Paracrine Effect of Mesenchymal Stromal Cells on Multifactorial Lung Injury in Neonatal Mice

By

Lannae Strueby

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Department of Physiology

University of Alberta

Abstract

Bronchopulmonary dysplasia (BPD) is a common complication of extreme prematurity. Preterm infants often require mechanical ventilation and supplemental oxygen for survival. These interventions increase the risk of developing BPD. Despite its frequency, there remains no effective treatment for BPD. Evidence suggests that mesenchymal stromal cells (MSCs) prevent oxygen-induced lung injury in rodent models of BPD. The benefits observed appear mediated via paracrine effects. We explored the ability of cell-free conditioned media (CDM) from human umbilical cord-derived MSCs (MSC-CDM) to prevent lung damage in a neonatal mouse model of BPD combining inflammation, ventilation-induced lung injury, and supplemental oxygen.

Neonatal mice (C57BL/6) were mechanically ventilated at postnatal day 9-10 for 8 hours with a tidal volume of 10µl/g, 180 breaths/minute and 40% oxygen. Inflammation was induced by intraperitoneal administration of lipopolysaccharide (LPS) 48 hours preceding ventilation. Age matched unventilated mice that did not receive LPS were used as controls. The treatment group received intratracheal MSC-CDM (3µl/g) immediately prior to mechanical ventilation. At completion of ventilation lungs were harvested for structural and molecular analysis.

Ventilated mice that received LPS exhibited alveolar simplification and reduced vascular density compared to controls as demonstrated by a significantly greater mean linear intercept and reduced number of vessels per high power field ($p<0.05$). These mice also exhibited elevated levels of macrophage inflammatory protein – 2 (MIP-2) and monocyte chemoattractant protein – 2 (MCP-1) compared

with controls ($p<0.05$). Intratracheal MSC-CDM treatment significantly attenuated structural lung injury and improved vascular density compared with untreated, ventilated mice ($p<0.05$), but did not significantly reduce levels of MIP-2/MCP-1.

Treatment with MSC-CDM attenuates structural lung injury and improves vessel density in a clinically relevant neonatal mouse model of BPD. By harnessing the beneficial effects of MSC-CDM, new therapies for treating BPD may be developed.

Preface

This Thesis is an original work by Lannae Strueby. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee, Project Name "Lung development and experimental therapies and breeding colony", No. Aup00000341, July 22, 2014.

Dr. R. Bland taught the small animal ventilation techniques used in creating our clinically relevant mouse model of BPD, described in chapter 4 of this thesis, to members of Dr. B. Thébaud's lab. Dr. M. O'Reilly, a member of Dr. B. Thébaud's lab, was experienced in these techniques and subsequently educated/assisted me in the performance of procedures required for development of our mouse model of BPD.

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List of Abbreviations

aMEM	alpha Minimum Essential Medium
ANOVA	one-way analysis of variance
BOOST	benefits of oxygen saturation targeting
BPD	bronchopulmonary dysplasia
CAP	caffeine for apnea of prematurity
CGA	corrected gestational age
CI	confidence interval
COIN	continuous positive airway pressure or intubation
COT	Canadian oxygen trial
CURPAP	prophylactic or early selective surfactant combined with NCPAP
DAB	3,3′-Diaminobenzidine
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
HFV	high frequency ventilation
HLA	human leukocyte antigen
HPF	high power field
IM	intramuscular
iNO	inhaled nitric oxide
IU	international units
LPS	lipopolysaccharide
mcg	micrograms
MCP-1	monocyte chemoattractant protein – 1
MIP-2	macrophage inflammatory protein – 2
MIST	minimally invasive surfactant therapy
MLI	mean linear intercept
MSC	mesenchymal stromal cell
NCPAP	nasal continuous positive airway pressure
NIH	national institutes of health
NIPPV	nasal intermittent positive pressure ventilation
NO	nitric oxide
OR	odds ratio
PDA	patent ductus arteriosus
PEEP	positive end expiratory pressure
PLV	pressure-limited ventilation
RCT	randomized controlled trial
RR	relative risk
SAEs	serious adverse events
SI	sustained inflation
SUPPORT	surfactant, positive pressure and oxygenation randomized trial
TAC	total antioxidant capacity
VTV	volume-targeted ventilation
vWF	von Willebrand Factor

Chapter 1:

Bronchopulmonary Dysplasia

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Prematurity is the leading cause of newborn death and the second leading cause of death in children less than 5 years of age. It is defined as birth prior to 37 completed weeks of gestation. In 2010 it is estimated that 14.9 million infants were born premature worldwide with 0.78 million of these infants being born extremely premature at less than 28 weeks gestation. In the same year, the preterm birth rate in the United States was 12%, accounting for 42% of all preterm births in the developed region. More than 90% of neonates born less than 28 weeks gestation in high-income countries survive.[1] Bronchopulmonary dysplasia (BPD) is one of the most common complications experienced by these extremely premature infants.[2] BPD rates in infants increase with decreasing gestational age. The estimated annual cost of treating premature infants in the United States in 2005 was $26.2 billion dollars, of which 10% was just for treating infants with BPD.[3]

