Human mesenchymal stem cells attenuate early damage in a ventilated pig model of acute lung injury

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ABSTRACT

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is a major cause of global morbidity and mortality. Mesenchymal stem cells (MSC) have shown promise in treating inflammatory lung conditions. We hypothesised that human MSC (hMSC) can improve ALI/ARDS through their anti-inflammatory actions. We subjected pigs (n = 6) to intravenous oleic acid (OA) injury, ventilation and hMSC infusion, while the controls (n = 5) had intravenous OA, ventilation and an infusion vehicle control. hMSC were infused 1 h after the administration of OA. The animals were monitored for additional 4 h. Nuclear translocation of nuclear factor-light chain enhancer of activated B cells (NF-κB), a transcription factor that mediates several inflammatory pathways was reduced in hMSC treated pigs compared to controls (p = 0.04). There was no significant difference in lung injury, assessed by histological scoring in hMSC treated pigs versus controls (p = 0.063). There was no difference in neutrophil counts between hMSC-treated pigs and controls. Within 4 h, there was no difference in the levels of IL-10 and IL-8 pre- and post-treatment with hMSC. In addition, there was no difference in hemodynamics, lung mechanics or arterial blood gases between hMSC treated animals and controls. Subsequent studies are required to determine if the observed decrease in inflammatory transcription factors will translate into improvement in inflammation and in physiological parameters over the long term.

1. Introduction

Acute respiratory distress syndrome (ARDS) is characterized by refractory hypoxemia in patients with bilateral lung infiltrates. The Berlin definition of ARDS is based on the degree of hypoxemia: mild (200 mm Hg $PaO_2/FiO_2 \leq 300$ mm Hg), moderate (100 mm Hg $PaO_2/FiO_2 \leq 200$ mm Hg), and severe ($PaO_2/FiO_2 \leq 100$ mm Hg). In addition, the condition must develop within one week of a known clinical insult or new/worsening respiratory symptoms. Bilateral opacities on chest imaging that is not fully explained by effusions, lobar/lung collapse or nodules and the respiratory failure must not be fully explained by cardiac failure or fluid overload (Ranieri et al., 2012). A National Institutes of Health study estimated the incidence of ARDS to be 75/100,000 population in the USA, while in Australia the incidence is estimated at 30/100,000 admissions (Ware and Matthay, 2000).

ARDS may be caused by direct or indirect injuries to the lung including aspiration of gastric contents and sepsis (Petty and Ashbaugh, 1971). The early phase of lung injury is characterized by epithelial and endothelial cell damage leading to a compromised alveolar-capillary barrier and exudation of fluid into the alveolar space, followed by infiltration of inflammatory cells such as neutrophils. Progression from acute lung injury (ALI) to a fibro-proliferative phase observed 5–7 days after the injury may be reversible or persistent (Leaver and Evans, 2007).

Many strategies have been directed at reducing lung injury and augmenting tissue repair in ALI/ARDS with limited beneficial outcomes. Low tidal volume ventilation and prone positioning have proven to be effective in reducing mortality (Slutsky and Ranieri, 2000; Guerin et al., 2013). However, a randomised control trial of "Conventional ventilatory support vs extracorporeal membrane oxygenation (ECMO) for severe adult respiratory failure (CESAR)" showed that ECMO had limited success in preventing lung injury (Ware and Matthay, 2000; Peek et al.,

2006). Given the significant mortality associated with ARDS, alternative therapies aimed at reducing lung injury are needed.

Stem cell therapy has raised the possibility of augmenting lung repair and restituting damaged lung tissue. Human bone marrow derived mesenchymal stem cells (hMSC) have improved a variety of animal models of acute lung injury and ex vivo models of human lung injury (Ortiz et al., 1999; Lee et al., 2009; Asmussen et al., 2014). Although there have been significant advances in MSC therapy including the commencement of clinical trials, there are still critical areas that need further elucidation. There is a paucity of studies testing MSC in large animal models of ALI. In addition, there is conflicting data regarding the onset of benefit of MSC. Several studies have shown beneficial physiological effects of MSC at time points of 24–48 h post-infusion (Asmussen et al., 2014; Devaney et al., 2015), whilst Rojas et al. (2014) showed beneficial effects within 2 h of MSC infusion (Rojas et al., 2014). Therefore, determining the early effects of MSC on inflammation and lung physiology in a large animal model of ALI would inform clinical studies about the timelines to expect improvement and the impact of MSC on the early pathogenesis of the disease.

There are several proposed mechanisms by which hMSC act including the release of anti-inflammatory mediators such as nitric oxide, IL-1R antagonist, angiopoietin and tumour necrosis factor-inducible gene (TSG-6) (Moodley et al., 2013). Recently, MSC were shown to release exosomes including donating mitochondria that then improve the energetics of damaged tissue thus augmenting repair (Islam et al.). Based on the pathogenesis of ALI/ARDS and the mechanisms of action of MSC, we hypothesized that MSC would improve the early inflammatory and physiological changes in this condition. In this study, we treated an oleic acid (OA) induced-ventilatory pig model of ALI with hMSC and measured physiological and inflammatory outcomes during the initial 4 h after treatment.

