Intravenous; Allogeneic
Hogan, SE	2019	United States	Mouse	Pulmonary Arterial Hypertension	Human bone marrow	Tangential flow filtration, Size exclusion chromatography; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD81, TSG101, Alix, Flotillin-1, Annexin V, EpCAM, ICAM	GM130	2e7 particles in 200uL; 1 or 9 doses; Intravenous; Xenogeneic
Zhang, S	2020	China	Rat	Pulmonary Arterial Hypertension	Human umbilical cord	Ultracentrifugation; TEM; 30-150 nm (small EVs)	Yes	CD63, CD81, TSG101, Alix	Not described	25ug; 3 doses; Intravenous; Xenogeneic
Klinger, JR	2020	United States	Rat	Pulmonary Arterial Hypertension	Human bone marrow	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, TSG101	Albumin	100ug/kg; 3 doses; Intravenous; Xenogeneic
Pulmonary Fibrosis										
Choi, M	2014	South Korea	Mouse	Pulmonary Fibrosis	Human bone marrow	Isolation kit / PEG; Not described; Not described	Unclear	Not described	Not described	10ug; 2 doses; Intravenous; Xenogeneic

(Continues)

TABLE 1 (Continued)

First author	Year	Country	Animal model	Human disease	MSC source	EV isolation and size / morphology analysis	Terminology consistency	Positive surface markers	Negative surface markers	Dosage regimen (dose, no. of doses, route and immuno-compatibility)
Gao, Y	2016	China	Rat	Pulmonary Fibrosis	Rat adipose tissue	Ultracentrifugation; NTA, TEM; 30–1000 nm (both small/large EVs)	Yes	CD63, TSG101, Alix	GM130	2.5-2.8e10 particles; 1 dose; Intratracheal; Allogeneic
Bandeira, E	2018	Brazil	Mouse	Pulmonary Fibrosis	Mouse adipose tissue	Ultracentrifugation; NTA, SEM, TEM; 30–1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, Lamp1	Not described	Two doses: 1) 1e5 MSC equivalent over 24h; 2) 1e6 MSC equivalent over 24h; 1 dose; Intratracheal; Allogeneic
Mansouri, N	2019	United States	Mouse	Pulmonary Fibrosis	Human bone marrow	IDX density gradient ultracentrifugation; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, Alix, Flotillin-1	GM130	5e6 MSC equivalent; 1 dose; Intravenous; Xenogeneic
Dinh, PC	2020	United States	Rat	Pulmonary Fibrosis	Human bone marrow	Ultrafiltration; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101	Not described	10e9 particles/kg; 7 doses; Inhalation; Xenogeneic
Other Models of Lung Injury										
Lei, X	2020	China	Mouse	Radiation-Induced Lung Injury	Human placenta	Not described; NTA, TEM; 30-150 nm (small EVs)	No	CD63, CD9, TSG101, Alix	GM130	100ug/dose; 4 doses; Intravenous; Xenogeneic
Xu, B	2020	China	Rat	Smoke Inhalation Lung Injury	Rat bone marrow	Isolation kit / PEG; NTA, TEM; 30–150 nm (small EVs)	Yes	CD63, CD9, CD81	Not described	250ug; 1 dose; Intravenous; Allogeneic

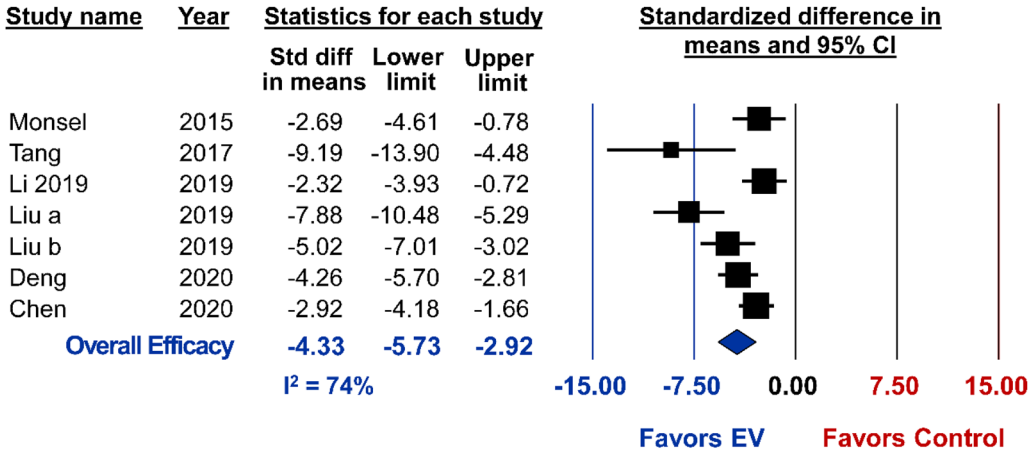

FIGURE 2 Meta-analysis for all included studies of acute lung injury that reported the primary outcome of lung injury score. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes < 0 favours EV treatment and > 0 favours control. Subscript denotes a separate study or article that was published within the same year. The 'Overall Efficacy' represents a pooled estimate of MSC-EV effect on lung injury score from all studies combined. I^2 value represents the statistical heterogeneity

3.2 | EV methodology and interventional characteristics

The animal source of MSCs used included human (N = 34; 65%), mouse (N = 11; 21%), rat (N = 8; 15%) and pig (N = 1; 2%) (Figure S3.2). MSCs were derived from a variety of tissue sources including bone marrow (N = 31; 60%), umbilical cord (N = 11; 21%), adipose tissue (N = 6; 12%), placenta (N = 3; 6%) and induced pluripotent stem cells (iPSCs) (N = 2; 4%) (Figure S3.2). Studies administered either xenogeneic EVs (N = 34; 65%) or allogeneic EVs (N = 20; 38%). Based on the ISCT guidelines, 30 studies (58%) met all three criteria for MSC characterization. In vitro trilineage differentiation and the positive/negative markers used in each study are described in Figure S3.2.

The most common techniques used for EV isolation were ultracentrifugation (N = 34; 65%), isolation kits (N = 7; 13%) and tangential flow filtration (N = 6; 12%) (Figure S3.3). EVs were characterized by quantification (N = 47; 90%), size distribution (N = 45; 87%), morphological analysis (N = 45; 87%) and/or surface marker expression (N = 41; 79%) in most studies (Figure S3.3). Based on the MISEV 2018 guidelines, 10 studies (19%) adequately characterized their EV therapies. Importantly, 52% (N = 27) of studies did not report assessment of negative markers to demonstrate specific isolation of EVs. Regarding EV nomenclature, we assessed whether the terminology used was consistent with reported size distribution of EVs (e.g. small, large or both small/large EVs combined). Thirty-five (67%) studies utilized consistent terminology, whereas 8 studies (15%) used discordant terms and 9 studies (17%) did not provide size analysis. More details regarding EV isolation and characterization, including specific techniques and markers used, can be found in Figure S3.3.