Despite the frequency of BPD, current therapies offer limited benefit or have unacceptable long-term side effects. Extensive research investigating new strategies to prevent or treat BPD has identified stem cell-based therapies as a possible new therapeutic option. Of the stem cells investigated, mesenchymal stromal cell (MSC)-based therapies demonstrate the most potential for preventing or treating BPD. The research comprising this thesis contributes to the current body of knowledge by exploiting the known benefits of MSCs and investigating the paracrine potential of MSC conditioned media to prevent lung injury in a clinically relevant model of BPD in newborn mice.

The diagnostic dilemma

Northway and colleagues first defined BPD in 1967. They described a chronic pulmonary syndrome occurring in infants with severe respiratory distress syndrome requiring prolonged ventilation and high-inspired oxygen concentrations. BPD was characterized using the clinical, radiologic and pathologic changes present in these infants at 1 month of age.[4] The definition of BPD has since undergone multiple modifications.

Through the 1970s and early 1980s, the definition evolved to include infants requiring oxygen at 1 month of age with an abnormal chest radiograph. In 1988, Shennan and colleagues proposed BPD be defined as the requirement for supplemental oxygen at 36 weeks corrected gestational age (CGA). They recognized that few infants were following the radiographic pattern described by Northway and colleagues. Additionally, with increasing prematurity, oxygen requirement at 1 month of age was not accurately identifying infants at increased risk of abnormal pulmonary outcome.[5]

A subset of the National Institutes of Health (NIH) organized a workshop in 2000 focusing on BPD. A new severity-based definition for BPD was proposed. Infants born at less than 32 weeks gestational age and requiring supplemental oxygen at 28 days of life were categorized as mild, moderate or severe BPD based on assessment at 36 weeks CGA. Infants breathing room air at 36 weeks CGA were classified as mild BPD, those needing less than 30% oxygen as moderate BPD and those requiring greater than 30% oxygen or needing positive pressure as severe BPD (Table 1-1).[6] The validity of this definition was assessed in a retrospective

review and found to more accurately identify the spectrum of risk for adverse pulmonary and neurodevelopmental outcomes at 18-22 months CGA.[7]

The above methods of diagnosing BPD primarily focus on quantity of supplemental oxygen provided. This results in significant variance in the incidence of BPD secondary to institutional differences in saturation targets for preterm infants. This limitation was addressed by the physiologic definition of BPD proposed by Walsh and colleagues. They propose BPD be defined as requiring mechanical ventilation, continuous positive airway pressure or greater than 30% oxygen at 35-37 weeks CGA. Additionally, infants receiving less than 30% oxygen undergo a timed

Table 1-1. National Institutes of Health severity-based definition of BPD for infants born at less than 32 weeks gestational age and requiring supplemental oxygen at 28 days of life.[6]

Severity of BPD	**Oxygen requirement at 36 weeks CGA or discharge home**
Mild	Breathing room air
Moderate	Requiring < 30% oxygen
Severe	Requiring ≥ 30% oxygen and/or positive pressure
BPD: Bronchopulmonary dysplasia; CGA: Corrected gestational age	

stepwise reduction to room air and those failing this reduction are diagnosed with BPD.[8] This physiologic definition of BPD has been shown to reduce the overall reported incidence of BPD and decrease the institutional variation.[9]

The multitude of definitions currently used in the literature present a significant confounder to future therapeutic studies and site comparisons, particularly since a change in the BPD definition has been shown to alter the incidence of BPD by a mean of 10%, equal to the therapeutic effect of some interventions.[9] A consensus on the most accurate diagnostic criteria for BPD is required for future research and comparisons.