2. Materials and methods

2.1. Preparation of hMSC

Human MSC were isolated from donor bone marrow as previously described (Herrmann et al., 2012), and culture expanded in Dulbecco's modified eagle medium (DMEM; Gibco, Life Technologies) containing 10% fetal bovine serum (FBS; HyClone, Thermo Scientific). Bone marrow for MSC manufacturing was collected under the approval from the Royal Perth Hospital Ethics Committee for clinical therapy and the need for consent to use the cells for research was waived because the cells manufactured were surplus to requirements.

The batch of hMSC used in our study expressed the cell surface markers CD105, CD90 and CD73, but not CD45, CD34, CD11b, and the human leucocyte antigen (HLA)-DR. Cells were harvested at $P3$ and cryopreserved at 5×10^6 cells/ml in 10% dimethyl sulfoxide (DMSO; Wakchemi), 10% porcine serum (HyClone, Thermo Scientific) and 80% sodium chloride solution (0.9%) in OriGen cryogenic bags using a controlled rate freezer. Cells were then stored at −196 °C in vapour nitrogen. Cell aliquots containing 40×10^6 cells along with control bags (same vehicle but without cells) were prepared at the Cell and Tissue Therapies WA (CTTWA) facility, cryopreserved in cryobags, shipped on dry ice to the University of Manitoba and stored at −80 °C in a secure fridge.

2.2. Ventilation of pigs

After receiving approval from the University of Manitoba Animal Care Ethics Committee (submitted, Sep. 2012), a total of 12 farm bred female pigs (20–22 kg) were fasted overnight with free access to water. An intramuscular injection of ketamine, midazolam and atropine (10/0.1/0.02 mg/kg) was given for sedation. Anaesthesia was induced with isoflurane by inhalation via a nosecone. The animals were intubated with a 6.0 cuffed endotracheal tube and mechanically ventilated with a tidal volume = 10 ml/kg, inspired oxygen concentration = 50%, positive end-expiratory pressure (PEEP) = 5 cm H_2O and respiratory rate adjusted to maintain $PaCO_2$ in the normal range. Anaesthesia was maintained with 1.5 to 2 MAC Isoflurane during surgical preparation. An intravenous infusion of Ringer's Lactate (10 ml/kg/h) was established in an ear vein. Through a cut-down, a 5 ft Swan Ganz catheter was floated into the pulmonary artery via the external jugular vein to measure pulmonary artery pressure, pulmonary capillary wedge pressure, right atrial pressure and cardiac output by thermodilution in triplicate. A femoral artery cannula was placed for continuous arterial pressure measurement and blood gas sampling. A femoral venous cannula was advanced in the IVC above the diaphragm for OA administration.

Anaesthesia was then switched to a continuous infusion of propofol/ketamine/rocuronium, 10/2.5/0.5 mg/kg/h to allow for ventilation with an Esprit ® ventilator, which ensured more precise control of tidal volume, PEEP and respiratory mechanic measurements for the experimental period. Muscle relaxation was required to prevent spontaneous ventilator efforts during low tidal volume ventilation which interfere with accurate measurements of lung mechanics. The animal was allowed to stabilize for 15 min after surgical preparation, then baseline hemodynamics, arterial blood gases and lung function (peak and mean airway pressures, total respiratory system compliance, by interrupter method, and dead space) were determined.

2.3. Oleic acid induced injury

ALI was then induced with a well-established protocol (Froehlich et al., 2008). OA was infused at 0.2 ml/kg/h until static respiratory compliance decreased by at least 40% and arterial PaO_2 decreased to <100 mm Hg at an FIO_2 of 50%. Dopamine was infused at 5–10 mcg/kg/min during OA infusion to maintain arterial blood pressure > 60 mm Hg, then discontinued. When a stable injury had been achieved, (no further change in blood gases or lung compliance for 15 min) ventilation was maintained with a low tidal volume strategy (V_T = 7 ml/kg, PEEP 10, FiO_2 50%) for the duration of experiments. The animals were allowed to stabilize for 1 h and measurements repeated. The animals were then randomly assigned to a sham treatment group (n = 5) or hMSC group (n = 6).

2.4. MSC administration

The dose of MSC of 2×10^6 cells/kg administered intravenously was based on our studies in other disease cohorts (Forbes et al., 2014). In preparation for infusion, the cryobag was thawed in a water bath of sterile saline at 37 °C. An equal volume of sterile Ringer's Lactate was added to the bag to stabilize the cells. The cells (2×10^6 cells/kg) or vehicle control were infused immediately following preparation over 10 min. DMSO, used in the cryopreservation of the MSC was infused with the cells and DMSO present in the vehicle control was administered to controls in equivalent volumes to the experimental group but without the cells. Cell viability was assessed using the Vi-Cell XR cell analyser (trypan blue based determination) post cryopreservation and in our laboratory was 87% viability.

