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Combined mesenchymal stromal cell therapy and ECMO in ARDS: A controlled experimental study in sheep

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Running title

Combined MSC therapy and ECMO in experimental ARDS

Subject category classification

4.1 ALI/ARDS: Biological Mechanisms

Manuscript word count

3,086 words

At a glance commentary

Scientific knowledge on the subject:

Mesenchymal stromal cell (MSC) therapy is a promising novel intervention for ARDS. Trials to date have failed to explore the safety of cell therapy during ECMO. Pre-clinical investigations have found that MSC therapy during ECMO attenuate the efficacy of ECMO.

What this study adds to the field:

This study, employing a 24-hour, large animal model of ARDS and ECMO, examined the safety and efficacy of MSC therapy. We found that endobronchially administered MSCs adhere to, and impair, membrane oxygenators *in-vivo*. MSCs did not improve oxygenation or other ventilatory parameters but did reduce the severity of histological evidence of lung injury. Our data also suggest that MSCs may reduce circulatory shock associated with ARDS. There were no adverse effects of MSC administration on renal or liver function.

Online data supplement

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abstract

Rationale

Mesenchymal stromal cell therapy is a promising intervention for ARDS, although trials to date have not investigated its use alongside ECMO. Recent pre-clinical studies have suggested that combining these interventions may attenuate the efficacy of ECMO.

Objectives

To determine the safety and efficacy of mesenchymal stromal cell therapy in a model of ARDS and ECMO.

Methods

ARDS was induced in 14 sheep, after which they were established on veno-venous ECMO. Subsequently, they received either, endobronchial iPSC-derived human MSCs (hMSCs, n=7) or cell-free carrier vehicle (Vehicle control, n=7). During ECMO, a low tidal volume ventilation strategy was employed in addition to protocolized hemodynamic support. Animals were monitored and supported for 24 hours. Lung tissue, bronchoalveolar fluid, and plasma were analysed, in addition to continuous respiratory and hemodynamic monitoring.

Measurements and main results

The administration of hMSCs did not improve oxygenation ($\text{PaO}_2/\text{FiO}_2$ mean difference -146 mmHg, $p = 0.076$) or pulmonary function. However, histological evidence of lung injury (Lung Injury Score mean difference -0.07, $p = 0.04$) and BAL IL-8 were reduced. In addition, hMSC treated animals had a significantly lower cumulative requirement for vasopressor. Despite endobronchial administration, animals treated with hMSCs had a significant elevation in trans-membrane oxygenator pressure gradients. This was accompanied

by more pulmonary artery thromboses and adherent hMSCs found on explanted oxygenator fibers.

Conclusions

Endobronchial hMSC therapy in an ovine model of ARDS and ECMO can impair membrane oxygenator function and does not improve oxygenation. These data do not recommend the safe use of hMSCs during VV-ECMO.

Abstract word count

250

Keywords

Acute Respiratory Distress Syndrome

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Mesenchymal stromal cells

Models, Animal

Introduction

The quest for an effective pharmacological treatment for the Acute Respiratory Distress Syndrome (ARDS) has been unsuccessful. Recently, mesenchymal stromal cells (MSCs) have attracted attention as a candidate therapy for ARDS (1).

MSCs are multipotent adult stem cells found in tissues of mesodermal origin such as bone marrow (2). Therapeutic interest in these cells has arisen because of their pleiotropic immunomodulatory abilities. During acute inflammation, MSCs appear to be immunosuppressive, influencing both innate and adaptive immune responses (3). In ARDS, their beneficial effects are believed to be mediated in a variety of ways, including; secretion of anti-inflammatory paracrine factors (4), restoration of epithelial and endothelial integrity (5), enhancement of alveolar fluid clearance (6), direct antimicrobial activity (7), and by mitochondrial transfer (8). In pre-clinical models of acute lung injury, MSCs have been shown to reduce mortality (9). A phase 2 study has been conducted in patients with ARDS with no reported infusion-related adverse events (10).

To date, trials of MSCs in ARDS have excluded patients supported with extracorporeal membrane oxygenation (ECMO). The use of ECMO in acute severe respiratory failure has increased substantially in the last decade and is now an established tool for supporting those with refractory illness (11). The use of MSCs during ECMO, while potentially attractive, raises some unique considerations. Firstly, MSCs are large cells, with an average diameter between 10-30 μm (12), which when administered therapeutically may pose a risk to the patency of a membrane oxygenator. Secondly, a defining characteristic of MSCs is avid plastic adherence (13); this too may threaten membrane oxygenators, which are constructed largely from plastics. Recent *ex-vivo* and small animal experimentation has confirmed these concerns (14, 15).

Conversely, immunomodulation by MSCs may provide additional benefits for ECMO patients, where the institution of extracorporeal support results in an additional inflammatory insult (16).

Given the paucity of evidence to support the safe use of MSC therapy during ECMO, we conducted a controlled trial of clinical-grade induced pluripotent stem cell (iPSC) derived human MSCs (hMSCs) in an ovine model of ARDS, supported with veno-venous ECMO (VV-ECMO). The primary objective was to assess the safety of MSC therapy and to investigate its effect on physiologic and biologic markers of pulmonary and systemic injury.