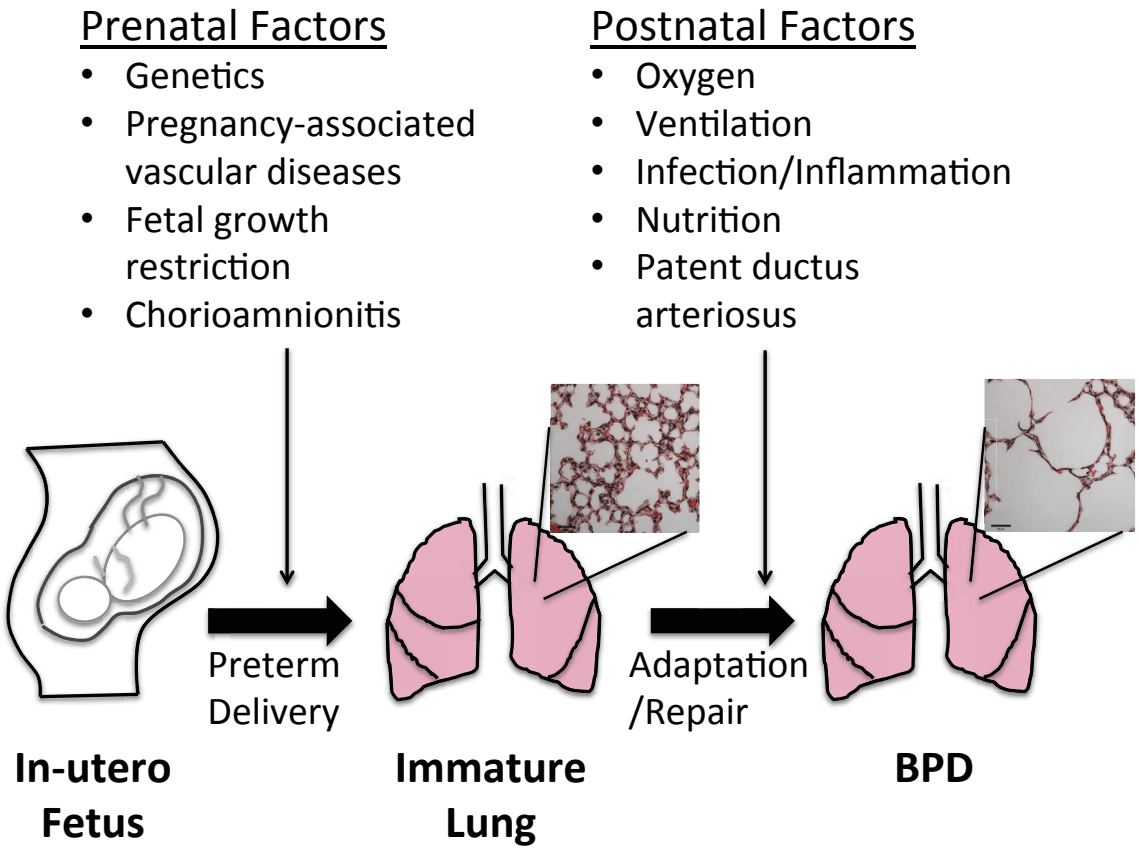
Pathogenesis of BPD

BPD has been difficult to precisely define as the clinical and pathologic characteristics of the disease have evolved since the initial description in 1967, mainly because of the increased survival of extremely preterm infants. The classic BPD described by Northway *et al.* typically occurred in infants greater than 30 weeks gestational age. Currently, BPD primarily affects infants less than 28 weeks gestational age who are born with immature lungs in the late canalicular and early saccular phases of development.[10] In the pre-surfactant era, classic BPD findings were airway injury, inflammation, muscular hypertrophy and parenchymal fibrosis. The histologic hallmarks of the "new" BPD are impaired alveolar and vascular development with minimal fibrosis and muscular hypertrophy. Impaired alveolar development results in fewer and larger alveoli[10, 11] while impaired vasculogenesis

in the immature lung results in a smaller vascular bed, with increased vascular tone and reactivity.[12]

BPD is a multifactorial disease process created by the synergistic combination of multiple clinical interventions and individual pre/postnatal factors superimposed on the immature lung (Figure 1-1). Supplemental oxygen and positive pressure ventilation have been recognized as significant, often unavoidable, risk factors for the development of BPD since Northway's seminal publication.[13]

Figure 1-1. Schematic representation of recognized prenatal and postnatal factors associated with the development of BPD.



In addition to inducing inflammation in the immature lung, oxygen exposure and mechanical stretch disrupt the extracellular matrix creating subsequent alterations in growth factor signaling and in endothelial and epithelial cells of the lung. These alterations contribute to the pathogenesis of BPD.[13] Additional post-natal factors associated with BPD include sepsis and increased pulmonary blood flow/patent ductus arteriosus (PDA).[14] Increasingly recognized is the importance of optimal postnatal nutrition in reducing the risk and severity of BPD. Adequate nutrition is necessary to avoid a catabolic state and to support lung growth and function, alveolar development and surfactant production.[15]

Prenatal factors linked to BPD include chorioamnionitis, pregnancy-associated vascular disorders and fetal growth restriction.[16-18] Chorioamnionitis increases the risk of preterm delivery, a crucial component in the development of BPD. However, chorioamnionitis may also induce inflammation in the developing lung and potentially blunt the favorable response to exogenous surfactant, contributing to the risk of BPD.[18] Processes that restrict fetal growth are also likely to be detrimental to fetal lung growth and maturation. Fetal growth restriction, associated with reduced oxygen and substrate supply to the fetus, may create a relatively hypoxic environment impairing lung development.[16] Pregnancy-associated vascular disorders can relate to an imbalance in pro- and anti-angiogenic factors and this imbalance may alter normal fetal lung angiogenesis, a critical determinant of lung development.[16, 17] A better understanding of the relationship between prenatal factors/fetal growth and the development of BPD is required as

evidence of causality as opposed to correlation is often lacking in clinical studies.[16, 18]