Blood gases and lung function measurements were determined immediately after infusion and then hourly for 4 h. Lung oedema fluid aspirate samples were obtained by gentle suction of a soft catheter wedged into a distal airway at baseline OA injury (1 h post OA infusion) and at 4 h of ventilation post lung injury. Samples were centrifuged at 3000 g for 10 min and the supernatant stored at −80 °C. The animals were sacrificed using Euthanyl 100 mg/kg with lung inflation maintained at 10 cm PEEP.

2.5. Histology and lung aspirate

Lung tissue samples were obtained from upper, middle and lower lobes and fixed in formalin. The lungs were then removed en bloc,

weighed and dried to determine the wet:dry weight ratio. Cytokine levels were assessed in paired aspirate samples obtained at time zero and 4 h post-MSC transfer. Following centrifugation, each was quantified in triplicate using Duoset ELISAs (R&D Systems) following the manufacturer's protocols but optimized to provide sensitivity of 16 pg/ml (IL-8) and 6 pg/ml (IL-10). Inter-assay variability was 5–10%. ELISA for measurement of cytokine responses in primary culture was done (Stefura et al., 2008).

Formalin-fixed paraffin-embedded pig lung tissue were stained with hematoxylin and eosin and examined under a light microscope (×400 magnification). About 120 random areas (about 40 random area for each upper, middle and lower right lobe for each sample) were evaluated. Histopathological lung injury scoring analysis was performed according to the ATS Workshop Report methodology (Matute-Bello et al., 2011; Voelker et al., 2014). The degree of microscopic injury was scored by two raters based on the following variables: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspace and alveolar septal thickening. Each element was graded according to a 3-point scale described (Table 1), and the result was expressed as a mean of the total score of the two raters. All counts and scores were done in a blinded fashion.

2.6. Immunofluorescence

Immunofluorescence of NF-κB was examined in formalin fixed pig lung tissues sections. Briefly, following deparaffinization and permeation with a PBS-Triton (0.1%, 5 min) solution, and saturation of non-specific sites with BSA (5%, 30 min), cells were incubated with primary antibodies of NF-κB (Abcam) overnight in a humidified chamber at 4 °C. Conjugated secondary antibodies were used at a dilution of 1:2000. Slides were mounted in Prolong-gold with DAPI (ProLong® Gold antifade regent with DAPI, Life Technologies). To take a picture of the immunofluorescence, we used the automatic slide scanner microscope (Olympus VS 120-L). The analysis of nuclear translocation was performed using the plugin (JACoP) for imageJ. Picture threshold used for the analyses was identical for all images.

2.7. Statistics

We used repeated measures ANOVA to demonstrate differences between OA treated and OA + MSC pigs. A p-value of <0.05, corrected for repeated measures was regarded as significant. Random effects linear regression models with maximum likelihood estimation (MLE) were used to investigate differences in injury scores (2 raters) post injury between OA and OA + MSC groups, as well differences in the pattern of IL-8 and IL-10 over time between the two groups via the interaction of group and time (pre and 4 h post injury). Injury scores were analysed with repeated measures to account for scoring by two raters. The

Table 1
Lung injury scoring system.

Parameter	Score per field		
	0	1	2
A. Neutrophils in the alveolar space	none	1–5	>5
B. Neutrophils in the interstitial space	none	1–5	>5
C. Hyaline membranes	none	1	>1
D. Proteinaceous debris filling the airspaces	none	1	>1
E. Alveolar septal thickening	<2×	2–4×	>4×

Score = [(20 × A) + (14 × B) + (7 × C) + (7 × D) + (2 × E)] / (number of fields × 100). Neutrophils scores were calculated using data obtained from lung injury scoring. Briefly, neutrophils are counted in the alveolar airspaces and interstitium. If neutrophils are not visible within the field, a score of zero is recorded for that field. One to five neutrophils give a score of one, and more than five neutrophils give a score of two. The result was expressed as a mean of the sum of individual scores for both alveolar space and interstitial space neutrophils.

Table 2
The airway pressure and arterial blood gas parameters and mechanics monitored following OA, ventilation and hMSC treatment.