Methods

Study design

Ethical approvals were obtained from University Animal Ethics Committees (QUT1600001108, UQPCH/483/17) and authorization for *in-vivo* use of hMSCs was granted by the Australian Department of Agriculture (2017/075). The study was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (17) and is reported in compliance with ARRIVE guidelines (18). Detailed methods and a comprehensive description of the experiments and analyses are provided in an online supplement. A schematic of the study protocol is provided in **Figure 1**.

Animal model

Fourteen healthy Border Leicester Cross ewes aged between 1-3 years and weighing between 46-55 kg (mean, 52.6 ± 3 kg), were randomly assigned to one of two groups; endobronchial iPSC-derived hMSC treatment (n=7) or endobronchial carrier vehicle only (n=7).

In brief, animals were anesthetized with a combination of ketamine, midazolam, and fentanyl. Continuous neuromuscular blockade was maintained by infusion of vecuronium. In a

supine position, animals were tracheostomized and ventilated using a low tidal volume strategy (6 mL/kg actual body weight (ABW)). After instrumentation, acute lung injury was induced by combining an intravenous infusion of oleic acid (OA; 0.06 mL/kg; O1008, Sigma-Aldrich, Castle Hill, NSW, Australia) with endobronchial *E. coli* lipopolysaccharide (LPS; 100 µg; O55:B5, Sigma-Aldrich, Castle Hill, NSW, Australia). Once a PaO₂/FiO₂ ratio < 100 mmHg (PEEP ≥ 10 cmH₂O) was obtained (T₀), animals were established on VV-ECMO via a right-sided jugular-jugular configuration (T₁) and positioned in sternal recumbency. VV-ECMO was combined with a lower tidal volume strategy (4 mL/kg ABW) for 22 hours, at which time (T₂₃) ECMO was stopped and a standardized recruitment manoeuvre was performed. Animals were returned to pre-ECMO ventilatory settings for one hour before being euthanized (T₂₄).

iPSC-derived hMSCs

After one hour of VV-ECMO (T₂), animals received a fixed dose of 3 x 10⁸ iPSC-derived hMSCs suspended in a carrier vehicle (hMSC) or carrier vehicle alone (Vehicle control). Cells were provided by Cynata Therapeutics Ltd. (CYP-001; Cynata Therapeutics Ltd., Melbourne, VIC, Australia). These cells were ≥ 99% positive for CD-73, CD-90, and CD-105, but negative for CD-31 and CD-45. The total volume of vehicle was 60 mL (57.5% Plasmalyte-A, 40% Flexbumin 25%, 2.5% Dimethyl sulfoxide). Cells were ≥ 97% viable prior to administration. The distribution of delivery is described in **Figure E1**.

Statistical analysis

An *a priori* sample size calculation, based on the primary outcome of PaO₂/FiO₂ ratio at 24 hours, is detailed in the online supplement. Data are expressed as mean (± SD) or median (IQR) if non-normally distributed. Analysis was undertaken in Graphpad Prism (v 8.1.2., GraphPad Software, San Diego, USA). Longitudinal data were analyzed by fitting a mixed model. This model uses a compound symmetry covariance matrix and is fit using restricted

maximum likelihood. Where a significant interaction was observed *post-hoc* comparisons were undertaken. Correction for multiple comparisons was made using the Benjamini-Hochberg method (false discovery rate restricted to 5%). Non-longitudinal data were compared using an unpaired t-test or a Mann-Whitney test as appropriate. Categorical data were compared using the chi-squared test. Statistical significance was assumed if $P < 0.05$.

Results

Baseline characteristics at injury (T_0) are shown in **Table 1** and in **supplementary Table E1**. All animals completed the study protocol and were euthanized at T_{24} .

Respiratory variables

The use of ECMO facilitated a lower tidal volume ventilation strategy (4 (4-4) mL/kg ABW). The median ECMO flow rate was 2.75 L/min (2.5-3.25 L/min), with a sweep gas flow of 3 L/min (2-3.5 L/min). During VV-ECMO, animals had a median PaO_2 of 109 mmHg (94-131 mmHg) and a $PaCO_2$ of 32 mmHg (30-35 mmHg). There were no significant differences in these parameters between groups (**supplementary Figure E2**). Animals were adequately anticoagulated during ECMO, as measured by activated partial thromboplastin time (aPTT) ratios. The dose of heparin was not significantly different between groups (**supplementary Figure E2**).

Because VV-ECMO controls gas exchange, native lung function was assessed one hour after cessation of extracorporeal flow and after the performance of a standardized lung recruitment manoeuvre (T_{24}). As shown in **Figure 2** both the PaO_2/FiO_2 ratio ($p = 0.076$) and the oxygenation index (OI) ($p = 0.153$) were numerically better in the carrier vehicle only group, the differences were not statistically significant.

The plateau airway and driving pressures were similar between groups at T₂₄ (**Figure 2**). Static lung compliance was also similar, both during and after ECMO (**Figure 2**). The use of a protocolized recruitment manoeuvre did not improve compliance after cessation of extracorporeal support in either group (**Figure 2**).