The two most common routes of administration for MSC-EV delivery included intravenous (N = 31; 60%) and intratracheal (N = 16; 31%) delivery (Figure S3.4). The units applied for dosing EVs varied considerably and included absolute protein amount (N = 23; 44%), particle number (N = 11; 21%) or amount of EVs released by a certain number of MSCs (N = 8; 15%), as well as EVs released over time or dosed by weight of animal (Figure S3.4). Most studies delivered a single dose of therapy (N = 35; 67%); studies with multiple administrations used a median value of 4 doses (ranging from 2–15 doses). EV modifications were conducted in 33 studies (63%) which included modifications before EV isolation (N = 17; 33%), modifications to EVs directly (N = 8; 15%) and co-treatments (N = 8; 15%). Lastly, dose-response and biodistribution analyses were examined in 8 (15%) and 7 (13%) studies, respectively (Figure S3.4).

3.3 | Meta-analyses of acute lung injury studies

Method of ALI induction included intratracheal endotoxin (e.g. lipopolysaccharide; N = 8), intratracheal *E. Coli* (N = 3), ischemia-reperfusion injury (N = 2), bleomycin injection (N = 2), smoke inhalation (N = 1), radiation (N = 1), viral induction (N = 1), shock (N = 1), phosgene (N = 1), burn (N = 1), trauma (N = 1) or intestinal ischemia-reperfusion (N = 1). Histological lung injury was reported in 7 studies investigating preclinical ALI. All 7 studies demonstrated a significant reduction in lung injury from MSC-EV therapy (pooled analysis, SMD -4.33, 95%CI -5.73 to -2.92, $I^2 = 74\%$) (Figure 2). Subgroup analyses demonstrated that small EVs consistently result in a significant attenuation of lung injury whereas the efficacy of large EVs is highly variable and similar to control treatments from pooled analysis (Figure 3). Studies that isolated EVs by ultracentrifugation did

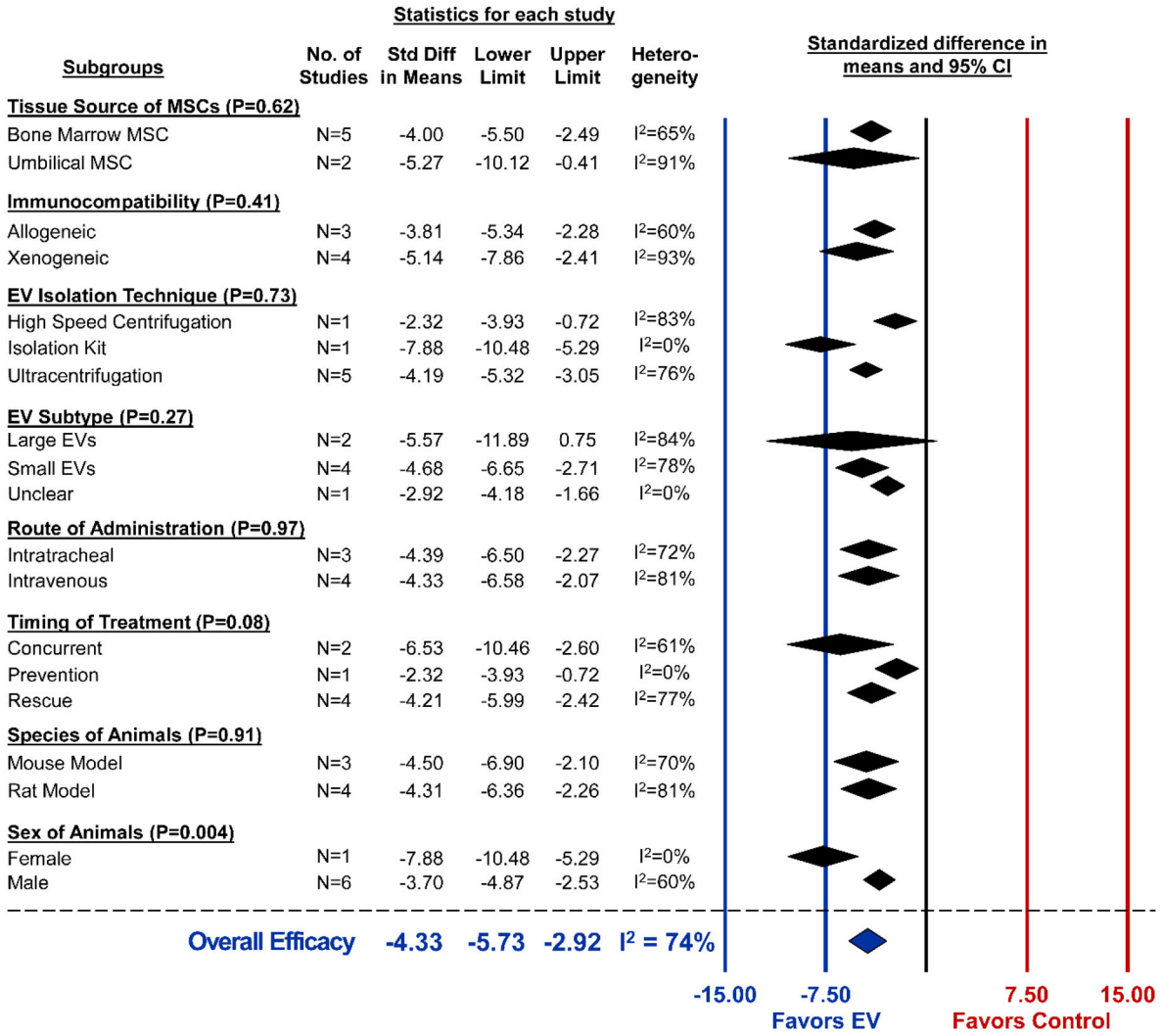

FIGURE 3 Subgroup analysis for all included studies of acute lung injury that reported the primary outcome of lung injury score. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I^2 value represents the statistical heterogeneity within each subgroup. Effect sizes < 0 favours EV treatment and > 0 favours control. The 'Overall Efficacy' is a pooled estimate effect of MSC-EVs on lung injury score from all studies combined

not demonstrate a difference in effect size as compared to the single study that utilized an isolation kit (Figure 3). Lastly, MSC-EVs may demonstrate greater benefits (P = 0.004) to attenuate lung injury in female rodents as compared to males, however only one study used female animals (Figure 3). No difference in effect size was observed between MSC tissue source (bone marrow vs. umbilical cord), species source of MSCs, immunocompatibility (allogeneic vs. xenogeneic), timing of treatment, route of administration or animal model (Figure 3).

Changes to alveolar-capillary barrier permeability measured by bronchoalveolar lavage fluid (BALF) protein concentration were reported in 28 studies. Overall, MSC-EV administration was associated with a significant attenuation of lung permeability (SMD -2.43, 95%CI -3.05 to -1.82, $I^2 = 83\%$) (Figure 4). From subgroup analysis, lung permeability was reduced to a greater extent ($P < 0.001$) by delivery of small EVs or both small/large as compared to large EVs alone (Figure 5). Allogeneic administration of MSC-EVs also showed a greater reduction in lung permeability compared to xenogeneic delivery ($P = 0.001$). Sex ($P = 0.009$) and species ($P = 0.03$) may be associated with greater EV efficacy as female animals and rat models demonstrated better outcomes (Figure 5). Lastly, the single study that used an isolation kit displayed a greater improvement ($P < 0.001$) to lung permeability as compared to studies that utilized ultracentrifugation. Similar to lung injury score, no difference in effect size was observed when considering MSC tissue source, route of administration or timing of treatment.