Parameter	Baseline		Post OA		Post MSC/serum		1 h		2 h		3 h		4 h	
	Control	MSC	Control	MSC	Control	MSC	Control	MSC	Control	MSC	Control	MSC	Control	MSC
Airway pressures														
Peak Paw (cm H₂O)	17.0 (0.6)	17.7 (1.0)	30.4 (2.3)	30.6 (1.8)	29.8 (1.0)	30.6 (1.7)	29.9 (1.7)	31 (2.3)	29 (2.6)	30.7 (2.2)	28.8 (2.5)	30.2 (1.8)	29.1 (2.2)	29.4 (1.6)
Mean Paw (cm H₂O)	8.1 (0.3)	8.3 (0.4)	16.5 (1.2)	16 (1.0)	16.5 (1.2)	16.3 (0.4)	16.5 (0.4)	16.5 (0.8)	16.2 (1.1)	16.5 (0.7)	16.1 (1.1)	16.4 (0.5)	16.2 (1.0)	16.4 (0.3)
PEEP (cm H₂O)	4.6 (0.2)	4.7 (0.2)	10.6 (0.6)	10.6 (0.4)	10.2 (0.7)	10.3 (0.5)	10.3 (0.1)	10.4 (0.4)	10.2 (0.3)	10.3 (0.4)	10.2 (0.3)	10.4 (0.4)	10.2 (0.4)	10.4 (0.4)
VT (ml/kg)	9.3 (0.5)	10.7 (0.7)	7.4 (0.4)	7.8 (1.0)	7.7 (0.6)	7.9 (0.9)	8.1 (1.2)	7.8 (1.0)	8.0 (0.7)	7.8 (1.0)	8.0 (0.7)	7.8 (1.0)	8.0 (0.9)	8.1 (1.0)
RR (b/min)	25 (0)	25 (0)	35 (0)	35 (0)	35 (0)	36 (2)	39 (2)	37 (3)	38 (3)	38 (3)	38 (3)	38 (3)	38 (3)	37 (3)
MV (ml/kg/min)	233 (13)	261 (18)	259 (16)	251 (35)	270 (21)	268 (33)	317 (55)	285 (59)	303 (24)	297 (60)	304 (25)	295 (59)	305 (26)	298 (59)
Arterial blood gases														
PaO₂ (mm Hg)	230 (22)	235 (10)	141 (18)	140 (23)	164 (11)	181 (21)	186 (17)	188 (1)	215 (27)	208 (10)	204 (13)	202 (17)	199 (22)	202 (14)
PaCO₂ (mm Hg)	39 (2)	37 (5)	60 (5)	56 (10)	66 (10)	59 (15)	60 (9)	60 (11)	57 (3)	53 (9)	53 (3)	53 (7)	53 (5)	55 (8)
pH	7.4 (0.1)	7.39 (0.05)	7.23 (0.1)	7.27 (0.05)	7.18 (0.1)	7.23 (0.1)	7.22 (0.1)	7.19 (0.1)	7.24 (0.1)	7.27 (0.1)	7.26 (0.1)	7.27 (0.1)	7.26 (0.1)	7.27 (0.1)
QS:QT	0.7 (0.6)	0 (1.8)	11.9 (8)	7.6 (2.5)	7.1 (3.6)	3.6 (2.0)	2.5 (1.4)	2.5 (1.3)	1.8 (1.2)	1.0 (0.4)	2.1 (1.8)	1.2 (0.5)	2.6 (2.5)	1.7 (0.9)
VD:VT	56.2 (2.5)	53.4 (5.8)	76.4 (4.5)	75.2 (5.1)	75.3 (5.5)	73.8 (6.0)	75.8 (2.8)	73.9 (4.8)	73.6 (2.4)	70.6 (5.5)	70.5 (1.2)	71.5 (4.7)	70.9 (2.1)	72.9 (5.4)

Table 3
The lung mechanics and hemodynamics monitored following OA, ventilation and hMSC treatment.

Parameter	Baseline		Post OA		Post MSC/serum		1 h		2 h		3 h		4 h	
	Control	MSC	Control	MSC	Control	MSC	Control	MSC	Control	MSC	Control	MSC	Control	MSC
Lung mechanics														
Compliance (RS) (ml/cm H_2O)	1.23 (0.15)	1.26 (0.08)	0.60 (0.09)	0.57 (0.06)	0.63 (0.09)	0.60 (0.07)	0.68 (0.13)	0.63 (0.13)	0.70 (0.1)	0.64 (0.17)	0.72 (0.1)	0.69 (0.16)	0.72 (0.1)	0.71 (0.17)
Resistance (cm H_2O/l/s)	12.8 (1.2)	12.5 (1.0)	12.5 (1.6)	11.8 (1.8)	13.68 (0.7)	12.7 (1.1)	13.4 (1.3)	14.2 (1.9)	13.2 (3.2)	13.7 (1.9)	13.0 (2.6)	14.2 (1.7)	14.5 (1.8)	14.6 (1.9)
Hemodynamics														
MAP (mm Hg)	96 (8)	108 (9)	94 (11)	99 (14)	98 (13)	108 (14)	97 (14)	113 (12)	99 (20)	108 (16)	101 (26)	94 (21)	89 (17)	83 (18)
CVP (mm Hg)	9.3 (0.8)	10.9 (0.8)	10.5 (2.2)	11.0 (1.1)	10.7 (1.6)	11.6 (1.1)	10.2 (2.4)	10.9 (0.8)	10.3 (1.9)	11.0 (0.7)	10.1 (1.9)	11.2 (0.8)	10.4 (1.9)	10.7 (1.0)
PPA (mm Hg)	26.9 (7.2)	24.5 (4.7)	39.5 (3.8)	41.1 (5.3)	40.0 (5.1)	41.8 (7.5)	37.1 (5.9)	38.9 (6.6)	35.2 (5.1)	37.2 (8.8)	34.9 (4.9)	37.5 (8.7)	35.2 (4.3)	37.0 (9.4)
PCWP (mm Hg)	15.5 (3.3)	14.6 (1.9)	20.7 (4.0)	21.0 (2.6)	19.0 (3.5)	19.2 (3.4)	18.0 (5.6)	17.8 (2.9)	19.0 (4.8)	17.7 (5.0)	17.8 (3.8)	17.9 (4.4)	17.2 (4.2)	17.7 (3.8)
HR (b/min)	105 (11)	107 (17)	132 (39)	132 (47)	170 (42)	152 (56)	172 (53)	155 (65)	172 (43)	146 (55)	179 (34)	153 (46)	192 (32)	156 (57)
CO (l/min)	3.5 (0.6)	3.1 (0.4)	2.7 (0.5)	2.0 (0.4)	3.2 (0.6)	2.0 (0.2)	2.4 (0.6)	1.6 (0.2)	2.4 (0.7)	1.6 (0.3)	2.5 (0.8)	1.6 (0.3)	2.6 (1.0)	1.6 (0.3)
TEMP (°C)	36.3 (0.2)	36.4 (0.5)	36.9 (0.3)	36.7 (0.4)	36.9 (0.5)	36.9 (0.5)	37.1 (0.5)	37.1 (0.3)	37.4 (0.7)	37.2 (0.4)	37.5 (0.7)	37.4 (0.6)	37.7 (0.7)	37.4 (0.7)