Hemodynamic variables

This model of acute lung injury was associated with the development of hyperdynamic shock, which worsened over time (**Figure 3**). The administration of hMSCs resulted in significantly lower cumulative vasopressor doses (**Figure 3**). At T₄, mean arterial pressure (MAP) was significantly higher in the hMSC treated group ($p = 0.001$), even though these animals received lower doses of noradrenaline (**Figure 3**). By T₁₄, MAP was again similar between groups, although vasopressor requirements continued to be lower in hMSC treated animals. In addition, there were lower arterial lactate concentrations, higher arterial base excesses, and lower mean pulmonary artery pressures from 12 hrs (T₁₄) post instillation in the hMSC group (**Figure 3**), however these were not statistically significant. Cumulative fluid balance at T₂₄ was similar in both groups (Vehicle control, 2713 ± 970 mL vs. hMSCs, 2992 ± 1237 mL, $p = 0.648$).

Histopathology and lung injury

The blinded assessment of lung tissue was conducted by an independent expert veterinary pathologist. Sections of the right lower lobe were prepared, and a lung injury score (LIS) was calculated (19). The administration of hMSCs resulted in significantly lower scores ($p = 0.04$) (**Figure 4**), principally mediated by a reduction in neutrophil infiltration.

There were no significant differences in lung wet/dry ratio or bronchoalveolar lavage (BAL) total protein concentration (**Figure 4**). BAL fluid inflammatory cell counts are detailed in **supplementary Table E4**. There were no significant differences in these counts over time.

Similarly, there was no difference in lung tissue homogenate gene expression (as assessed by qPCR) between groups (**supplementary Figure E3**).

In a *post-hoc* analysis, pulmonary arterial thrombosis was noted in 5 hMSC treated animals, but only one animal receiving carrier vehicle alone ($p = 0.031$).

Inflammatory cytokines

BAL and plasma cytokine concentrations were assessed longitudinally (**supplementary Figure E4 and supplementary Figure E5**). In BAL, statistically significant differences in IL-8 were observed at T₃, T₁₄, and T₂₃ ($p = 0.013$, 0.016 , and 0.028 respectively). In plasma, cytokine trajectories were similar between groups (**supplementary Figure E5**).

Hematological and biochemical measurements

A summary of hematological and biochemical values are provided in **supplementary Tables E2 and E3**. This lung injury model was associated with the development of acute kidney injury and abnormal liver function, although there were no significant differences in indices between groups. The administration of hMSCs resulted in a significantly lower lymphocyte count at T₂₄ ($p = 0.047$) (**supplementary Figure E5**).

Cell-ECMO membrane interaction and cell fate

The administration of hMSCs was associated with a significant increase in the trans-oxygenator pressure gradient, becoming apparent 4 hours after cell delivery (**Figure 5**). By T₂₃, the mean pressure gradient in the hMSC group reached 64 ± 37 mmHg vs. 17 ± 9 mmHg in the vehicle only group. The instillation of carrier vehicle alone was associated with a reduction in the ECMO pump speed to flow ratio over time, a finding not observed in the hMSC group (**Figure 5**). During the study, there were no instances of pump or oxygenator failure requiring

a component exchange. Likewise, there was no evidence of clotting on the oxygenator surface by visual examination in either group.

Membrane oxygenators from animals treated with hMSCs were isolated and preserved at the termination of ECMO, subsequent deconstruction and staining of the fiber bundles (n=7) revealed adherent cells exhibiting surface markers consistent with those of hMSCs (**supplementary Figure E6**). Similar cell populations were not apparent in vehicle only controls (n=3).

Discussion

We carried out a trial of clinical-grade iPSC-derived hMSCs, given endobronchially, for acute lung injury in sheep during VV-ECMO. The main findings of this study can be summarized as follows; (1) with regard to the primary outcome, hMSCs did not improve oxygenation at 24 hours. (2) hMSCs did not improve pulmonary mechanics but did improve the severity of histological lung injury and reduced the concentration of bronchoalveolar lavage IL-8. (3) in spite of endobronchial administration, hMSCs adhered to and impacted the function of a commercial membrane oxygenator *in-vivo* - with an increase in the trans-membrane oxygenator pressure gradient. In addition, more pulmonary arterial thromboses were noted in hMSC-treated lungs. (4) hMSCs reduced the depth and severity of shock.

This study was conducted in a large animal model of ARDS and ECMO, which replicates several important clinical features (20). The ‘double-hit’ injury applied in this study resulted in acute severe hypoxemic respiratory failure consistent with modern criteria for the use of VV-ECMO (11). To support the severe acute respiratory failure, we employed a commercial ECMO device which is in widespread clinical use. Additionally, our protocolized intensive care was consistent with clinical best practice standards (21). A common criticism of pre-clinical trials of MSCs has been the use of heterogeneous, non-clinical grade cell products

(1), however, we tested a commercial hMSC product which is under investigation in clinical trials.