Other secondary outcomes considered for ALI studies included mortality (RR 0.39, 95%CI 0.22 to 0.68, $I^2 = 14\%$) (Figure S4.1) and inflammatory response as measured by neutrophil infiltration in BALF (SMD -1.42, 95%CI -1.83 to -1.02, $I^2 = 66\%$) (Figure S4.2), both of which were significantly improved by MSC-EV therapy. Lastly, a dose-response analysis

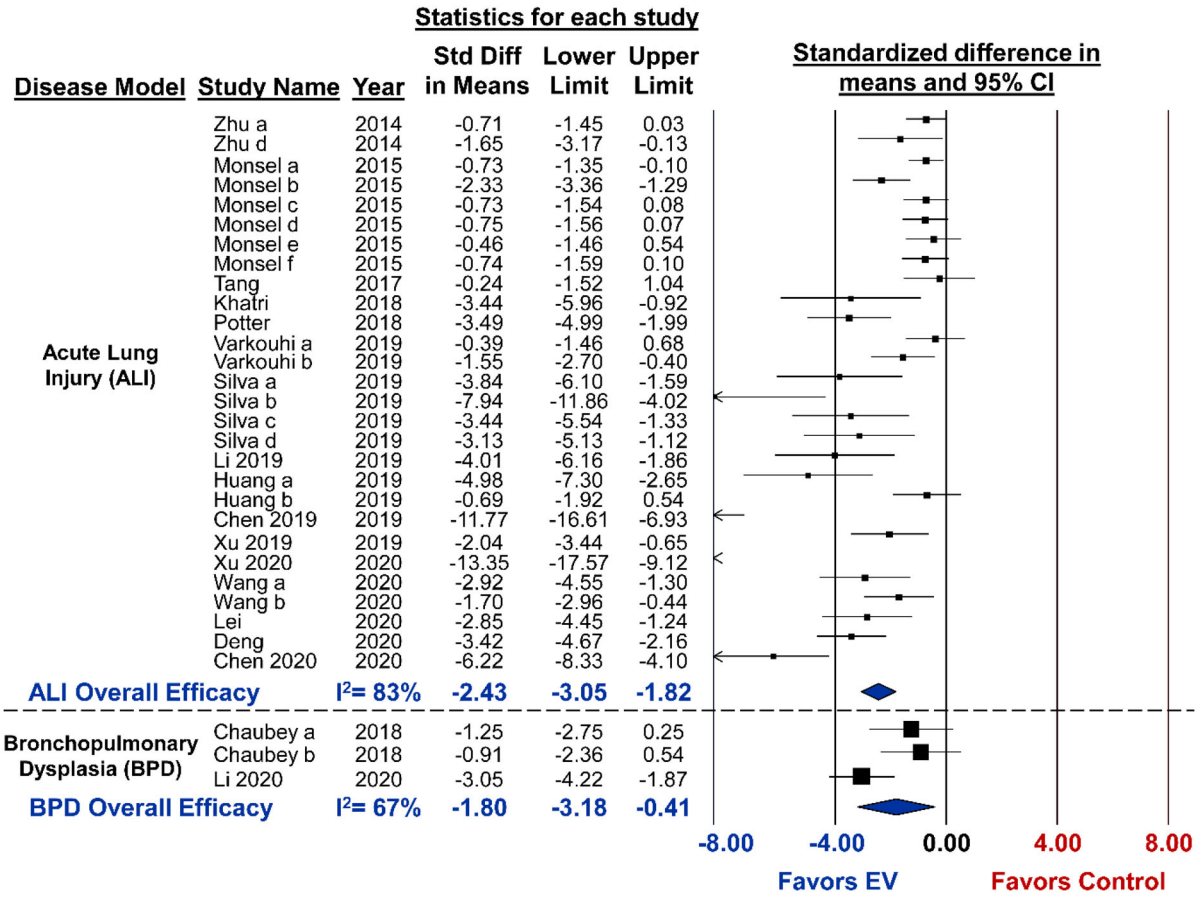

FIGURE 4 Meta-analysis for all included studies of acute lung injury and bronchopulmonary dysplasia that reported the secondary outcome of alveolar capillary permeability. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes < 0 favours EV treatment and > 0 favours control. Subscript denotes a separate study or article that was published within the same year. The 'Overall Efficacy' represents a pooled estimate of MSC-EV effect on lung vascular permeability from all studies combined. I^2 value represents the statistical heterogeneity

was conducted for studies administering EVs by protein amount and reporting lung vascular permeability as an outcome. Despite many studies delivering similar doses, the effect size varied considerably and was not correlated to dose ($P = 0.311$) (Figure S4.3).

3.4 | Meta-analyses of bronchopulmonary dysplasia studies

All animal models of BPD were induced by housing new-born mice or rats under hyperoxia conditions, ranging from 60–95% oxygen. Alveolarization was reported in 11 studies from 7 different articles and pooled analysis demonstrated marked improvements from MSC-EV administration (SMD -1.45, 95%CI -2.08 to -0.82, $I^2 = 63\%$) (Figure 6). Similar to ALI, subgroup analysis indicated that small EVs consistently improve alveolarization, whereas the combination of small/large EVs were highly variable and not statistically different as compared to control (Figure 7). One study that isolated EVs by TFF did not exhibit any benefits from EVs. However, when TFF was combined with density gradient ultracentrifugation or ultracentrifugation was used alone, MSC-EVs significantly improved lung alveolarization (P = 0.008) (Figure 7). Immunocompatibility (allogeneic, P = 0.045), route of administration (intraperitoneal, $P = 0.040$) and timing of treatment (concurrent, $P = 0.044$) may result in differential effects, whereas no differences in efficacy was observed when comparing tissue source of MSCs or animal species.

One study evaluated the survival benefits of MSC-EVs in experimental BPD; however, mortality was shown to be unaltered as compared to control (RR 0.86, 95%CI 0.41 to 1.80) (Figure S4.1). Other secondary outcomes of experimental BPD that were assessed include alveolar permeability (SMD -1.80, 95%CI -3.18 to -0.41, $I^2 = 67\%$) (Figure 4) and lung inflammation as measured by BALF neutrophil (SMD -2.48, 95%CI -3.41 to -1.75, $I^2 = 66\%$) (Figure S4.2), both of which demonstrated significant improvements from MSC-EV delivery. Furthermore, MSC-EV delivery resulted in marked reductions to both Fulton's Index (measure