association between neutrophils and IL-8 over time was also investigated using random effects linear regression with MLE. Differences in the association between OA and OA + MSC groups were tested using the three way interaction of group, neutrophil count and time. Analysis was conducted using Stata (StataCorp. 2013. *Stata Statistical Software*: *Release 13*. College Station, TX: StataCorp LP).

3. Results

3.1. Respiratory and hemodynamic parameters

A similar degree of lung injury was induced in both OA and hMSC groups. Total respiratory system compliance decreased by 50% with OA injury and was associated with a PaO_2 nadir of 99 (3) mm Hg and PaO_2/FiO_2 ratio 198 (5) in the control OA group vs a PaO_2 nadir of 90 (10) mm Hg and PaO_2/FiO_2 ratio of 181 (20) in the hMSC group. These values correlate with a moderate ALI score using the Berlin classification (Ranieri et al., 2012). This degree of lung injury was chosen to ensure viability for the duration of the study period. Post nadir, with PEEP increased to 10 cm H_2O, PaO_2 increased to 140 mm Hg in both groups with no change in respiratory system compliance (Tables 2 and 3), demonstrating recruitability in the model. The increase in PaO_2 over the subsequent 4 h with minimal improvement in respiratory system compliance suggests the presence of a significant hypoxic pulmonary vasoconstrictor response, despite the presence of significant lung infiltrates, as previously demonstrated in this porcine model (Graham et al., 2011).

There were no differences in airway pressures, arterial blood gases, lung mechanics, or hemodynamics between control and hMSC treated pigs at baseline, post OA injury, post hMSC administration or in the subsequent 4 h studied (Tables 2 and 3). Left lung wet:dry weight ratio was 5.2 (0.4) and 5.8 (0.8) in control and hMSC treated groups respectively, corroborating the modest degree of lung injury. No adverse events were documented with the administration of hMSC, with no subsequent changes in either temperature or any hemodynamic parameter over those seen with OA administration alone.

3.2. There is a reduction in NF-κB nuclear translocation following hMSC treatment

NF-κB is an important transcription factor for the activation of several inflammatory pathways. We examined NF-κB nuclear translocation to determine if there was a change in the expression of this transcription factor following hMSC treatment. The NF-κB positive nucleus was assessed by counting the number of positive cells on fluorescence microscopy of lungs obtained at 4 h post-hMSC infusion. NF-κB nuclear translocation was significantly reduced in lungs of pigs treated with hMSC as compared to controls (p = 0.04; Fig. 1).

3.3. There were no significant changes in lung injury following hMSC

Noting the fall in NF-κB nuclear translocation, we investigated the role of hMSC in treating lung injury and inflammation. The composite lung injury assessment was made by scoring histological sections of the lung following 4 h post-hMSC treatment. The composite lung injury score demonstrated a numerical reduction in lung injury in hMSC treated pigs versus controls but this did not reach statistical significance (p = 0.063; Fig. 2).

