

Online Data Supplement

Title: Mesenchymal Stem Cells enhance recovery and repair following Ventilator Induced Lung Injury **in the Rat**

Authors: Dr. Gerard Curley, MB, FCARCSI ^{1,2},
Dr Mairead Hayes, MB, FCARCSI^{1,2},
Dr Bilal Ansari, MB, FCARCSI^{1,2},
Ms Georgina Shaw ³,
Dr Aideen Ryan, PhD³,
Prof Frank Barry, PhD ³,
Prof Tim O'Brien, MD, PhD ³
Dr. Daniel O' Toole, PhD ^{1,2},
Prof. John G. Laffey, MD, FCARCSI ^{1,2,3}.

Institution: ¹Department of Anaesthesia, Clinical Sciences Institute; ²Lung Biology Group, National Centre for Biomedical Engineering Sciences; and ³Regenerative Medicine Institute, National University of Ireland, Galway, IRELAND.

Contact information: Prof John G Laffey, Anaesthesia, School of Medicine, Clinical Sciences Institute, National University of Ireland, Galway, Ireland.

E-mail: john.laffey@nuigalway.ie

Phone: 353 – 91 – 544908.

Fax: 353 – 91 – 544908.

Abbreviated Title: MSC's enhance repair following VILI

MATERIALS AND METHODS

All work was approved by the Animal Ethics Committee of the National University of Ireland, Galway and conducted under license from the Department of Health, Ireland. Specific-pathogen-free adult male Sprague Dawley rats (Charles River Laboratories, Kent, UK) weighing between 350–450g were used in all experiments.

Rodent MSC Isolation and Culture

Rodent MSCs (rMSCs) were isolated from rat femora and tibiae under sterile conditions as previously described (1) with additional modifications. Briefly, male Sprague Dawley rats (8-12 weeks old) were euthanized by inhalation of CO₂. Incisions were made on both lower limbs to expose the tibiae and femora. Both bones were removed from the hind limbs and placed in ice cold sterile Tyrode's solution (Sigma, St. Louis, MO). The marrow was then flushed into a dish containing rMSC complete culture medium (MEM- α Media (Gibco, Paisley, UK), F12-Ham Media (Gibco), 10% foetal bovine serum (PAA, Somerset, UK), 1% antibiotic/antimycotic (Sigma) and dispersed into a cell suspension. After centrifugation and filtration through a 100 μ m nylon mesh, a cell count was performed and the cells were transferred to a 175 cm² flasks containing 30 mls of rMSC complete medium, at a density of 9×10^5 cells/cm². On day 3 of culture in an atmosphere of 5% CO₂/90% humidity at 37°C, medium and non-adherent cells were removed and fresh medium was added to each flask. Cells were ready for subculture (usually after 16-17 days) when colonies began to exhibit a compact appearance and multi-layered growth or when the loosely formed colonies began to merge into a monolayer (<90% of confluence).

Thereafter, cells were ready to be passaged after 6/7 days culture, at 80% confluence. For passage, media was aspirated off and cells were washed with sterile PBS to remove any remaining serum. 8mls 0.25% trypsin/EDTA solution was added to the cells, which were incubated for 5 minutes at 37° C. The enzymatic reaction was stopped by adding the same volume of rMSC media to cells. Cells were centrifuged at 400g for 5 min. Media was aspirated off the cell pellet which was resuspended in 1ml and a haemocytometer count was undertaken. Cells were expanded to passage 4, whereupon they were used for experiments. (*Figure A, Online Repository.*)

Human MSC Isolation and Culture

Human MSCs were aspirated under sterile conditions from the iliac crests of healthy human volunteers. The obtained marrow was filtered with a 70 µm cell strainer (Falcon, USA) before centrifuging at 400 g for 10 min. Cell pellets were resuspended in media consisting MEM-α (Gibco), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% antibiotics (streptomycin and penicillin) (Gibco, USA), and cultured in 175 cm² flasks at 37 °C in a humidified atmosphere containing 5% CO₂. At day 4, the cultures were washed with PBS to remove the non-adherent cells and further expanded until >80% confluence, when they were harvested and expanded in 175 cm² flasks. After subculture, these cells were designated as passage 1.