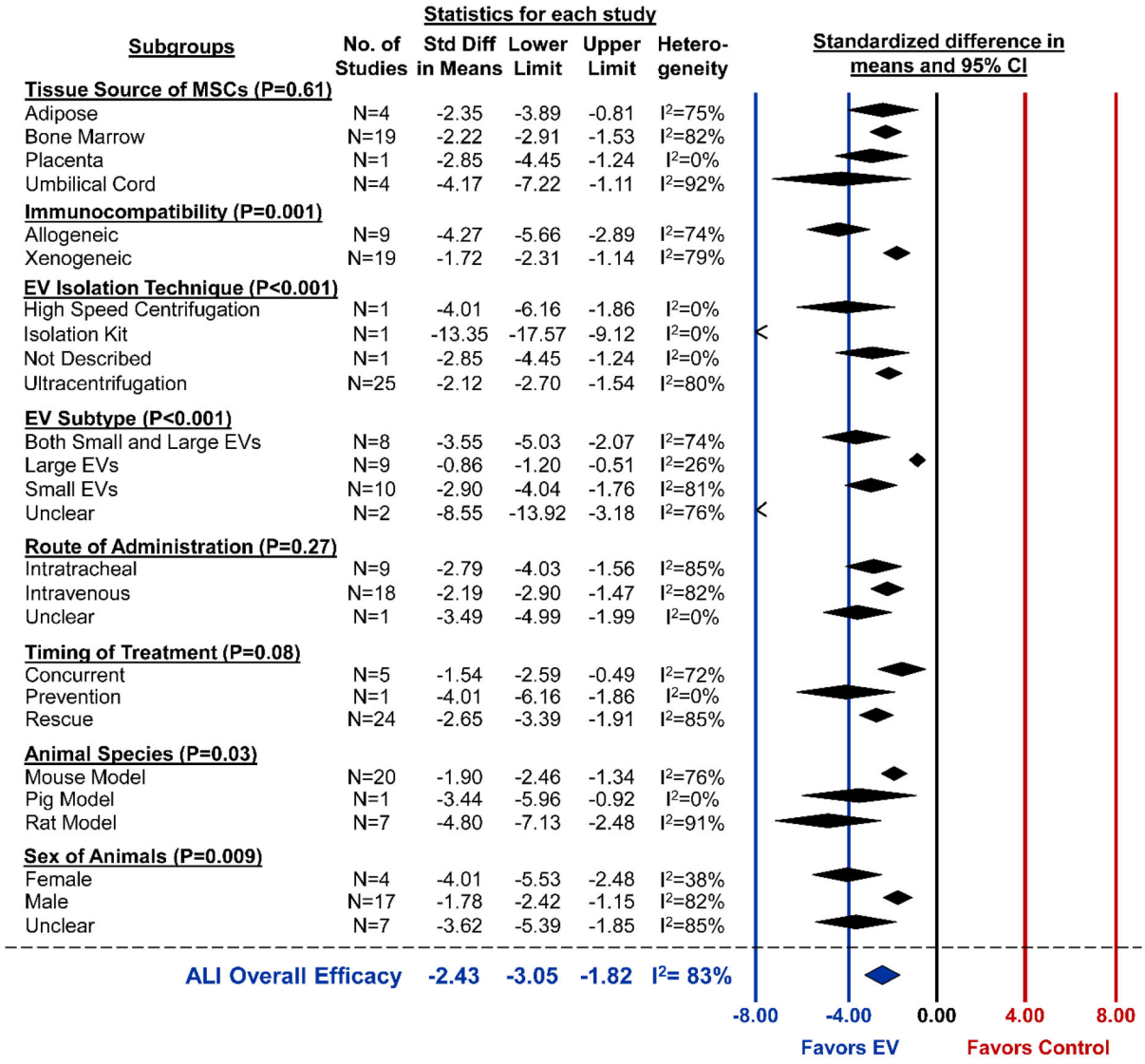

FIGURE 5 Subgroup analysis for all included studies of acute lung injury that reported the secondary outcome of alveolar capillary permeability. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I^2 value represents the statistical heterogeneity within each subgroup. Effect sizes < 0 favours EV treatment and > 0 favours control. The 'Overall Efficacy' is a pooled estimate effect of MSC-EVs on lung vascular permeability from all studies of acute lung injury combined

of right ventricular hypertrophy; SMD -0.99, 95%CI -1.49 to -0.49, $I^2 = 0\%$) (Figure S4.4) and RVSP (SMD -1.20, 95%CI -2.07 to -0.34, $I^2 = 0\%$) (Figure S4.5).

3.5 | Meta-analyses of pulmonary arterial hypertension studies

Method for PAH induction included monocrotaline injection (N = 5), chronic hypoxia(N = 2), or the combination of semaxanib (SU5416) and chronic hypoxia in rats (N = 2). Ten distinct studies from 5 articles reported RVSP, each of which demonstrated a significant reduction after MSC-EV administration (pooled analysis, SMD -4.16, 95%CI -5.68 to -2.64, $I^2 = 84\%$) (Figure 8). Exploratory subgroup analysis showed allogeneic MSC-EVs improved RVSP to a significantly greater extent than xenogeneic administration ($P < 0.001$) (Figure 9). Interestingly, in the single report of EVs isolated by combining TFF and size exclusion chromatography, EVs were less efficacious ($P < 0.001$) as compared to ultracentrifugation or ultracentrifugation/chromatography combined (Figure 9). Studies that did not report sex showed a significantly greater reduction in RVSP as compared to male-only studies ($P < 0.001$). Despite the well-known sex dependency of PAH, no preclinical study explored MSC-EV therapy in female animals. Unlike ALI or BPD, EV subtype did not demonstrate variable effects to RVSP. No differences in EV efficacy were observed when comparing MSC tissue source or timing of treatment (Figure 9). Lastly, 17 studies reported the secondary outcome

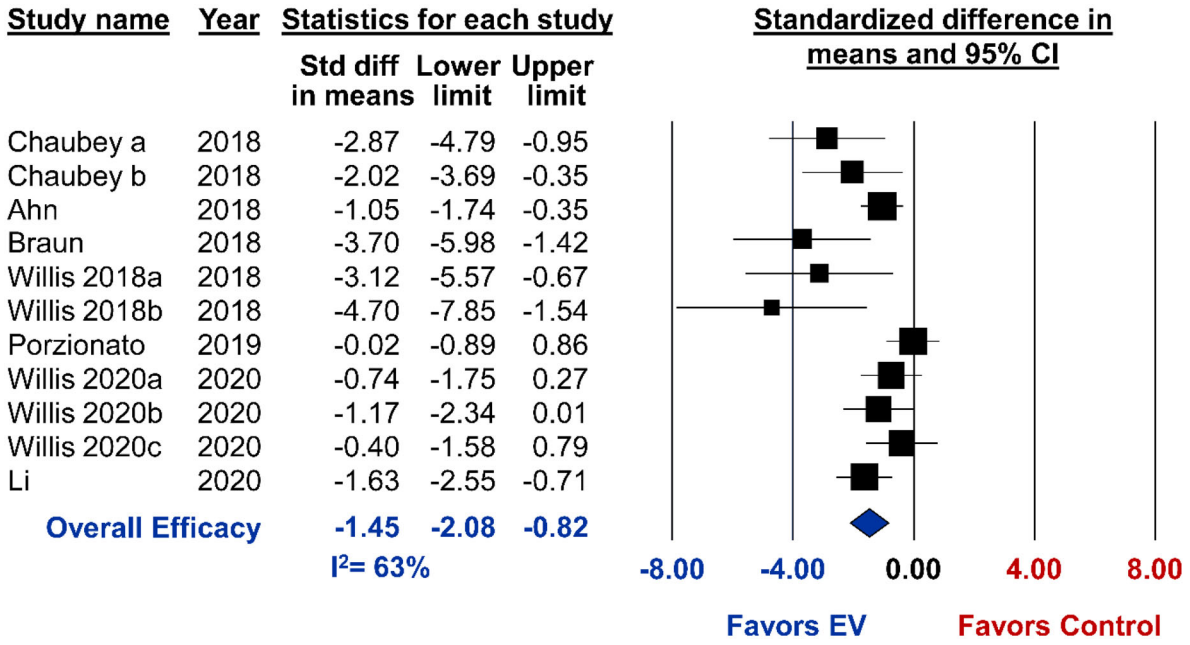

FIGURE 6 Meta-analysis for all included studies of bronchopulmonary dysplasia that reported the primary outcome of lung alveolarization. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes < 0 favours EV treatment and > 0 favours control. Subscript denotes a separate study or article that was published within the same year. The 'Overall Efficacy' represents a pooled estimate of MSC-EV effect on alveolarization from all studies combined. I^2 value represents the statistical heterogeneity