Neutrophilic inflammation was also assessed by counting neutrophils on histological sections of lung. There were no significant differences in the composite neutrophil counts which included upper, middle and lower lobes between control and hMSC groups (p = 0.36; Fig. 3a). There was a trend towards reduced alveolar neutrophil count in the upper lobes in hMSC group versus controls, however, this was not statistically significant (p = 0.07; Fig. 3b).
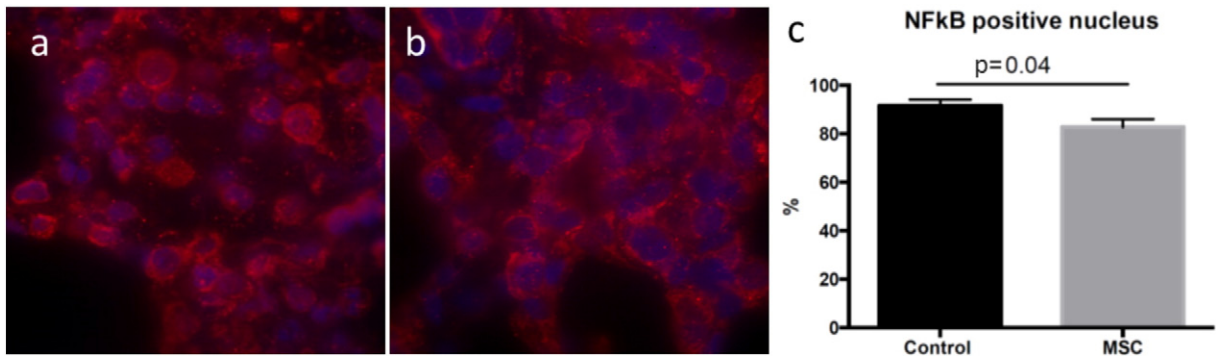
Fig. 1. There is a significant fall in NF-κB following OA + MSC versus OA alone. Immunofluorescence shows that NF-κB nuclear translocation in the lung tissue. A: OA injured lung. B: OA + MSC injured lung. C: The NF-κB positive nuclei percentage was calculated by counting at least 200 DAPI-positive cells per sample were counted (×400 magnification). Red is NF-κB and blue is DAPI. There is a significant decrease in NF-κB positive nuclear cells in hMSC treated pigs versus controls.

3.4. IL-8 correlated with tissue neutrophils

There was no significant difference detected in the change in aspirate IL-10 levels or IL-8 levels over time between hMSC groups and controls ($p = 0.18$ and $p = 0.23$ respectively; Fig. 4). The three way interaction of time, group and neutrophils in the IL-8 model was almost significant ($p = 0.06$). This indicates that the change in the slope from pre to post test in the control group differs from the change in the slope from pre to post in the stem cell group. This suggests a close association in the change of IL-8 and neutrophils.

4. Discussion

The present study characterises the early effects and safety profile of hMSC therapy in a large animal model of ALI. After 4 h of treatment, we have demonstrated that hMSC reduced tissue NF-κB, an important transcription factor that mediates inflammation while no significant effects on hemodynamics, lung mechanics, gas exchange, inflammatory mediators or lung injury scores were evident. These data suggest that hMSC therapy is safe over the short term. Subsequent studies are required to determine if the observed decrease in inflammatory transcription factors translates into improvements in inflammation and in physiological parameters over the long term.

The beneficial effects of MSC have been described in several animal models (Rojas et al., 2014; Curley et al.). In two studies using a rat model of ventilator-induced lung injury, there were improvements in lung injury within 4 h of MSC treatment (Chimenti et al., 2012; Hayes et al., 2015). However, in ALI/ARDS there is a relative paucity of cell therapy studies in large animal models. Pre-clinical studies in large animal models are more informative about potential efficacy as compared to small rodents, with their significant differences in biology and

physiology to humans. Our results suggest that the early benefits seen in small animal models are not evident in a larger porcine model.

There are few studies that reported the physiological parameters in the early period post stem cell therapy. In a ventilator induced lung injury model in rats, MSC improved oxygenation, oxygen alveolar-arterial gradient, static compliance and microvascular permeability at 48 h post-treatment. The shorter time-points were not analysed (Curley et al.). Asmussen et al. (2014) used a cotton smoke inhalation model and infection with *Pseudomonas aeruginosa* to determine if hMSC would improve a sheep model of pneumonia. At 24 h post-hMSC treatment, pulmonary artery pressures were reduced and PaO$_2$/FIO$_2$ ratio increased with no differences in airway pressure between controls and hMSC treated sheep. Notably there was no difference in any physiological parameters between controls and hMSC treated groups at time-points before 24 h (Asmussen et al., 2014). Both these studies are in-keeping with our findings of no physiological changes at 4 h. In contrast, Rojas et al. (2014) found that autologous CD45 negative bone marrow mononuclear cells (BMC) significantly reduced pulmonary vascular resistance and improved oxygenation at 2 h post-treatment in an endotoxin-induced porcine model of lung injury. The BMC preparation would include MSC and vascular cells. The difference in injury model and cell therapy chosen may explain the difference between these results and the present study, despite the use of pigs in both studies. These studies support the need for monitoring the effect of MSC at longer time-points (Asmussen et al., 2014; Rojas et al., 2014; Curley et al.).