Characterization of MSCs

In accordance with the position statement for the minimal criteria to define an MSC (2), cells were labeled with monoclonal antibodies against CD31, CD34,

CD44, CD45, CD54, CD73, and CD90 (Santa Cruz Biotechnology, Santa Cruz, CA) and analyzed with a FACScan (Becton Dickinson, Franklin Lakes, NJ) and CellQuest software as described (3).

Osteogenic differentiation was induced by culturing rMSCs or hMSCs for up to 4 weeks in rMSC or hMSC Complete Medium supplemented with dexamethasone, ascorbic acid and β -glycerophosphate as previously described (4). To observe calcium deposition, cultures were stained with Alizarin Red stain (Sigma, St Louis, MA). To induce adipogenic differentiation, rMSCs were cultured for up to 4 weeks in medium supplemented with dexamethasone and insulin; adipocytes were discerned by staining with Oil Red O (Sigma). Chondrogenic capacity was assessed by addition of TGF-beta (Invitrogen) and staining with Toluidine blue (*Figure B panels a to c, Online Repository*).

Fibroblast isolation and Culture

Adult male Sprague Dawley rats were euthanized by CO₂ inhalation. The ventral surface of the rat was shaved and sprayed with 70% ethanol. Skin and subcutaneous tissue was removed and placed into 70% ethanol for 30 seconds. Fat and subcutaneous tissue was removed and the skin strips were placed in 0.25% trypsin (Sigma) overnight. The epidermis was then peeled from the dermal layer, and the dermal layer was placed on a scored 6 well plate (Sarstedt, Wexford, Ireland) in F-12/MEM- α medium supplemented with fetal calf serum (10%) and penicillin/streptomycin (1%).

Primary human lung fibroblasts were obtained from American Type Culture Collection (ATCC).

Conditioned Medium

Allogeneic human or rodent MSC, or human or rodent fibroblasts (2×10^6) were washed with PBS and cultured without serum for 24 h. The cells were again washed and the medium was then replaced, and the subsequent medium without serum for the next 24 h was used as the conditioned medium (CM). All conditioned medium was sterile filtered through a 22 μ m filter to remove cellular debris. For the *in vivo* experiments 15 mls of this medium was concentrated using a 3000 kDa centrifugal concentrating filter (Amicon, Billerica, MA, USA) to give 500 μ L.

Rodent Ventilator Induced Injury Protocol

Anesthesia was induced with intraperitoneal ketamine 80 mg.kg⁻¹ (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 mg.kg⁻¹ (Xylapan, V  toquinol, Dublin, Ireland). After confirmation of depth of anesthesia by paw clamp, intravenous access was obtained via tail vein, laryngoscopy was performed and the animals were intubated with a size 14G intravenous catheter (BD Insyte  , Becton Dickinson Ltd, Oxford, UK). The lungs were ventilated using a small animal ventilator (CWE SAR 830 AP, CWE Inc, Pennsylvania, USA). Anesthesia was maintained with repeated boli of Saffan   (alfaxadone 0.9% and alfadadolone acetate 0.3%; Schering Plough, Welwyn Garden City, UK) and muscle relaxation was achieved with cis-atracurium besylate 0.5mg.kg⁻¹ (GlaxoSmithKline, Dublin, Ireland). The animals were then subjected to a high stretch mechanical ventilation protocol (FiO₂ 0.3, inspiratory pressure 35 cmH₂O, respiratory rate 18 min⁻¹, and positive end-expiratory pressure 0 cmH₂O). When static compliance

had decreased by 50%, high stretch ventilation was discontinued and animals were extubated, allowed to regain consciousness, and entered into the treatment protocol.

Assessment of Injury and Repair

At 48 hours following VILI induction, animals were re-anesthetized. A tracheostomy was inserted and carotid arterial access established (22G, BD Insyte), and the lungs were mechanically ventilated at a respiratory rate of 80 min⁻¹, tidal volume 6 ml.kg⁻¹ and positive end-expiratory pressure 2 cmH₂O as previously described (5-7). Intra-arterial blood pressure, peak airway pressures and rectal temperature were recorded continuously. Static inflation lung compliance measurements were performed as previously described (8, 9). After 20 minutes, the inspired gas was altered to a FiO₂ of 1.0 for 15 min, and a final arterial blood sample was taken. Heparin (400 IU.kg⁻¹, CP Pharmaceuticals, Wrexham, U.K.) was then administered intravenously, and animals were killed by exsanguination.