of Fulton's Index. In these studies, MSC-EV administration led to a significant reduction in right ventricular hypertrophy (SMD -2.80, 95%CI -3.68 to -1.91, $I^2 = 78\%$) (Figure S4.4).

3.6 | Studies of other respiratory conditions

3.6.1 | Asthma / allergic airway inflammation (N = 5 studies)

Disease was induced by intraperitoneal delivery of ovalbumin (N = 3) (De Castro et al., 2017; Fang et al., 2020a; Kim et al., 2020), *Aspergillus fumigatus* hyphal extract (N = 1) (Cruz et al., 2015), or IL-33 (N = 1) (Fang et al., 2020b). MSC-EVs were administered by intravenous injection in 4 studies, with one study using intranasal delivery for more localized lung distribution. Four studies assessed in vivo outcomes, all of which reported a significant reduction to BALF neutrophils, eosinophils and pro-inflammatory cytokines from MSC-EV delivery. Three studies reported a marked attenuation in lung inflammation score. One study showed improvements to lung mechanics, and another demonstrated a reduction in lung hyperresponsiveness to allergens (e.g. Aspergillus hyphal). Lastly, one study assessed differential gene expression in lung tissues post-EV administration and found the most upregulated genes were those related to antioxidation (Pon1), mitochondrial function (Bex2) and lung epithelium protection (Scgb1c1). Of note, the inhibition of miR-146a abolished the therapeutic efficacy of MSC-EVs, thereby illustrating its critical role within EVs for immunomodulation.

3.6.2 | Pulmonary fibrosis (N = 5 studies)

Fibrosis was induced by intratracheal silica (N = 2) (Bandeira et al., 2018; Choi et al., 2014), bleomycin (N = 2) (Dinh et al., 2020; Mansouri et al., 2019), or fine particulate matter (N = 1) (Gao et al., 2020). All studies delivered MSC-EVs as a rescue treatment which led to reduced lung collagen content in three studies, one of which was still significantly elevated as compared to control. In the other two studies, MSC-EV treated animals did not attenuate collagen deposition, whereas MSCs themselves or lung spheroid cell exosomes led to marked improvements. The effect of MSC-EVs on BALF macrophage expression was variable. Two studies reported no changes in number of lung macrophages, whereas one study observed a rescued expression of anti-inflammatory macrophages. Most studies demonstrated reduced expression of pro-inflammatory cytokines including IL-1β, TGFβ1 and TNFα. Lastly, one study found that transplantation of bone marrow derived monocytes preconditioned with MSC-EVs attenuated both pulmonary fibrosis and lung inflammation.

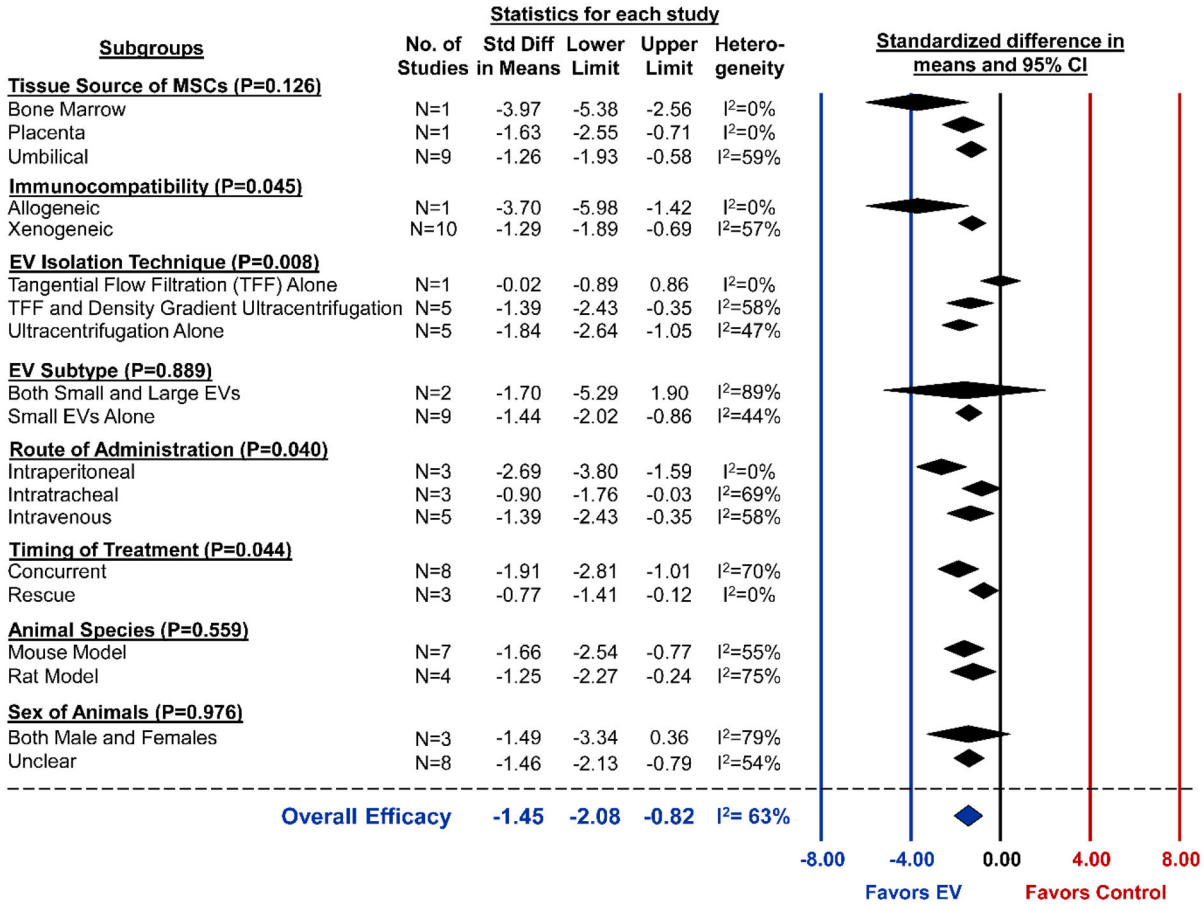

FIGURE 7 Subgroup analysis for all included studies of bronchopulmonary dysplasia that reported the primary outcome of lung alveolarization. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I^2 value represents the statistical heterogeneity within each subgroup. Effect sizes < 0 favours EV treatment and > 0 favours control. The 'Overall Efficacy' is a pooled estimate effect of MSC-EVs on alveolarization from all studies combined