We used a well characterized method of OA induced lung injury in ventilated pigs. OA (cis-9-octadecenoic acid) is the most common free fatty acid in mammals, comprising 60% of the free fatty acid pool and is, through as yet unknown mechanism, directly toxic to endothelial cells within one minute of administration (Beilman, 1995). It causes necrosis and severe vacuolation of endothelial cells followed by epithelial injury (Hussain et al., 1998). OA and mechanical injury mimics early
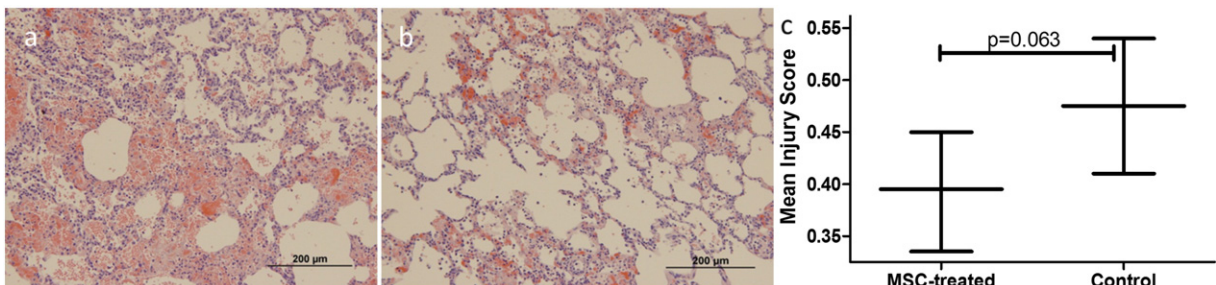

Fig. 2. There is a numerical reduction in the inflammatory score between OA and OA + MSC. The injury score of (a) OA injured lung and (b) OA + MSC injured lung was based on lung tissue sections obtained 4 h post-hMSC treatment and controls. (c) There was a reduction in the score for hMSC treated animals but this did not reach significance.
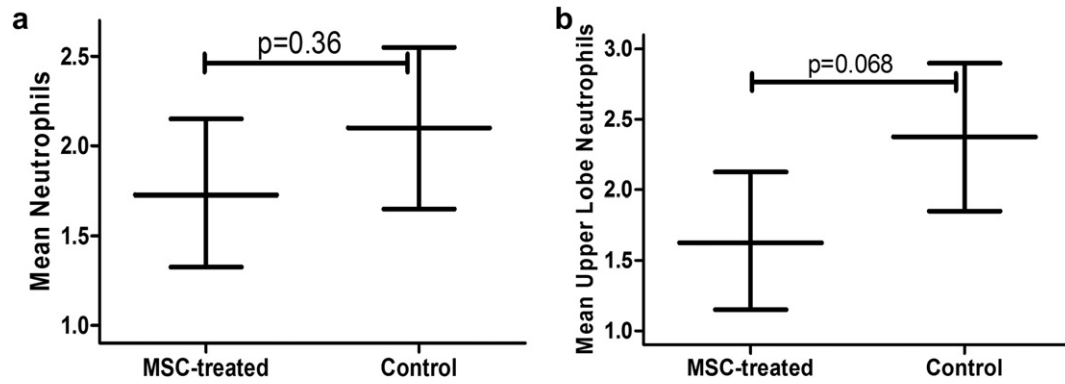
Fig. 3. There is a mild reduction in alveolar neutrophils in the upper lobe between OA and OA + hMSC. Tissue sections obtained from lung were scored for neutrophil infiltration of the alveolar space and interstitium. (a) There was no difference in total neutrophil scores between hMSC treated animals and controls across the upper, middle and lower lobe of the lung. (b) There was a reduction in alveolar neutrophil infiltration in the upper lobe of hMSC treated animals versus controls which almost reach significance.

ALI/ARDS and fat embolism with neutrophil infiltration and hyaline membrane formation leading to the physiological changes including increased capillary permeability, hypoxia from ventilation-perfusion mismatching and shunting, decreased lung compliance and increased dead space (Voelker et al., 2014).

Since inflammation is reduced by hMSC, we examined the expression patterns of the transcription factor NF-κB which is central to the development of inflammation and found in high concentrations in ARDS (Vlahopoulos et al., 1999; Bajwa et al., 2011; Fudala et al., 2011). NF-κB is a transcription factor found in all cells and constitutes a family of DNA-binding proteins that are responsible for the gene transcription of pro-inflammatory cytokines, chemokines and adhesion molecules (Perkins, 2007). NF-κB responds to different stimuli including oxidants and cytokines (Perkins, 2007). Following activation of cytokine receptors such as tumour necrosis factor associated receptor, the complex activates NF-κB inducing kinase (NIK) which results in the phosphorylation of IκB. The phospho-IκB, destined for the ubiquitinization/proteosome pathway, dissociates from NF-κB thereby allowing NF-κB to translocate into the nucleus for gene transcription (Zhang et al., 2000).