MSCs have been administered to patients with respiratory failure on ECMO (22, 23). These reports, which include only three patients, did not describe infusion-related adverse events although failed to fully characterize the interaction between MSCs and the extracorporeal device. Kocyildirim *et al.* have conducted the only other pre-clinical study involving both MSCs and ECMO for respiratory failure (24). Their ovine based, 6-hour pilot study did not report on the impact of MSC administration on the performance of ECMO.

hMSCs and pulmonary function

In this study, the administration of hMSCs failed to improve oxygenation at T₂₄. Furthermore, animals receiving hMSCs had a trend for worse oxygenation index values. In a phase 2a study of 60 patients with ARDS, the intravenous administration of hMSCs did not significantly improve PaO₂/FiO₂ ratio, although there was a signal toward improvement in oxygenation index in a *post-hoc* analysis (10). Pre-clinical studies of MSCs in ARDS have reported improvements in oxygenation, although few have produced lung injury as severe (25). In a recent systematic review of pre-clinical models combining ARDS and ECMO, only four achieved PaO₂/FiO₂ values <100 mmHg (20). The degree of lung injury in this model may explain why oxygenation is impaired in the treated group, despite improvements in inflammation and lung injury. Emerging research has highlighted the pro-coagulant effects of transplanted MSCs. These appear to be primarily mediated by MSC expression of tissue factor (26), but also by the secretion of pro-coagulant microvesicles (27) and by direct enhancement of platelet deposition (28). In pre-clinical experiments MSCs have been associated with the development of pulmonary emboli *in-vivo* (29). In this study, despite the use of heparin, almost all hMSC animals (n=6) had histological evidence of pulmonary arterial thrombosis at post-

mortem. The presence of exogenous hMSCs within the disordered pulmonary vasculature may have contributed to impairments in oxygenation, tempered by the fact that there was no increase in mean pulmonary artery pressure in treated animals.

Animals receiving hMSCs had improved composite histologic lung injury scores at post-mortem. The components of the score most influenced by hMSCs were neutrophil numbers in the alveolar and interstitial space. Multiple studies of MSCs in pre-clinical models of ARDS have demonstrated their ability to reduce neutrophil infiltration (30) and neutrophil extracellular trap formation (31). In this study, BAL neutrophil counts did not differ between groups. This may reflect the technical challenges of obtaining and assessing BAL cell counts. In a recent porcine model of ARDS and MSC therapy, a reduction in neutrophil infiltration was correlated with a reduction in BAL IL-8 concentrations (32), a finding confirmed in this study.

hMSCs and the systemic inflammatory response

Multiple pre-clinical models (33) and recent clinical trials (34-37) have examined the use of MSCs in the treatment of septic shock. Animals receiving hMSCs required less vasopressor support throughout the experiment to achieve an equivalent or higher MAP. A similar, early but non-sustained, reduction in vasopressor requirement has previously been described in a large-animal model of septic shock treated with MCSs (38). hMSCs did not alter plasma concentrations of pro-inflammatory cytokines over extended time periods in this study, a finding which has previously been identified in other pre-clinical (39) and clinical studies (34). A recent Phase I dose escalation study of MSCs in patients with septic shock demonstrated that the maximum effect of cell therapy on plasma cytokine levels occurred at 4 hours post-administration and declined with time (36). In this study, that time period coincides with the maximum separation in vasopressor dose, MAP, and levels of IL-1 β and IL-6 between

groups. This may indicate that repeat dosing of MSCs will be required for optimal therapeutic efficacy.

hMSCs and ECMO

The risk posed by MSCs to membrane oxygenators has been postulated for some time (1), but has only recently been shown to have an experimental basis. Our group has previously reported the ability of hMSCs to tightly adhere to the membrane fibers of a commercial oxygenator. This may have been the result of the known plastic avidity of MSCs (13). Recently, Cho *et al.* reported the loss of systemically administered MSCs in an *ex-vivo* model of veno-arterial ECMO (15).

Given the emerging signal that systemically administered MSCs may interact with membrane oxygenators, we decided to test endobronchial instillation in this study. Cardenes *et al.* have used 18F-fluorodeoxyglucose labelling to track the fate of both systemically and endobronchially administered MSCs in an ovine model of ARDS (40). While systemically administered cells have a wide biodistribution in the first 5 hours, endobronchially administered cells were retained at the site of instillation. There are key differences in our approach, including the means of inducing lung injury and its severity.