Immediately post-mortem, the heart-lung block was dissected and bronchoalveolar lavage (BAL) collection was performed as previously described (10, 11). BAL differential cell counts were performed. Protein concentration was determined using a Micro BCA™ Protein assay kit (Pierce, Rockford, IL, USA).(12) BAL IL-1β, IL-6, TNF-α and IL-10 concentrations were determined using quantitative sandwich enzyme-linked immunosorbent assays (R and D Systems, Abingdon, UK) (13). The detection limit of these assays was 62.5 pg/ml for the IL-6 assay and 31.2 pg/ml for TNF-α and IL-10 assays.

Wet:dry lung weight ratios were determined using the lowest lobe of the right lung as previously described (14). The left lung was isolated and fixed for morphometric examination, and the extent of histologic lung damage was determined using quantitative stereological techniques as previously described (10, 14).

Wound Repair Experiments

A549 cells were purchased from the European collection of cell cultures (Porton Down, UK) as cryopreserved 90-passage culture and used at passages 91-95. All cells were seeded at a density of 5×10^5 cells per ml on plastic 24 well plates (Corning Ltd, New York, US) at 37°C in a humidified incubator saturated with a gas mixture containing 5% CO₂ in air. Once the cells had grown to confluence, single wounds were made in each well, by scraping off cells with a 1000µL pipette tip, as previously described (18). Wells were washed with PBS and exposed to different conditions as per group allocation. 48 hours later, the monolayers were fixed with 4% paraformaldehyde in PBS (w/v), and stained with hematoxylin and eosin. The extent of epithelial restitution was determined by imaging each plate on a flatbed scanner and assessing the area of each wound using edge-finding software (Photoshop v8.0, Adobe Systems Inc, San Jose, California).

Experimental series

Series 1: Determination of potential for MSCs to enhance repair post VILI

Following recovery after induction of VILI, animals were randomly allocated to receive a tail vein injection of either: (1) 2×10^6 allogeneic rat MSCs suspended in 500 μ L PBS; or (2) 500 μ L PBS alone, and returned to cages. 24 hours later they were re-anesthetized, a second injection of MSCs or vehicle was administered intravenously, and the animals returned to their cages. The extent of repair following VILI assessed at 48 hours.

Series 2: Determination of the mechanism by which MSCs enhance repair

Following induction of VILI, animals were randomly allocated to receive a tail vein injection of: (1) 2×10^6 MSCs suspended in 500 μ L PBS; (2) 500 μ L PBS; (3) 2×10^6 rat dermal fibroblasts suspended in PBS; or (4) 500 μ L of MSC conditioned medium, and were returned to cages. 24 hours later they received a second injection of MSCs, vehicle, fibroblasts or medium. The extent of recovery and repair following VILI **was** assessed at 48 hours.

Series 3: Effects of MSCs and MSC-CM on epithelial wound repair

Pulmonary epithelial (A549) monolayers were subjected to scratch wound injury, incubated in: (i) MEM- α medium, (ii) human fibroblast conditioned medium, (iii) human MSC conditioned medium, or (iv) co-cultured with MSCs (Corning HTS transwell, pore size 0.4 μ m, Corning, NY, USA). In the case of co-cultures, MSCs were seeded at 1×10^3 cells/cm² in the inserts and maintained in human MSC medium for 3 days prior to co-culture, which allowed the mesenchymal stem cells to reach 70–80% confluence. Mesenchymal stem cell containing inserts were washed and then added to the A549 wells and flooded with fresh serum free medium. Similarly, as controls, human fibroblasts were

seeded at 1×10^3 cells in a co-culture insert and maintained in MSC medium for 3 days prior to co-culture.

Wound closure was assessed at 48 hours.

Series 4: Determination of the contribution of MSC secreted growth factors to wound repair.

