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## **The administration of mesenchymal stem cells during simulated ECMO results in a rapid decline in oxygenator performance.**

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### **Declaration of Interest**

Cynata Therapeutics Ltd. provided mesenchymal stem cells in kind to this study. The company was not involved in the commission, design or analysis of the study.

### **Individual Contributions**

JEM, DFM, and JFF conceived and designed the study. VvB, and MM designed and modified the ex-vivo ECMO model. JEM, VvB, and MM conducted the ex-vivo experiments. KKK, MAR, NB, and JYS analysed generated samples. All authors have participated in the drafting and critical revision of the manuscript.

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To the Editor,

There is growing interest in the potential of mesenchymal stem cells (MSCs) as a therapy for the acute respiratory distress syndrome (ARDS). Phase I studies have been reported (1, 2), while a larger phase II study has recently completed recruitment (NCT02097641). Several more are underway (NCT02611609, NCT03042143, NCT02804945). However, these trials have excluded patients supported by extracorporeal membrane oxygenation (ECMO), which is increasingly used in patients with severe ARDS (3). While the use of MSCs during ECMO has been described in case reports (4), the compatibility of MSCs and ECMO has not been systematically evaluated. Here, we present data to suggest that the intravascular administration of MSCs during ECMO may have consequences for oxygenator function.

## **Methods**

The study was approved by the Metro North Ethics Committee (HREC/16/QPCH/221). *Ex-vivo* ECMO was conducted based on our previously described simulated model (5). Briefly, permanent life support (PLS) circuits (Maquet, Germany) incorporating, a Quadrox D Oxygenator, a RotaFlow pump, and Bioline™ tubing, were used. Circuits were primed with 500 mL 0.9% sodium chloride (Baxter, Australia), after which, this was exchanged for fresh human whole blood (final volume,  $420 \pm 50$  mL). Circulation was commenced at 2000 rpm, using a smooth transition clamp to provide a resistance permitting a blood flow of  $4 \text{ L min}^{-1}$ . After baseline sampling, 10 mL of calcium chloride (CSL, Australia), 12 mL of 8.4% sodium bicarbonate (Baxter), and 400 units of sodium heparin (Pfizer, Australia), were added to the circuit to achieve a pH between 7.3 – 7.5 and an activated clotting time (ACT)  $\geq 180$  seconds. Pressure across the oxygenator was measured using a silicone based pressure transducer (Omega Engineering, USA). Circulating blood temperature was maintained at  $37 \pm 0.5$  °C. A solution of saline, adenine, glucose, and mannitol (MacoPharma, Australia) was infused at a

rate of 4 mL hr<sup>-1</sup> to ensure pressure equilibrium within the circuit. Fresh gas flow was set at 2.5 L min<sup>-1</sup> (5% CO<sub>2</sub>, 21% O<sub>2</sub>, 74% N<sub>2</sub>).

Clinical-grade induced pluripotent stem cell (iPSC) derived human MSCs (Cynata Therapeutics Ltd., Australia) were obtained and stored in the vapor-phase of liquid nitrogen until use. Cells were suspended in a vehicle composed of Plasmalyte-A (Baxter) (57.5%), Flexbumin 25% (Baxter) (40%), and DMSO (2.5%). iPSC derived MSCs used in these experiments were between passage 3 and 5. Fourteen ex-vivo ECMO experiments were undertaken, divided as follows; circuits injected with 40 x 10<sup>6</sup> MSCs, circulated for 4 hours or until flow reached 0 L min<sup>-1</sup> (n=4), circuits injected with 20 x 10<sup>6</sup> MSCs, circulated for 4 hours or until flow decreased by 25% from baseline (n=4), and control circuits, circulated for 4 hours (n=6). When physiological conditions were obtained (PaO<sub>2</sub> ≥ 80 mmHg, PaCO<sub>2</sub> 30-50 mmHg), MSCs in vehicle were thawed to room temperature (>95% viability), agitated to eliminate clumping, and then immediately administered to the circuit, after the oxygenator and before the pump head, by slow injection over 30 seconds.

Whole blood samples were collected from the circuit at 30 seconds, 15, 30, 60, 120, and 240 minutes. Erythrocytes were lysed, and residual cells were washed as described previously (6). Fc receptors were blocked using Human TruStain FCx (Biolegend, USA), and, MSCs were stained with mouse anti-human monoclonal antibodies: CD45-PECy7 (Biolegend), CD73-PerCP, CD90-APC, and CD105-FITC (Abcam, UK), according to the manufacturer's protocol. Precision Count Beads (Biolegend) were then added to determine the fate of circulating MSCs, quantified using a two laser FACSCanto I flow cytometer (BD Biosciences, USA).

After termination of each experiment, oxygenators were flushed with 1000 mL 0.9% NaCl, perfused with 500 mL paraformaldehyde (Merck, Germany) and rinsed again with 1000 mL 0.9% NaCl. Samples of the heat and gas exchange fibers were then retrieved.

For immunohistochemistry analysis, fiber sections were blocked for 2 hours in PBS containing 2% HISS (Sigma, Australia) and 0.5% triton X-100 (Sigma). Samples were incubated overnight at 4 °C with primary antibodies: mouse Ab to CD105-FITC (Abcam, 1:10), or mouse Ab to CD90-PE (Abcam, 1:50) and rabbit antibody to  $\beta$ 1 tubulin-488 conjugated (Abcam, 1:50). The fibres were washed with PBS followed by 1 hour incubation with secondary antibodies to boost the fluorescent signal followed by additional PBS washes. Images were acquired with a widefield Nikon deconvolution (TiE) microscope, and confocal z-stacks were acquired with Zeiss LSM710 AiryScan.