Limitations

This study has some limitations. First, while our model of injury replicates several relevant features including severe respiratory and hemodynamic failure, clinical ARDS is usually caused by infection and develops over several days, often in patients with other comorbidities (41). Second, MSCs are known to exhibit different functional responses dependent on the contemporary milieu, which in some circumstances may be detrimental (42). This study may have modelled only one phase of acute lung injury and so hMSCs may have exerted an effect which may differ in other phases. Third, while our model extended 21 hours

post cell or vehicle delivery, this may have been too short a period to observe some beneficial effects of the intervention. For example, it may be that the favourable effect of hMSCs on histological injury may have translated to improvements in oxygenation over a longer time period. Conversely, an extension of the study period in the face of rising trans-oxygenator pressure gradients in the hMSC group may have ultimately led to circuit failures. Fourth, the use of a lung recruitment maneuver and the assessment of native lung function off ECMO may have had several adverse effects and we cannot be certain that these effects did not differ between groups. This approach was taken due to the challenge of assessing native lung function during ECMO, particularly where a lower tidal volume ventilatory strategy has been adopted. The study protocol was designed prior to the publication of the ART randomized controlled trial (43). Fifth, the addition of an uninjured control group may have provided further insights into the distribution of hMSCs during VV-ECMO. Finally, the dose and method of delivery of hMSCs remain a matter of conjecture. Based on the findings of our previous work (14), we chose not to investigate intravenous administration. Likewise, based on clinical trial experience, we opted to administer a single, fixed-dose of hMSCs. It is possible that varying the dose and/or route of administration of hMSCs may alter their efficacy and safety profile during ECMO.

Conclusion

In a 24-hour, ovine model of ARDS and VV-ECMO, we found that hMSC therapy was associated with impairment of the membrane oxygenator. The use of cell therapy did not result in improvements in oxygenation, the primary outcome of this study, but was associated with a reduction in histological evidence of lung injury and inflammation in the lung. Given these data we cannot currently recommend the administration of hMSCs during ECMO.

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References

1. Millar JE, Fraser JF, McAuley DF. Mesenchymal stromal cells and the acute respiratory distress syndrome (ARDS): challenges for clinical application. *Thorax* 2015; 70: 611-612.
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284: 143-147.
3. Curley GF, McAuley DF. Stem cells for respiratory failure. *Curr Opin Crit Care* 2015; 21: 42-49.
4. Devaney J, Horie S, Masterson C, Elliman S, Barry F, O'Brien T, Curley GF, O'Toole D, Laffey JG. Human mesenchymal stromal cells decrease the severity of acute lung injury induced by E. coli in the rat. *Thorax* 2015; 70: 625-635.
5. Fang X, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. *J Biol Chem* 2010; 285: 26211-26222.
6. McAuley DF, Curley GF, Hamid UI, Laffey JG, Abbott J, McKenna DH, Fang X, Matthay MA, Lee JW. Clinical grade allogeneic human mesenchymal stem cells restore alveolar fluid clearance in human lungs rejected for transplantation. *Am J Physiol Lung Cell Mol Physiol* 2014; 306: L809-815.
7. Krasnodembskaya A, Song Y, Fang X, Gupta N, Serikov V, Lee J-W, Matthay MA. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010; 28: 2229-2238.
8. Jackson MV, Morrison TJ, Doherty DF, McAuley DF, Matthay MA, Kissenpfennig A, O'Kane CM, Krasnodembskaya AD. Mitochondrial Transfer via Tunneling Nanotubes is an Important Mechanism by Which Mesenchymal Stem Cells Enhance Macrophage

- Phagocytosis in the In Vitro and In Vivo Models of ARDS. *Stem Cells* 2016; 34: 2210-2223.
9. McIntyre LA, Moher D, Fergusson DA, Sullivan KJ, Mei SH, Lalu M, Marshall J, McLeod M, Griffin G, Grimshaw J, Turgeon A, Avey MT, Rudnicki MA, Jazi M, Fishman J, Stewart DJ, Canadian Critical Care Translational Biology G. Efficacy of Mesenchymal Stromal Cell Therapy for Acute Lung Injury in Preclinical Animal Models: A Systematic Review. *PLoS One* 2016; 11: e0147170.
10. Matthay MA, Calfee CS, Zhuo H, Thompson BT, Wilson JG, Levitt JE, Rogers AJ, Gotts JE, Wiener-Kronish JP, Bajwa EK, Donahoe MP, McVerry BJ, Ortiz LA, Exline M, Christman JW, Abbott J, Delucchi KL, Caballero L, McMillan M, McKenna DH, Liu KD. Treatment with allogeneic mesenchymal stromal cells for moderate to severe acute respiratory distress syndrome (START study): a randomised phase 2a safety trial. *Lancet Respir Med* 2018; 7(2):154-162.
11. Combes A, Hajage D, Capellier G, Demoule A, Lavoue S, Guervilly C, Da Silva D, Zafrani L, Tirot P, Veber B, Maury E, Levy B, Cohen Y, Richard C, Kalfon P, Bouadma L, Mehdaoui H, Beduneau G, Lebreton G, Brochard L, Ferguson ND, Fan E, Slutsky AS, Brodie D, Mercat A. Extracorporeal Membrane Oxygenation for Severe Acute Respiratory Distress Syndrome. *N Engl J Med*; 378: 1965-1975.
12. Hoogduijn MJ, van den Beukel JC, Wiersma LCM, Ijzer J. Morphology and size of stem cells from mouse and whale: observational study. *Br Med J* 2013; 347: f6833.
13. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8: 315-317.