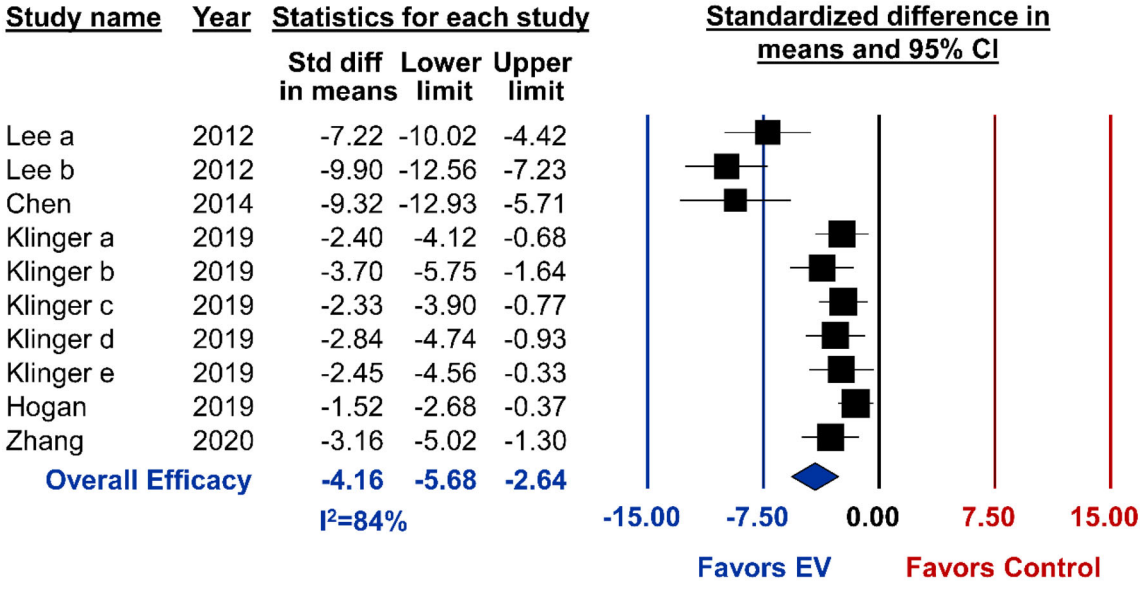

FIGURE 8 Meta-analysis for all included studies of pulmonary arterial hypertension that reported the primary outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes < 0 favours EV treatment and > 0 favours control. Subscript denotes a separate study or article that was published within the same year. The 'Overall Efficacy' represents a pooled estimate of MSC-EV effect on right ventricular systolic pressure from all studies combined. I^2 value represents the statistical heterogeneity

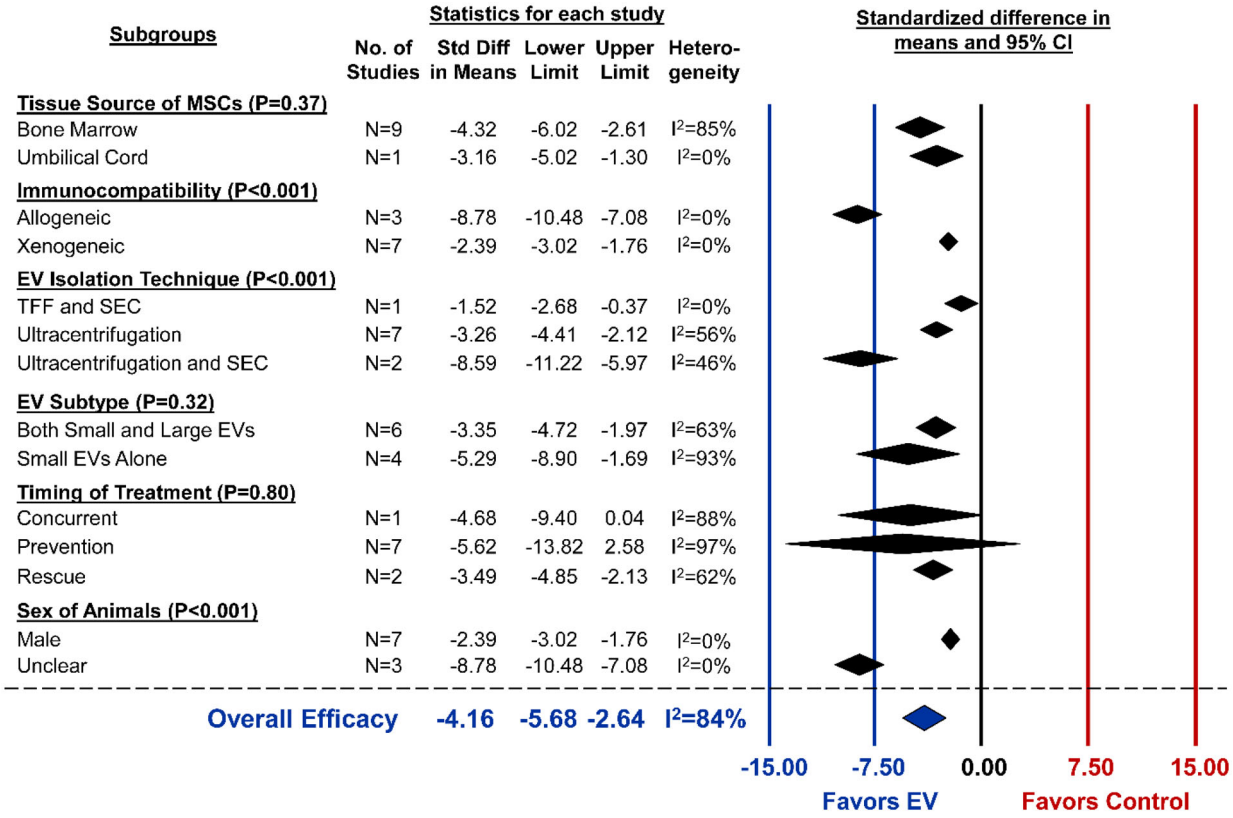

FIGURE 9 Subgroup analysis for all included studies of pulmonary arterial hypertension that reported the primary outcome of right ventricular systolic pressure. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I^2 value represents the statistical heterogeneity within each subgroup. Effect sizes < 0 favours EV treatment and > 0 favours control. The 'Overall Efficacy' is a pooled estimate effect of MSC-EVs on right ventricular systolic pressure from all studies combined

3.6.3 | Chronic obstructive pulmonary disease (COPD)

Two articles (4%) studied the effects of MSC-EVs in a cigarette smoke induced mouse model of COPD (Harrell et al., 2020; Maremanda et al., 2019). In one study, the combination of MSCs and exosomes were more effective than either treatment alone to reduce BALF cell count and cytokine levels (Maremanda et al., 2019). The mechanism of action was found to be recovery of mitochondrial transfer and mitochondrial fusion gene expression. The second study of COPD found intraperitoneal delivery of MSC-EVs resulted in marked reductions to plasma pro-inflammatory cytokines, histological lung injury and lung influx of inflammatory immune cells (Harrell et al., 2020).