## **Results**

In all circuits to which MSCs were administered, blood flow through the ECMO oxygenator decreased by at least 25% within 4 hours, with a corresponding increase in the trans-oxygenator pressure gradient (Table 1). When  $40 \times 10^6$  MSCs were administered, flow was reduced to  $< 1.5 \text{ L min}^{-1}$  in all circuits by 4 hours, and in one case within 30 minutes. ACT was maintained  $\geq 180 \text{ s}$  throughout all experiments. Microscopy of deconstructed oxygenators demonstrated the widespread adherence of MSCs to plastic fibers (Figure 1).

## **Discussion**

This is the first study to directly address the feasibility of MSC therapy during ECMO. Our data suggest that intravascular administration of MSCs during ECMO may have important consequences for oxygenator function, as well as for their efficacy as a therapy for severe ARDS in this setting. This may have occurred due to the characteristic plastic adhesiveness of MSCs. A limitation of our study is that we did not assess gas exchange across the oxygenator.

MSC use during ECMO has been described previously but has either been administered before the commencement of ECMO (7), by intra-tracheal administration (8), or during a pause

in flow (4). These methods of administration may not always be possible in severely ill ARDS patients who are reliant on continuous high flow ECMO for oxygenation.

At a circuit concentration of  $48 - 95 \times 10^3$  MSCs mL<sup>-1</sup>, our study may have underestimated the effect of MSCs on oxygenator performance. Previous studies in ARDS have used up to  $10 \times 10^6$  MSCs kg<sup>-1</sup> (1), which assuming an average blood volume of 70 mL kg<sup>-1</sup> (9) and equal distribution, would result in a higher circulating cell concentration (approximately  $143 \times 10^3$  MSCs mL<sup>-1</sup>). This must be weighed against the possibility that indirect intravascular administration, peripherally or after the membrane oxygenator, may reduce the number of MSCs reaching the oxygenator surface, most likely as a result of entrapment in the pulmonary circulation. These data also support further investigation of MSC therapy during extracorporeal carbon dioxide removal (ECCO<sub>2</sub>R) or cardiopulmonary bypass, both of which share functionally similar membrane gas exchange devices with ECMO (10).

Alternative routes of administration, such as intra-bronchial, may have advantages in the setting of ECMO, when prolonged bronchoscopy is possible. This should be evaluated in any future study. In light of these results, further investigations using MSCs in ECMO should explore safety considerations in an intact animal model before progression to clinical trials.

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|   | <b>40 x 10<sup>6</sup> IPSC-MSCs</b><br>(n=4) | <b>20 x 10<sup>6</sup> IPSC-MSCs*</b><br>(n=4) | <b>Control</b><br>(n=6) |
|---|---|--|-------------------------|
| <b>Time to 25% decrease in blood flow</b> (minutes ± SE)      | 68 ± 32                                       | 99 ± 21  | -                       |
| <b>Time to 50% decrease in blood flow</b> (minutes ± SE)      | 85 ± 39                                       | -  | -                       |
| <b>Blood flow at 2000 rpm</b> (L min <sup>-1</sup> ± SE)      |   |  |                         |
| 30 s  | 4.05 ± 0.02                                   | 4.04 ± 0.02                                    | 44.03 ± 0.05            |
| 15 min  | 3.79 ± 0.35                                   | 4.01 ± 0.04                                    | 4.03 ± 0.05             |
| 30 min  | 2.83 ± 0.98                                   | 4.12 ± 0.07                                    | 3.98 ± 0.06             |
| 60 min  | 1.94 ± 1.14                                   | 3.95 ± 0.20                                    | 4.12 ± 0.03             |
| 120 min   | 1.39 ± 0.92                                   |  | 4.09 ± 0.05             |
| 240 min   | 0.60 ± 0.35                                   |  | 4.12 ± 0.03             |
| <b>Trans-oxygenator pressure gradient</b> (mmHg ± SE)         |   |  |                         |
| 30 s  | 20 ± 6  | 21 ± 2   | 119 ± 3                 |
| 15 min  | 27 ± 12                                       | 21 ± 3   | 21 ± 2                  |
| 30 min  | 51 ± 24                                       | 22 ± 3   | 25 ± 4                  |
| 60 min  | 55 ± 31                                       | 28 ± 4   | 24 ± 3                  |
| 120 min   | 50 ± 18                                       |  | 225 ± 4                 |
| 240 min   | 101 ± 9                                       |  | 21 ± 4                  |
| <b>MSCs detectable in blood</b> (cells μL <sup>-1</sup> ± SE) |   |  |                         |
| 30 s  | 21.8 ± 1.9                                    |  |                         |
| 30 min  | 9.4 ± 4.2                                     |  |                         |
| 60 min  | 6.9 ± 3.4                                     |  |                         |
| 120 min   | 4.1 ± 3.8                                     |  |                         |
| 240 min   | 0.3 ± 0.2                                     |  |                         |

**Table 1. Conditions during *ex-vivo* ECMO**

\* Circuits terminated after a 25% reduction in blood flow (3 L min<sup>-1</sup>), performed to optimise conditions for microscopy. - Did not occur.

**Figure 1. ECMO oxygenator fibres after the administration of iPSC-derived human**

**MSCs.** (A) Wide field microscopy of CD105-stained MSCs (green) with a bright field overlay, of a section of polyurethane (PU) heat exchange fibres. MSCs can be seen adherent between adjacent fibre strands. (B) A confocal image of a MSC bridging two PU fibres. (A-B) DAPI-stained nuclei (blue)

(C) A sample of polymethylpentene (PMP) gas exchange fibres, taken from the core of the oxygenator bundle and stained for CD105. (D) A similar section of PMP gas exchange taken from the periphery of the oxygenator and stained for CD90 with DAPI-stained nuclei.