14. Millar JE, von Bahr V, Malfertheiner MV, Ki KK, Redd MA, Bartnikowski N, Suen JY, McAuley DF, Fraser JF. Administration of mesenchymal stem cells during ECMO results in a rapid decline in oxygenator performance. *Thorax* 2018.
15. Cho HJ, Hong H, Kim DW, Lee KS, Han HS, Kim GH, Choi KS, Kim YS, Qayumov M, Ki KK, Suen J, Fraser J, Jeong IS. Viability of Mesenchymal Stem Cells in an Ex Vivo Circulation System. *ASAIO J* 2019. doi: 10.1097/MAT.0000000000001025. [Epub ahead of print].
16. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF. The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology. *Crit Care* 2016; 20: 387.
17. Council NHaMR. Australian code for the care and use of animals for scientific purposes. In: Council NHaMR, editor, 8th Edition ed. Canberra; 2013.
18. Kilkeny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *Osteoarthritis Cartilage* 2012; 20: 256-260.
19. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol* 2011; 44: 725-738.
20. Millar JE, Bartnikowski N, von Bahr V, Malfertheiner MV, Obonyo NG, Belliato M, Suen JY, Combes A, McAuley DF, Lorusso R, Fraser JF. Extracorporeal membrane oxygenation (ECMO) and the acute respiratory distress syndrome (ARDS): a systematic review of pre-clinical models. *Intensive Care Med Exp* 2019; 7: 18.
21. Griffiths MJD, McAuley DF, Perkins GD, Barrett N, Blackwood B, Boyle A, Chee N, Connolly B, Dark P, Finney S, Salam A, Silversides J, Tarmey N, Wise MP, Baudouin

- SV. Guidelines on the management of acute respiratory distress syndrome. *BMJ Open Respir Res* 2019; 6: e000420.
22. Simonson OE, Mougiakakos D, Heldring N, Bassi G, Johansson HJ, Dalen M, Jitschin R, Rodin S, Corbascio M, El Andaloussi S, Wiklander OP, Nordin JZ, Skog J, Romain C, Koestler T, Hellgren-Johansson L, Schiller P, Joachimsson PO, Hagglund H, Mattsson M, Lehtio J, Faridani OR, Sandberg R, Korsgren O, Krampera M, Weiss DJ, Grinnemo KH, Le Blanc K. In Vivo Effects of Mesenchymal Stromal Cells in Two Patients With Severe Acute Respiratory Distress Syndrome. *Stem Cells Transl Med* 2016; 5: 845.
23. Lin H-C, Wang C-C, Chou H-W, Wu E-T, Lu FL, Ko B-S, Yao M, Wang P-Y, Wu M-H, Chen Y-S. Airway Delivery of Bone Marrow-Derived Mesenchymal Stem Cells Reverses Bronchopulmonary Dysplasia Superimposed with Acute Respiratory Distress Syndrome in an Infant. *Cell Med* 2018; 10: 2155179018759434.
24. Kocyildirim E, Cardenes N, Ting A, Caceres E, Bermudez C, Rojas M. The Use of GMP-Produced Bone Marrow-Derived Stem Cells in Combination with Extracorporeal Membrane Oxygenation in ARDS: An Animal Model. *ASAIO J* 2017; 63: 324-332.
25. McIntyre LA, Moher D, Fergusson DA, Sullivan KJ, Mei SH, Lalu M, Marshall J, McLeod M, Griffin G, Grimshaw J, Turgeon A, Avey MT, Rudnicki MA, Jazi M, Fishman J, Stewart DJ. Efficacy of Mesenchymal Stromal Cell Therapy for Acute Lung Injury in Preclinical Animal Models: A Systematic Review. *PloS One* 2016; 11: e0147170.
26. Christy BA, Herzig MC, Montgomery RK, Delavan C, Bynum JA, Reddoch KM, Cap AP. Procoagulant activity of human mesenchymal stem cells. *J Trauma Acute Care Surg* 2017; 83: S164-s169.
27. Fiedler T, Rabe M, Mundkowski RG, Oehmcke-Hecht S, Peters K. Adipose-derived mesenchymal stem cells release microvesicles with procoagulant activity. *Int J Biochem Cell Biol* 2018; 100: 49-53.