3.7 | Quality of reporting in MSC-EV research

All 52 lung articles were assessed for their completeness of reporting study design domains based on the NIH Principles and Guidelines for Reporting in Preclinical Research (NIH 2014). These guidelines identify parameters that should be included in preclinical publications to enhance scientific rigor, reproducibility and transparency. No articles used established reporting guidelines (e.g. Animal Research: Reporting In Vivo Experiments, ARRIVE) (Figure S6). Findings were substantiated under a range of conditions (e.g. different dosages, routes of administration, modifications) in 31 studies (60%). Forty studies (77%) stated N-values for at least one in vivo outcome and 31 studies (60%) for all outcomes. A priori sample size determination was described in seven studies (13%); however, all seven studies had inadequate information necessary to reproduce calculations. The total number of animals used in all experiments was reported in 17 studies (33%) (Figure S6).

A primary outcome was not explicitly stated in any of the 52 studies (Figure S6). The number of measurements per subject was described for at least one outcome in 30 studies (58%), two of which reported the number of replicates in all findings. Randomization of experimental groups was mentioned in less than half of the articles (N = 24); however, the method of randomization was not described in any article. No studies reported blinding of the personnel conducting experiments. Blinding of data analysis was described for all outcomes in two studies (4%) and some outcomes in 16 studies (31%), with no blinding of outcome assessment

mentioned for 33 studies (66%). Lastly, the NIH recommends reporting the criteria for data exclusion to minimize selection bias. Seven studies (13%) provided reasoning for data omission, which included excluding animals with heart rates < 300 beats per minute for hemodynamic measurements (Willis et al., 2018), low expression of genes in RNA sequencing (Hogan et al., 2019), or statistical outliers (Varkouhi et al., 2019). Forty-five studies (87%) did not provide any criteria for data exclusion (Figure S6).

3.8 | Risk of bias assessment

A majority of studies were deemed as having an 'unclear' risk of bias across most domains (Figure 10). Although 24 studies (46%) reported that animals were randomized to experimental groups, no studies provided details regarding method of *randomization* (i.e. to reduce selection bias) which is essential to assess adequate random sequence generation. Thirty-two studies (62%) displayed a low risk for reporting *baseline characteristics* (i.e. to reduce selection bias). However, the risk of bias was unclear across all studies for the domains of *allocation concealment* (i.e. selection bias), *random housing* (i.e. selection bias), *blinding of personnel* (i.e. performance bias) and *selective outcome reporting* (i.e. reporting bias). Seventeen studies reported *blinded assessments* (i.e. detection bias) for some outcomes (e.g. histological/pathological injury) and 2 studies were blinded for all outcomes. *Incomplete outcome data* (i.e. attrition bias) was assessed as whether the N-value was consistent between the methods and results sections. Fourteen studies (27%) exhibited low risk, 25 studies (48%) had unclear risk and 13 studies (25%) were deemed high risk. Lastly, *other potential sources of bias* considered included source of funding, sample size calculation and conflict of interests. Eleven studies (21%) had a high risk in at least one of these additional categories. (Figure 10).

4 | DISCUSSION

Our systematic review provides a comprehensive synthesis of the preclinical efficacy, methodology, study design and preclinical reporting of MSC-EV studies of acute and chronic respiratory diseases. Pooled estimates from meta-analyses indicate that MSC-EV administration significantly improves primary outcomes in ALI (i.e. lung injury score), BPD (i.e. alveolarization) and PAH (i.e. right ventricular systolic pressure). With the exception of mortality for BPD, MSC-EVs were also markedly effective at improving secondary outcomes including survival (ALI), lung vascular permeability (ALI and BPD), BALF neutrophil count (ALI and BPD), RVSP (BPD) and Fulton's Index (BPD and PAH). These findings demonstrate the therapeutic efficacy of MSC-EVs to treat both acute and chronic lung diseases.

Our previous systematic analysis of all animal studies investigating MSC-EVs found high heterogeneity in how EVs are being isolated and characterized (Tieu et al., 2020). Moreover, there were significant inconsistencies in nomenclature being used to describe EV therapy and variability in interventional characteristics including tissue source of MSCs and treatment regimen. After updating our search and limiting our review to publications related to lung disorders, we still observed similar heterogeneity. When assessing whether groups were characterizing their MSCs and EVs according to international guidelines (e.g. ISCT criteria and MISEV 2018) (Dominici et al., 2006; Théry et al., 2018), we found that 58% and 19% of studies met the recommendations, respectively. This is a significant increase from our previous reports of only 2% adherence to MISEV 2018 (Tieu et al., 2020) which highlights the impact of these guidelines for improving EV characterization. EV nomenclature remains inconsistent despite the suggestions from MISEV 2018 to describe EV therapies by physical parameters (e.g. small EVs 30–150 nm, large EVs 150–1000 nm). Based on size alone, we found that over 30% of studies are using discordant terminology. This finding may be problematic for two reasons: (1) inconsistent terms will hinder between study comparisons of different EV populations, and (2) our finding likely is an underestimate as we only considered size and did not include analysis of EV cellular origin (e.g. exosomes originate as intraluminal vesicles within late endosomes). Greater standardization in reporting of size and applying accurate terminology in the EV field can help identify specific EV populations that serve as a more effective therapeutic product.

Given the many variables involved in developing an MSC-EV therapy, we conducted exploratory subgroup analyses to determine if specific EV parameters may be associated with improved efficacy. Across studies of ALI, BPD and PAH, the different tissue sources of MSCs (e.g. bone marrow, umbilical cord, placenta) and routes of administration (e.g. intravenous, intraperitoneal, intratracheal) were equally efficacious, despite beliefs that intratracheal delivery may allow for greater lung distribution. Moreover, umbilical cord tissue is known to be more efficient in producing EVs with potentially less variability from donor demographics (e.g. age, sex, comorbidities) and serves as a non-invasive, abundant source for MSCs given its designation as biohazardous waste (Curley et al., 2017; Fong et al., 2012; Hua et al., 2014). Hence, the similar efficacy and ease in collection of umbilical cord tissue suggests some potential advantages when considering clinical translation. EVs isolated by TFF appeared to be less effective than those enriched by ultracentrifugation for preclinical BPD and PAH (no studies used TFF in ALI). However, these findings were from only two studies that utilized TFF and more direct comparisons of TFF and ultracentrifugation are warranted to establish the relative in vivo efficacy of EVs, especially given TFF's greater efficiency in EV yield and potential for clinical scaleup (Haraszti et al., 2018). Lastly, we conducted subgroup analysis by EV subtype/size to investigate whether distinct EV populations may exhibit variable outcomes. Small EVs or the combination of small/large EVs were found to be more