The hypothesis that BPD risk is influenced by genetic susceptibility is supported by studies comparing concordance rates in monozygotic and dizygotic twins. The observed concordance for BPD among monozygotic twins is significantly greater than anticipated. After adjusting for major covariates and depending on the definition of BPD employed, genetic factors accounted for 52% to 82% of the variance in liability for BPD.[19, 20] The recognition that BPD is, in part, a genetic disorder challenges previous paradigms related to pathophysiology and opens new, much needed, research avenues to advance our understanding of BPD.[19] The alterations in lung structure and function created by the interaction of recognized and unrecognized determinants of the "new" BPD may have long-lasting health implications for infants afflicted with BPD.

Outcomes of BPD

BPD has been associated with multiple health consequence including increased mortality, cerebral palsy, cognitive delay[21, 22] and growth failure.[23] BPD is also recognized as an independent predictor of poor neurodevelopmental outcome.[23] Pulmonary hypertension is a frequently occurring comorbidity, present in 25-40% of infants with established BPD. Increased mortality and morbidity is seen in infants with BPD associated pulmonary hypertension.[12, 24]

Long-term respiratory complications continue to be exhibited by infants with BPD, despite the apparently less severe lung pathology of the "new" BPD.[25] As many

as 50% of infants with BPD will be rehospitalized prior to 2 years of age and respiratory illness is the most frequent reason for readmission. Premature infants are also more likely to develop severe symptoms secondary to respiratory infection. They represent up to 30% of patients under 2 years of age admitted to the pediatric intensive care unit for respiratory illness.[26] Premature infants are significantly more likely to have chronic respiratory symptoms, receive asthma medications and have abnormal lung function on pulmonary function testing at school age, when compared to preterm infants without BPD.[27, 28] Welsh and colleagues examined exercise capacity and ventilation characteristics in school age children who were born at less than 25 weeks gestational age. In the study population, 71% of the children had a prior diagnosis of BPD. Children born extremely premature demonstrated significantly lower Z-scores for forced expiratory volume in 1 second, gas transfer and reduced peak oxygen consumption compared with controls. No differences in physical activity were noted, but children born extremely premature demonstrated higher breathing frequencies and lower tidal volumes during peak exercise.[29] Clemm *et al.* reported reduced maximal oxygen consumption, shorter exercise distance and reduced maximal heart rate in children and adolescents born at less than 28 weeks gestational age.[30] Even more concerning are reports of early onset emphysema in young adults (17-33 years of age) who were born preterm and diagnosed with moderate-to-severe BPD.[31] However, new imaging techniques estimating alveolar size indicate that children (10-14 years of age) born preterm and diagnosed with BPD have similar alveolar dimensions compared with children born at term. This suggests that preterm survivors with BPD may be able to

compensate for early abnormal alveolar development with continued late alveolarization.[32] Long-term follow-up of these infants into adulthood is crucial because of the potential risk of early decline in lung structure and function.[33]

Epidemiology of BPD

Remarkable advances have been made in the field of neonatology over the past few decades, resulting in the improved survival of premature infants. Unfortunately, this has not translated into a reduction in the overall incidence of BPD, but it has altered the affected population as infants diagnosed with BPD are becoming increasingly more premature. In Northway's era, the average gestational age of the infants diagnosed with BPD was 32 weeks.[4] BPD is now a disease primarily affecting infants born extremely premature.[10]

The reported incidence of BPD in infants born at less than 28 weeks gestational age varies between 35 and 50%.[2, 14, 23, 34] The incidence increases with earlier gestational age. Shah *et al.* completed an observational retrospective comparison of neonatal outcomes between 1996-1997 and 2006-2007 from fifteen Canadian centers. Infants in the later cohort were more likely to receive antenatal steroids and had a lower acuity of illness on admission. Results adjusted for major confounding factors revealed an increase in BPD in the later cohort (odds ratio (OR): 1.88; 95% confidence interval (CI): 1.60 to 2.20).[34]

Fanaroff *et al.*, using data from the National Institute of Child Health and Human Development Neonatal Research Network, compared outcomes during three separate time frames over a 15-year period (1987-2000). They reported no

difference in survival free of major morbidity or BPD alone.[35] Payne *et al.* analyzed outcomes from a 9-year (1998-2006) quality improvement project occurring in eight centers in the USA and survival free of BPD remained unchanged, but the rate of BPD increased (OR: 1.3; 95% CI: 1.1 to 1.6).[36] Tommiska *et al.* reported outcomes from a nationwide study in Finland over a 5-year period (1996-2000) and also found no change in the incidence of BPD.[37]