28. Gleeson BM, Martin K, Ali MT, Kumar AH, Pillai MG, Kumar SP, O'Sullivan JF, Whelan D, Stocca A, Khider W, Barry FP, O'Brien T, Caplice NM. Bone Marrow-Derived Mesenchymal Stem Cells Have Innate Procoagulant Activity and Cause Microvascular Obstruction Following Intracoronary Delivery: Amelioration by Antithrombin Therapy. *Stem Cells* 2015; 33: 2726-2737.
29. Tatsumi K, Ohashi K, Matsubara Y, Kohori A, Ohno T, Kakidachi H, Horii A, Kanegae K, Utoh R, Iwata T, Okano T. Tissue factor triggers procoagulation in transplanted mesenchymal stem cells leading to thromboembolism. *Biochem Biophys Res Commun* 2013; 431: 203-209.
30. Curley GF, Hayes M, Ansari B, Shaw G, Ryan A, Barry F, O'Brien T, O'Toole D, Laffey JG. Mesenchymal stem cells enhance recovery and repair following ventilator-induced lung injury in the rat. *Thorax* 2012; 67: 496-501.
31. Pedrazza L, Cunha AA, Luft C, Nunes NK, Schimitz F, Gassen RB, Breda RV, Donadio MV, de Souza Wyse AT, Pitrez PMC, Rosa JL, de Oliveira JR. Mesenchymal stem cells improves survival in LPS-induced acute lung injury acting through inhibition of NETs formation. *J Cell Physiol* 2017; 232: 3552-3564.
32. Moodley Y, Sturm M, Shaw K, Shimbori C, Tan DB, Kolb M, Graham R. Human mesenchymal stem cells attenuate early damage in a ventilated pig model of acute lung injury. *Stem Cell Res* 2016; 17: 25-31.
33. Lalu MM, Sullivan KJ, Mei SH, Moher D, Straus A, Fergusson DA, Stewart DJ, Jazi M, MacLeod M, Winston B, Marshall J, Hutton B, Walley KR, McIntyre L. Evaluating mesenchymal stem cell therapy for sepsis with preclinical meta-analyses prior to initiating a first-in-human trial. *eLife* 2016; 5.
34. McIntyre LA, Stewart DJ, Mei SHJ, Courtman D, Watpool I, Granton J, Marshall J, Dos Santos C, Walley KR, Winston BW, Schlosser K, Fergusson DA. Cellular

- Immunotherapy for Septic Shock. A Phase I Clinical Trial. *Am J Crit Care Med* 2018; 197: 337-347.
35. Galstian GM, Parovichnikova EN, Makarova PM, Kuzmina LA, Troitskaya VV, Gemdzhian E, Drize NI, Savchenko VG. The Results of the Russian Clinical Trial of Mesenchymal Stromal Cells (MSCs) in Severe Neutropenic Patients (pts) with Septic Shock (SS) (RUMCESS trial). *Blood* 2015; 126: 2220.
36. Schlosser K, Wang J-P, Dos Santos C, Walley KR, Marshall J, Fergusson DA, Winston BW, Granton J, Watpool I, Stewart DJ, McIntyre LA, Mei SHJ, Canadian Critical Care Trials G, the Canadian Critical Care Translational Biology G. Effects of Mesenchymal Stem Cell Treatment on Systemic Cytokine Levels in a Phase 1 Dose Escalation Safety Trial of Septic Shock Patients. *Crit Care Med* 2019; 47: 918-925.
37. Perlee D, van Vught LA, Scicluna BP, Maag A, Lutter R, Kemper EM, van 't Veer C, Punchard MA, Gonzalez J, Richard MP, Dalemans W, Lombardo E, de Vos AF, van der Poll T. Intravenous Infusion of Human Adipose Mesenchymal Stem Cells Modifies the Host Response to Lipopolysaccharide in Humans: A Randomized, Single-Blind, Parallel Group, Placebo Controlled Trial. *Stem Cells* 2018; 36(11):1778-1788.
38. Laroye C, Lemarié J, Boufenzer A, Labroca P, Cunat L, Alauzet C, Groubatch F, Cailac C, Jolly L, Bensoussan D, Reppel L, Gibot S. Clinical-grade mesenchymal stem cells derived from umbilical cord improve septic shock in pigs. *Intensive Care Med* 2018; 6: 24-24.
39. Laroye C, Boufenzer A, Jolly L, Cunat L, Alauzet C, Merlin JL, Yguel C, Bensoussan D, Reppel L, Gibot S. Bone marrow vs Wharton's jelly mesenchymal stem cells in experimental sepsis: a comparative study. *Stem Cell Res Ther* 2019; 10: 192.
40. Cardenes N, Aranda-Valderrama P, Carney JP, Sellares Torres J, Alvarez D, Kocydirim E, Wolfram Smith JA, Ting AE, Lagazzi L, Yu Z, Mason S, Santos E, Lopresti BJ, Rojas

- M. Cell therapy for ARDS: efficacy of endobronchial versus intravenous administration and biodistribution of MAPCs in a large animal model. *BMJ Open Respir Res* 2019; 6: e000308.
41. Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, Herridge M, Randolph AG, Calfee CS. Acute respiratory distress syndrome. *Nat Rev Dis Primers* 2019; 5: 18.
42. Islam D, Huang Y, Fanelli V, Delsedime L, Wu S, Khang J, Han B, Grassi A, Li M, Xu Y, Luo A, Wu J, Liu X, McKillop M, Medin J, Qiu H, Zhong N, Liu M, Laffey J, Li Y, Zhang H. Identification and Modulation of Microenvironment Is Crucial for Effective Mesenchymal Stromal Cell Therapy in Acute Lung Injury. *Am J Crit Care Med* 2018; 199: 1214-1224.
43. Cavalcanti AB, Suzumura EA, Laranjeira LN, Paisani DM, Damiani LP, Guimaraes HP, Romano ER, Regenga MM, Taniguchi LNT, Teixeira C, Pinheiro de Oliveira R, Machado FR, Diaz-Quijano FA, Filho MSA, Maia IS, Caser EB, Filho WO, Borges MC, Martins PA, Matsui M, Ospina-Tascon GA, Giancursi TS, Giraldo-Ramirez ND, Vieira SRR, Assef M, Hasan MS, Szczeklik W, Rios F, Amato MBP, Berwanger O, Ribeiro de Carvalho CR. Effect of Lung Recruitment and Titrated Positive End-Expiratory Pressure (PEEP) vs Low PEEP on Mortality in Patients With Acute Respiratory Distress Syndrome: A Randomized Clinical Trial. *JAMA* 2017; 318: 1335-1345.