There was a decrease of NF-κB nuclear translocation on immunofluorescence of lung tissue at 4 h, in hMSC treated pigs versus untreated controls. The number of cells with positively staining nuclei for NF-κB was significantly lower in the treatment group compared to controls. This suggests that the hMSC reduce NF-κB expression within 4 h of hMSC injection. We did not determine the mechanisms of NF-κB reduction in our model. Studies have suggested that the production of both transforming growth factor-β and TSG-6 by MSC are shown to reduce NF-κB and may play a role in our study (Wen et al., 2014).

Noting the reduction in NF-κB nuclear translocation, we assessed the degree of lung injury by scoring of histological sections at 4 h post-hMSC infusion. The injury score utilized in this study incorporated hyaline membrane formation, alveolar septal thickening, debris in the alveolar spaces and neutrophil infiltration (Matute-Bello et al., 2011). These features mimic the histologic changes that occur with ARDS. The difference in injury scores between controls and hMSC treated pigs did not reach statistical significance.

We separately assessed neutrophil infiltration of the lung following hMSC treatment. Neutrophils are the first cells recruited to sites of injury following the release of chemokines from macrophages, endothelial and epithelial cells (Leonard and Yoshimura, 1990). These cells release several mediators such as oxidants and proteinases that have potent anti-microbial properties but at excessive levels cause bystander damage to normal tissue (Palmgren, 1992). In ALI/ARDS, there are many lines of evidence that points to an important role for neutrophils in the pathogenesis of this condition (Curley et al.; Abraham, 2003). In addition, increased neutrophils in aspirates correlate with reduced survival (Yang et al., 2003). In our study there was no difference in total neutrophil infiltration of the lung between controls and hMSC treated animals as determined by histological scores of upper, middle and lower lobes of lungs at 4 h post treatment. Furthermore, we investigated the changes in the cytokine IL-8 by ELISA in aspirate since this cytokine is raised in ARDS and acts as a chemokine for neutrophils (Ware and Matthay, 2000; Miller et al., 1992), and is consistently the closest association to disease severity and survival in ARDS (Struyf et al., 2005; Goodman et al., 1996). There was no difference in IL-8 between controls and hMSC treated groups. In addition, there was no difference pre- and post-hMSC treatment. However, there was a significant relationship
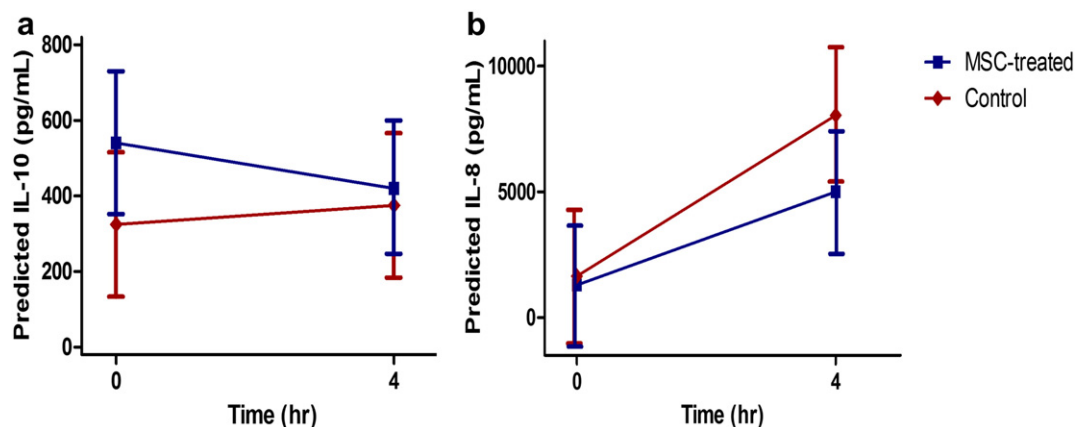

Fig. 4. There is no change in IL-10 and IL-8 levels following hMSC treatment. Cytokines (a) IL-10 and (b) IL-8 were measured by ELISA in aspirate samples following OA instillation pre-hMSC treatment and 4 h post-hMSC treatment. There was no change in IL-10 and IL-8 levels pre-hMSC and 4 h post-MSC treatment.

between the change in neutrophils and the change in IL-8 in both controls and hMSC groups from over time (from baseline to 4 h) confirming the association of this cytokine with neutrophil infiltration in this model.

Limitations to our study include a model which may not mimic all the steps in ALI/ARDS. In addition, this was considered a pilot study, approved as a safety and feasibility trial by the Ethics Committee. The results are underpowered due to the limited number of animals allowed. We conclude that the observed reduction in the inflammatory transcription factor NF-κB, coupled with trends toward decreases in inflammation and lung injury provide evidence to warrant further large animal studies which include a greater number of subjects monitored for a longer time period to determine if hMSC treatment may provide a role in the management of ALI/ARDS.

Acknowledgements

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