These findings are concerning as the perception of improved outcomes with increased survival appears misleading. In recent years, new strategies have been implemented and the rate at which these strategies are adopted in neonatal intensive care units varies. The multifactorial and differential nature of BPD makes it difficult to effectively treat with a single therapeutic modality.[38]

Conclusion

BPD currently is, and in coming years will remain, a significant problem facing infants born extremely premature. In order to accurately assess the incidence of BPD, and benefits provided by therapies, a single best definition of the disease must be identified and used. The most clinically useful and precise definition is the NIH severity-based criteria with the addition of a physiologic test for infants on less than 30% oxygen at 36 weeks CGA. Widespread use of these criteria would reduce the observed variance in the rates of BPD.

There are likely multiple variants of BPD resulting from alterations in the degree of lung immaturity, timing and types of injury and ante- and postnatal factors/interventions the premature lung is exposed to. Genetic factors are

recognized as influencing the response of the lung to these exposures and additionally complicate the understanding of the pathogenesis of BPD. With continued advances in perinatal medicine, survival of infants at the limit of viability will continue to improve and this may perpetuate the current trend toward an increasing or unchanged incidence of BPD. The multifactorial and evolving nature of BPD makes finding one single therapy that will dramatically reduce its incidence unlikely.

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Chapter 2:

Current and Investigational Therapies for Bronchopulmonary Dysplasia

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Currently, there are limited therapeutic options to treat BPD and existing therapies offer limited benefit or are accompanied by undesirable side effects. Pharmacologic approaches presently employed in the management of BPD include methylxanthines, corticosteroids, and vitamin A supplementation. Supportive therapies, including the increased use of non-invasive ventilation and careful oxygen delivery, strive to reduce injury inflicted on the developing lung. New ventilation strategies and the non-invasive administration of surfactant are currently being investigated as techniques for the prevention of BPD.

Current therapeutic strategies

Antenatal corticosteroid therapy and surfactant have improved outcomes of preterm infants with respiratory distress syndrome[1] and altered the course of BPD such that it is now infrequently seen in more mature infants.[2] Therapeutic strategies for the high-risk extremely premature population that have demonstrated modest improvements in BPD include methylxanthines, postnatal systemic corticosteroids, non-invasive ventilation and careful oxygen delivery. Inhaled nitric oxide (iNO) has recently received considerable attention as a possible therapy for BPD but multiple trials do not support its use for the prevention or treatment of BPD.

Methylxanthines

Methylxanthines are potent central nervous system stimulants that have been used for more than 30 years to treat apnea of prematurity. Methylxanthines include aminophylline, theophylline, and caffeine. Caffeine is the preferred

medication as it has a wider therapeutic range, less frequent dosing regime and monitoring of therapeutic levels is not required. Caffeine has been shown to increase minute ventilation, CO_2 sensitivity, neural respiratory drive and diaphragmatic contractility.[3] Methylxanthines increase the likelihood of successful extubation of premature infants.[4] The "caffeine for apnea of prematurity" (CAP) trial had a primary outcome of death or neurodevelopmental disability at a corrected age of 18-21 months and incidence of BPD as a secondary outcome. This trial has provided evidence for the beneficial effects of caffeine on BPD. The CAP trial randomized infants, during the first 10 days of life, with a birth weight of 500-1250 g to receive caffeine or placebo. Positive airway pressure was discontinued 1 week earlier and rates of BPD were reduced in infants receiving caffeine compared with infants receiving placebo (36% vs 47%; adjusted OR: 0.63; 95% CI: 0.52 to 0.76).[5] A *post-hoc* analysis of the CAP trial suggested that earlier initiation of caffeine might be associated with greater benefits.[6] Caffeine therapy has demonstrated to be safe in preterm infants, with 5-year follow-up indicating no differences in death or neurodevelopmental disability.[7]

Caffeine is a standard of care for the treatment of apnea of prematurity and demonstrates beneficial effects on reducing the incidence of BPD. These benefits are presumed to be secondary to a reduction in the duration of invasive and non-invasive ventilation. Currently, animal studies provide conflicting evidence regarding the effect of caffeine on pulmonary inflammation and alveolar development.[8, 9]

Systemic Corticosteroids

Inflammation is a key contributor to the development of BPD. Corticosteroids possess anti-inflammatory effects and have been extensively investigated as a therapy for BPD. Unfortunately, many of the studies performed are of poor methodological quality or have been terminated early limiting the reliability of the reported results.[10]