MSC conditioned medium was incubated with specific monoclonal antibodies to inactivate keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and transforming growth factor-beta (TGF- β) (Abcam) respectively. The concentration of antibody used was sufficient to ensure effective blockade based on the expected amounts of the specific growth factor in conditioned medium. In the case of KGF, used $2\mu\text{g/ml}$ of this antibody given that $0.56\text{-}0.85\mu\text{g/ml}$ of this antibody is required to yield 50% inhibition of the biological activity of 25 ng/ml hKGF. Measurement of the concentration of KGF in the MSC and fibroblast conditioned medium, using quantitative sandwich enzyme-linked immunosorbent assays (R and D Systems, Abingdon, UK) confirmed that the concentration of neutralizing antibody used was sufficient. A549 wounds were exposed to MSC conditioned medium with and without antibodies to each candidate mediator, and the extent of wound closure assessed at 48 hours.

Statistical Analysis

The distribution of all data was tested for normality using Kolmogorov-Smirnov tests. Results are expressed as mean (\pm SD) for normally distributed data, and as median (interquartile range, IQR) where non-normally distributed. Data were

analyzed by one-way ANOVA followed by Dunnett's test, with the vehicle group as the control group in each analysis. Comparisons between 2 groups were made using unpaired, two-tailed Student's t tests or using a Mann-Whitney U test as appropriate. Underlying model assumptions were deemed appropriate on the basis of suitable residual plots. A two-tailed p value of <0.05 was considered significant.

FIGURE LEGENDS

Figure A: Characterization of surface markers on rat MSCs

The rat MSCs used in these studies were CD29, CD90, CD44H, CD73 positive and CD45RA, CD71, CD80, MHCI, MHCII, CD106 low or negative. Shown are FACS histograms of Sprague Dawley MSCs (passage 3) stained with antibodies against surface markers as indicated (colored) or with appropriate isotype controls (gray). Each colored line indicates replicates.

Figure B: Differentiation of rat MSCs

Passage 2 bone marrow (BM)-derived adherent cells (Figure B-a), after culture in differentiation medium, were stained for alizarin red (Osteogenic staining, Figure B-b), oil red O (Adipocyte staining, Figure B-c) and safranin O (Chondrocyte staining, data not shown). All experiments were performed in triplicate.

References

1. Meirelles Lda S, Nardi NB. Murine marrow-derived mesenchymal stem cell: Isolation, in vitro expansion, and characterization. *Br J Haematol* 2003;123:702-711.
2. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006;8:315-317.
3. Rochefort GY, Delorme B, Lopez A, et al. Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. *Stem Cells* 2006;24:2202-2208.
4. Phinney DG, Kopen G, Isaacson RL, et al. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: Variations in yield, growth, and differentiation. *J Cell Biochem* 1999;72:570-585.
5. Curley G, Contreras M, Higgins B, O'Kane C, McAuley DF, O' Toole D, Laffey JG. Evolution of the inflammatory and fibroproliferative responses during resolution and repair following ventilator induced lung injury in the rat. *Anesthesiology* 2011.
6. Higgins BD, Costello J, Contreras M, et al. Differential effects of buffered hypercapnia versus hypercapnic acidosis on shock and lung injury induced by systemic sepsis. *Anesthesiology* 2009;111:1317-1326.
7. O'Croinin DF, Nichol AD, Hopkins N, et al. Sustained hypercapnic acidosis during pulmonary infection increases bacterial load and worsens lung injury. *Crit Care Med* 2008;36:2128-2135.

8. Costello J, Higgins B, Contreras M, et al. Hypercapnic acidosis attenuates shock and lung injury in early and prolonged systemic sepsis. *Crit Care Med* 2009;37:2412-2420.
9. Higgins BD, Costello J, Contreras M, et al. Differential effects of buffered hypercapnia versus hypercapnic acidosis on shock and lung injury induced by systemic sepsis. *Anesthesiology* 2009;111:1317-1326.
10. O'Croinin DF, Hopkins NO, Moore MM, et al. Hypercapnic acidosis does not modulate the severity of bacterial pneumonia-induced lung injury. *Crit Care Med* 2005;33:2606-2612.
11. O'Croinin DF, Nichol AD, Hopkins N, et al. Sustained hypercapnic acidosis during pulmonary infection increases bacterial load and worsens lung injury*. *Crit Care Med* 2008;36:2128-2135.
12. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Analytical biochemistry* 1985;150:76-85.
13. Adamson IY, Bakowska J. Relationship of keratinocyte growth factor and hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin. *Am J Pathol* 1999;155:949-954.
14. Laffey JG, Honan D, Hopkins N, et al. Hypercapnic acidosis attenuates endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* 2004;169:46-56.