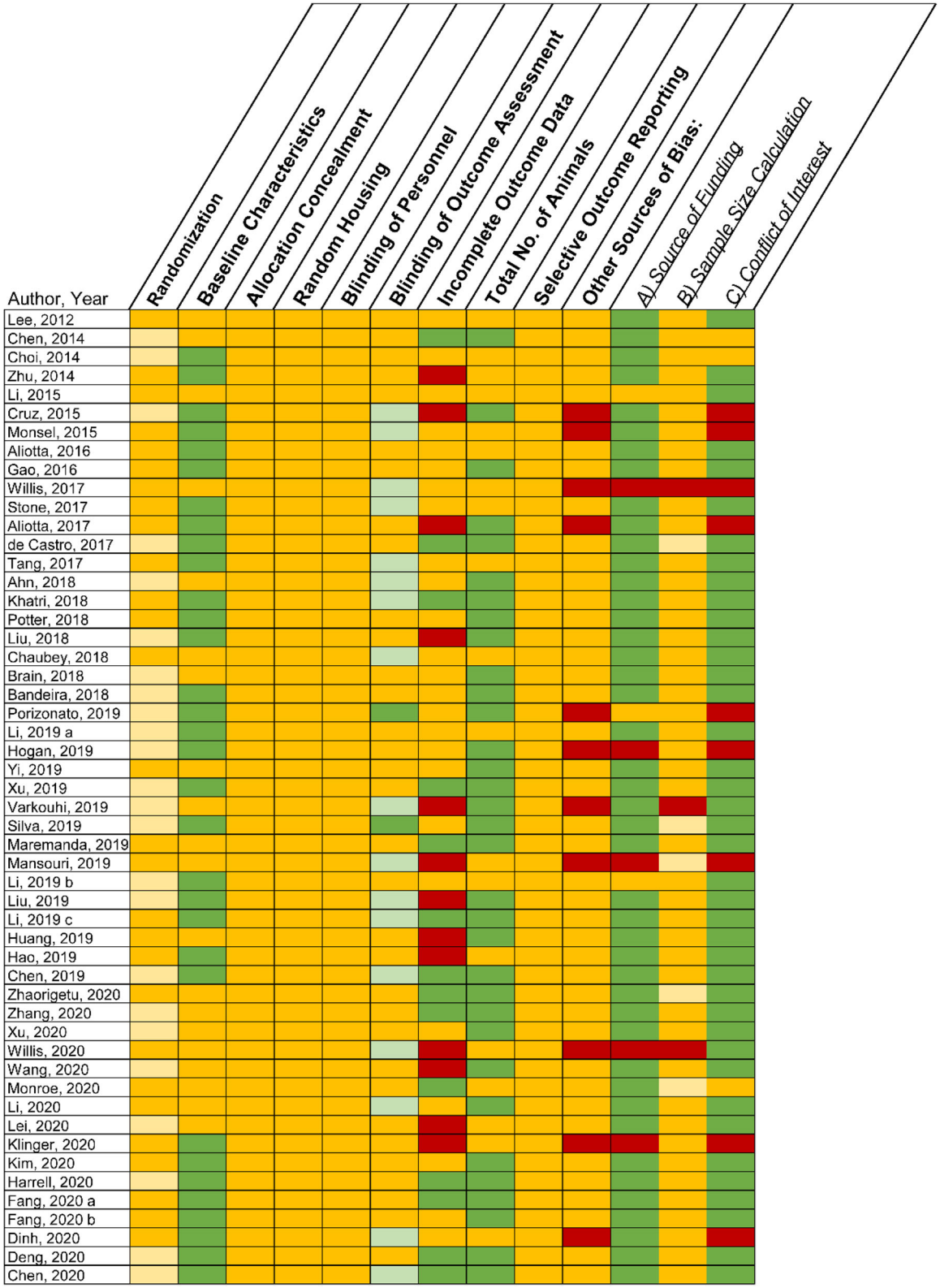

FIGURE 10 Risk of bias assessment in accordance with the SYRCLE tool. Yellow represents unclear, green represents low risk of bias and red represents high risk of bias. Light yellow represents a study that reported randomization or sample size calculations, but did not provide detailed methodology on how these parameters were achieved. Light green under 'blinding of outcome' represents studies that blinded some outcomes, whereas dark green represents blinding of all outcomes

consistently beneficial as compared to large EVs in ALI and BPD (no studies used large EVs in PAH). Moreover, small EVs led to a significant improvement in lung vascular permeability as compared to large EVs. These results may indicate that there are differential effects between small and large EVs, which may not be dependent on acuity/chronicity of the disease state.

Our study had limitations which warrant discussion. Firstly, the findings from subgroup analyses should be considered exploratory. We show that specific EV characteristics are associated with greater efficacy in these investigations. However, some subgroup categories were limited by the number of available studies and/or the sample size. Hence, updated analyses when more studies are published will be required to generate definitive conclusions. Our findings identify the need for robust data from head-to-head comparisons of EV methodology and/or subtypes to identify the most effective approaches for clinical translation. Next, data generated from studies with a low risk of bias may serve as a more accurate indicator for the efficacy of MSC-EVs. Unfortunately, most studies included in our analysis demonstrated an "unclear" risk across a majority of design parameters, which is commonly observed in many fields of preclinical in vivo research (Avey et al., 2016; Bailey et al., 2021a, 2021b; Begley & Ioannidis, 2015; Fergusson et al., 2019a, 2019b). Future analyses will provide greater insights once researchers and journals adopt more complete reporting of preclinical study design (Collins & Tabak, 2014; Henderson et al., 2013; Landis et al., 2012). Lastly, although no adverse events were reported, none of the included studies conducted formal experiments to investigate MSC-EV safety.

Although MSC-EV therapy demonstrates great promise, several questions still need to be answered to advance the field. We found dose-response and biodistribution studies to be infrequently conducted in respiratory diseases, which is also the case for the MSC-EV field as a whole (Tieu et al., 2020). These experiments are urgently needed to better understand the optimal approach for dosing EVs before attempting clinical translation. Moreover, there are no standardized guidelines on how EV dose should be measured and reported (e.g. protein, particle or RNA amount). We now know that MSC-EVs contain many bioactive factors including mRNAs, microRNAs and proteins that modulate critical cell processes ranging from inflammation to apoptosis to angiogenesis (Tieu et al., 2020). Although many studies show the importance of specific molecules by inhibiting their expression, few have attempted to compare whether overexpressing one bioactive factor is more important than others. Lastly, quality of reporting and potential risk of bias in study design is consistently unclear in preclinical EV research. Greater rigor and transparent detailing of experimental parameters including randomization, blinding, and sample size estimates will generate more robust evidence for EV efficacy to guide clinical translation.

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CONFLICT OF INTEREST

Duncan J. Stewart is an advisor to Northern Therapeutics (Montreal, QC, Canada). The remaining authors have no competing interests to declare.

ORCID

Alvin Tieu https://orcid.org/0000-0002-8733-6097
Joshua Montroy https://orcid.org/0000-0002-6611-0056
Dean A. Fergusson https://orcid.org/0000-0002-3389-2485
David S. Allan https://orcid.org/0000-0003-3261-8289
Duncan J. Stewart https://orcid.org/0000-0002-9113-8691
Bernard Thébaud https://orcid.org/0000-0003-1844-7145
Manoj M. Lalu https://orcid.org/0000-0002-0322-382X

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SUPPORTING INFORMATION

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