Multiple studies have assessed early (≤ 7 days) postnatal dexamethasone therapy in preterm infants and demonstrated a significant reduction in the incidence of BPD.[1, 11] Dexamethasone therapy may also confer benefits in pulmonary function assessed at school age.[12] Important short-term adverse effects of dexamethasone include gastrointestinal bleeding, intestinal perforation, hypertension and hyperglycemia. Of greater concern is the reported increased risk of adverse neurodevelopmental outcomes.[1, 11] Follow-up at school age demonstrates significantly poorer motor skills, motor coordination and lower full IQ scores in children treated with dexamethasone compared with controls.[13] Preterm infants treated with early dexamethasone also have a higher prevalence of cerebral palsy.[1,14] Current evidence indicates that early dexamethasone therapy has unacceptable long-term consequence and should not be used in the prevention of BPD.[15]

A Cochrane meta-analysis of late (> 7 days) dexamethasone therapy included nineteen randomized controlled trials (RCTs) with a total of 1345 patients. Beneficial effects observed included reduced extubation failure, reduced incidence of BPD and a reduced mortality at 28 days. Late dexamethasone therapy

demonstrated fewer long-term complications compared to early therapy. No significant difference in the combined outcome of death or cerebral palsy was found. A trend to increased cerebral palsy or abnormal neurologic exam was noted.[16] Insufficient evidence exists to make recommendations regarding late [15] or low-dose dexamethasone therapy for BPD.[17]

The adverse effects of dexamethasone have lead researchers to investigate alternate corticosteroids including betamethasone,[18] methylprednisone[19] and hydrocortisone. Hydrocortisone is the most extensively investigated alternative corticosteroid for the treatment or prevention of BPD. It is biologically different from dexamethasone. It holds both mineralocorticoid and glucocorticoid properties.[20, 21] Hydrocortisone is identical to endogenous cortisol[20] and can be metabolized by 11β-hydroxysteroiddehydrogenase-2 to an inactive form. Dexamethasone is a synthetic glucocorticoid with no equivalent enzyme.[20, 21] These differences may be of significance as hydrocortisone has demonstrated less adverse neurologic effects when compared to dexamethasone.[22]

Doyle and colleagues conducted a meta-analysis of eight RCTs including a total of 880 infants treated with early low dose hydrocortisone for prevention of BPD. No effect was found on the rate of BPD or death; increased gastrointestinal perforation was noted in hydrocortisone treated infants.[23] A recent pilot RCT of late low-dose hydrocortisone (cumulative dose 17 mg/kg) in ventilator-dependent extremely low birth weight infants demonstrated no effect on survival without severe BPD, days on positive pressure or days on supplemental oxygen. As a marker of neurologic effects, brain volumes at 38 weeks CGA were assessed and did not

significantly differ between treated and placebo groups.[20] Watterberg *et al.* performed a multicenter RCT examining the effect of early low-dose hydrocortisone on infants < 1000 g with a specified particular interest in infants exposed to chorioamnionitis. Survival without BPD was not improved in the total study population, but the portion of infants exposed to histologic chorioamnionitis demonstrated a significant decrease in mortality and increased survival without BPD. The study was terminated early due to increased gastrointestinal perforation in the hydrocortisone group.[24] Higher dose hydrocortisone regimes are under investigation for effectiveness in preventing BPD.[25] Kersbergen *et al.* retrospectively assessed the adverse neurologic effect of hydrocortisone initiated at postnatal age ≥ 7 days with a 22-day dosing scheme resulting in a cumulative dose of 72.5 mg/kg. They found no reduction in brain tissue or cerebellar volumes at term-equivalent age in infants who received hydrocortisone for treatment of BPD.[21] There is currently insufficient evidence to recommend the use of hydrocortisone as a therapy for BPD.[15, 17]

Non-invasive Ventilation

Mechanical ventilation and associated volu- and barotrauma have been implicated in the pathogenesis of BPD since Northway's initial description.[26] As no effective pharmacologic therapies are available for BPD, modifications to interventions such as mechanical ventilation have received considerable interest. More recently, one approach has been the increased use of non-invasive ventilation including nasal continuous positive airway pressure (NCPAP) and nasal intermittent

positive pressure ventilation (NIPPV). NCPAP provides a set continuous distending pressure while NIPPV superimposes an intermittent elevation in pressure on NCPAP at a specified rate.[27]

A recent meta-analysis compared non-invasive versus invasive respiratory support at birth in preterm infants born 24-30 weeks gestational age. The four RCTs included were the continuous positive airway pressure or intubation (COIN) at birth trial,[28] the surfactant, positive pressure and oxygenation randomized trial (SUPPORT),[29] the prophylactic or early selective surfactant combined with NCPAP (CURPAP) in very preterm infants trial[30] and the Delivery Room Management Trial.[31] A total of 2782 infants were included and NCPAP demonstrated a significant benefit in the combined outcome of BPD or death, or both (relative risk (RR): 0.90; 95% CI: 0.83 to 0.98). NCPAP conferred a borderline significant reduction in BPD alone (RR: 0.91, 95% CI: 0.81 to 1.01).[32] Individual studies have demonstrated an increased incidence of pneumothorax with non-invasive ventilation,[28] this meta-analysis found no significant increase in pneumothorax with non-invasive ventilation (RR: 1.26; 95% CI: 0.51 to 3.09).[32] Limitations to generalizing the results of the meta-analysis include variations in study design, lack of blinding to the intervention and limited enrollment of infants born less than 25 weeks gestational age, the population at greatest risk of developing BPD. Additionally, the requirement for antenatal consent preselects for more stable pregnancies.[32]