Figure Legends

Figure 1. Study schematic.

^a Adjusted to maintain pH 7.30-7.45. Permissive hypercapnia was tolerated to a minimum pH of 7.15.

^b PaO₂ 55 – 80 mmHg. If despite an FiO₂ of 1.0 oxygenation targets were not met, PEEP was increased, maintaining plateau pressure ≤ 32 cmH₂O.

^c Total PEEP (extrinsic PEEP + intrinsic PEEP) did not exceed 20 cmH₂O. PEEP was permitted to be reduced to 5 cmH₂O to maintain plateau pressure ≤ 30 cmH₂O. If despite a PEEP of 5 cmH₂O, plateau pressure > 30 cmH₂O, tidal volume was reduced in 1 mL/kg steps until set at 4 mL/kg.

Figure 2. Oxygenation and respiratory parameters.

a. PaO₂/FiO₂ ratio. **b.** Oxygenation index. **c.** Airway pressures and lung compliance. Data are presented as mean (± 95% confidence interval). Where error bars intersect the x axis the 95% CI includes zero.

Figure 3. Hemodynamic variables.

a. Mean arterial pressure and vasopressor dose (mean). **b.** Mean pulmonary artery pressure. **c.** Cumulative vasopressor dose. **d.** Cardiac index, base deficit, and arterial lactate. Data are presented as mean (± 95% confidence interval). Where error bars intersect the x axis the 95% CI includes zero. ** - p < 0.01.

Figure 4. Histopathology and lung injury.

a. Representative images of lung parenchyma. All animals showed evidence of diffuse alveolar damage however the frequency and degree of injury differed between groups. **Panel 1:** extensive alveolar edema with interstitial leukocyte infiltration. **Panel 2:** marked leukocyte infiltration within alveolar spaces and larger airways. **Panel 3:** some loss of alveolar structure with edema however a reduction in interstitial and alveolar leukocytes. **Panel 4:** preservation

of alveolar architecture with few leukocytes in the alveolar spaces. **Panel 5 & 6:** representative images of pulmonary arterial and arteriolar emboli (black arrows) in animals receiving hMCSs.

b. Composite lung injury score (LIS), lung wet/dry ratio (right lower lobe). **c.** BAL total protein concentration. Data are presented as mean (\pm 95% confidence interval). * - $p = < 0.05$.

Figure 5. Cell-ECMO interaction.

a. Trans-membrane oxygenator pressure gradient. **b.** Pump revolutions per minute (RPM)/flow ratio. Data are presented as mean (\pm 95% confidence interval). Where error bars intersect the x axis the 95% CI includes zero. * - $p = < 0.05$.

Table 1. Baseline physiological characteristics

	Overall (n=14)	Vehicle (n=7)	hMSCs (n=7)
Weight (kg)	52.6 ± 3	52.4 ± 3.2	52.9 ± 2.6
Pre-ECMO tidal volume (mL/kg)	6.0 ± 0.1	6.0 ± 0.1	6.0 ± 0.1
<i>At time of injury (T0)</i>			
Peak airway pressure (cmH₂O)	31.5 ± 4.2	31.9 ± 3.3	31.1 ± 4.9
Plateau pressure (cmH₂O)	26.5 ± 3.9	26.1 ± 3.9	26.9 ± 3.8
Driving pressure (cmH₂O)	16.5 ± 3.9	16.1 ± 3.9	16.9 ± 3.8
Static compliance (mL/cmH₂O)	21 ± 5.0	19 ± 4.6	23 ± 4.6
PaO₂/FiO₂	59 ± 20	58 ± 23	61 ± 17
PaCO₂ (mmHg)	41 (38-46)	38 (38-41)	44 (41-48)
pH	7.36 ± 0.05	7.38 ± 0.04	7.34 ± 0.05
Bicarbonate (mmol/L)	23.2 ± 1.5	23.6 ± 1.4	22.9 ± 1.5
Base deficit (mmol/L)	1.30 ± 1.31	0.94 ± 1.15	1.66 ± 1.37
Arterial lactate (mmol/L)	1.6 ± 0.7	1.8 ± 0.6	1.4 ± 0.7
Heart rate (bpm)	102 (96-117)	108 (98-128)	98 (95-103)
Mean arterial pressure (mmHg)	103 ± 19	96 ± 16	111 ± 19
Central venous pressure (mmHg)	13 (11-13)	12 (11-13)	13 (13-14)
Mean pulmonary artery pressure (mmHg)	25 (16-29)	20 (17-27)	28 (19-29)