Concern regarding the potential delay in surfactant therapy with initial non-invasive support lead to early studies examining prophylactic surfactant and early extubation versus selective surfactant therapy. A meta-analysis of these studies

demonstrated reduced BPD and air leaks with prophylactic surfactant.[33] These may no longer be valid conclusions; a recent Cochrane meta-analysis comparing prophylactic surfactant with selective surfactant therapy demonstrated an increased risk of BPD with the use of prophylactic surfactant (RR: 1.13; 95% CI: 1.00 to 1.28) and an increased risk of the combined outcome of BPD or death (RR: 1.13; 95% CI: 1.02 to 1.25).[34]

The use of NCPAP at birth to prevent intubation has a failure rate of 45-80% in extremely premature infants.[28, 29] This has resulted in increased interest in NIPPV. In a meta-analysis of fourteen RCTs NIPPV compared with NCPAP reduced the incidence of intubation (OR: 0.44; 95% CI: 0.31 to 0.63), increased the successful rate of extubation (OR: 0.15; 95% CI: 0.08 to 0.31) and had a better outcome indicated by decreased death and/or BPD (OR: 0.57; 95% CI: 0.3 to 0.88). Participants were preterm and term requiring support for apnea of prematurity or neonatal respiratory distress syndrome.[35] A recent RCT by Kirpalani *et al.* compared NIPPV or NCPAP at the time of first use of non-invasive support in 1009 infants with a birth weight < 1000 g. No significant difference was identified for the primary outcome of death before 36 weeks CGA or BPD indicating that NIPPV may not be superior to NCPAP at reducing the risk of BPD.[27]

Careful Oxygen Delivery

In recent years, a heightened awareness of the potential toxicity of oxygen has developed and recognition of the role oxidative stress plays in neonatal diseases, such as BPD, has increased. This has resulted in a more cautious approach

to the use of supplemental oxygen in preterm neonates.[36] Delivery room resuscitation of infants with room air compared with 100% oxygen reduces the risk of neonatal mortality.[37] Additionally, two recent RCTs have demonstrated a reduction in biomarkers of oxidative stress and a reduced incidence of BPD in infants resuscitated with lower initial oxygen concentrations (21-30%).[38, 39] The ideal post-natal oxygen saturation range that should be targeted for extremely preterm infants is unknown. A recent meta-analysis by Saugstad and Aune compared the effects of targeting a low (85-89%) versus high (91-95%) oxygen saturation range in infants born less than 28 weeks gestational age. The meta-analysis included five RCTs: SUPPORT, the three benefits of oxygen saturation targeting (BOOST) II studies and the Canadian oxygen trial (COT). A total of 4,911 infants were included and no difference was identified in the incidence of BPD (RR: 0.95; 95% CI: 0.86 to 1.04). However, they did demonstrate an increased risk of mortality (RR: 1.41; 95% CI: 1.14 to 1. 74) and necrotizing enterocolitis (RR: 1.25; 95% CI: 1.05 to 1.49) in the low-targeted oxygen saturation group.[36] The most recent European consensus guidelines recommend an initial oxygen concentration of 21-30% for delivery room resuscitation and targeting a post-natal oxygen saturation range of 90-95% in preterm infants.[40]

Inhaled Nitric Oxide

Endogenous nitric oxide (NO) is a biological signaling molecule generated in endothelial cells of the lung from L-arginine by NO synthases (NOS). NO plays an intimate role in regulating lung vascular tone.[41] In vascular smooth muscle cells, NO

increases the production of a critical second messenger responsible for smooth muscle relaxation resulting in pulmonary vasodilation.[42] NO has also been implicated in fetal lung liquid production, lung compliance and opposition of bronchoconstriction.[43] Inhaled NO is a selective pulmonary vasodilator currently approved for use in term infants with pulmonary hypertension.[44] Premature birth and respiratory failure have been associated with reduced NOS activity and NO in animal models.[43] In animal studies iNO reduced lung inflammation, improved surfactant function and enhanced lung growth.[45, 46]

Preclinical studies have supported the role for iNO in the management of BPD and prompted fourteen RCTs in premature infants.[45] The study populations and initiation, dosage and duration of iNO therapy are variable between the trials producing ambiguous results.[44] A 2010 Cochrane review analyzed the trials in three different *post-hoc* groupings depending on entry criteria. Data from nine trials of early rescue treatment based on oxygenation criteria demonstrated no significant effect of iNO on mortality or BPD (RR: 0.94; 95% CI: 0.87 to 1.01). Routine early use of iNO in intubated infants was assessed in three studies. No significant reduction in death or BPD was identified (RR: 0.93; 95% CI: 0.86 to 1.01). Data from two trials on late iNO treatment for infants at increased risk of BPD demonstrated no significant benefit on BPD or mortality (RR: 0.90; 95% CI: 0.80 to 1.02).[47]

Donohue *et al.* performed an analysis of the fourteen RCTs with the addition of seven follow-up studies and one observational study. They looked at the effect of iNO on both short- and long-term outcomes. The authors found a small difference in favor of iNO for the composite outcome of death or BPD (RR: 0.93; 95% CI: 0.87 to

0.99) but no reduction in BPD alone (RR: 0.93; 95% CI: 0.86 to 1.003). There was no evidence to suggest a difference in the incidence of cerebral palsy (RR: 1.36; 95% CI: 0.88 to 2.10), neurodevelopmental impairment (RR: 0.91; 95% CI: 0.77 to 1.12) or cognitive impairment (RR: 0.72; 95% CI: 0.35 to 1.45).[48]

A NIH consensus statement on iNO therapy for premature infants concluded that the available body of evidence does not support iNO use in the care of premature infants. Future research directions were identified including clinical trials to investigate the effects of treatment-related variables and differences in subpopulations of respiratory failure.[49] Additional trials investigating the use of iNO and non-invasive iNO on prevention of BPD in premature neonates are ongoing.[50,51] Current evidence does not support the use of iNO for the prevention or treatment of BPD in premature infants.

Investigational therapeutic strategies

The limited and inconsistent effects of current therapies for BPD have made BPD an area of active interest. Extensive research into possible therapeutic modalities for BPD is ongoing. This includes research exploring supplemental vitamin A as a potential therapy, the non-invasive delivery of surfactant and using new ventilation strategies targeted at preventing BPD.

Vitamin A

Vitamin A refers to a group of fat-soluble compounds including retinol, retinaldehyde and retinoic acid.[52] Vitamin A is involved in maintaining the integrity

of the epithelial cells of the respiratory tract.[53] In the fetal lung, it is required for cellular differentiation and surfactant synthesis. A recent animal study demonstrated the benefit of combination vitamin A and retinoic acid therapy in a hyperoxia mouse model of lung injury. Mice treated with vitamin A and retinoic acid were found to have attenuation of the hyperoxia-induced alveolar simplification and abnormal lung function.[54]

Preterm infants have low plasma concentrations of vitamin A and retinol binding protein.[52] A blinded RCT by Tyson *et al.* studied intramuscular (IM) vitamin A at a dose of 5000 international units (IU) three-times weekly for 4 weeks in 807 infants with a birth weight < 1000 g. Infants treated with vitamin A were at reduced risk of BPD or death compared to controls (55% vs 62%; RR: 0.89; 95% CI: 0.80 to 0.99).[55] Long-term follow-up of the same population to 18–22 months of age found no reduction in pulmonary morbidity after discharge. Pulmonary morbidity included the use of pulmonary medications, home oxygen and rehospitalization as recalled by caregiver. However, this study was not adequately powered for follow-up outcomes.[56] Moreira *et al.* conducted a retrospective cohort study in infants with a birth weight ≤ 1000 g receiving early prophylactic surfactant followed by NCPAP. The addition of 12 IM injections of vitamin A to routine care was associated with an 11% decrease in the incidence of BPD.[57] A Cochrane review analyzing nine RCTs of infants with birth weights ≤ 1500 g determined vitamin A to be beneficial in reducing BPD defined as oxygen need at 36 weeks CGA (RR: 0.87; 95% CI: 0.77 to 0.98). No differences were noted in neurodevelopmental outcome at 18–22 months.[53]

Despite the reported benefit of vitamin A, it is not routinely used in the care of extremely low birth weight neonates.[52] Additional guidance regarding this therapy will be provided by the proposed multicenter, international RCT termed "NeoVitaA" investigating oral administration of vitamin A in infants with birth weights ≤ 1000 g. NeoVitaA randomizes infants to receive either vitamin A 5000 IU/kg/day for 28 days or placebo. All infants will receive basic vitamin A supplementation at 1000 IU/kg/day. The primary combined endpoint of BPD or death will be assessed at 36 weeks CGA.[58]

Non-invasive Surfactant Administration

Surfactant administration traditionally requires intubation and positive pressure ventilation, interventions known to increase the risk of BPD. A non-invasive method of delivering surfactant would obviate the need for these interventions in the context of surfactant administration. A recent Cochrane analysis assessing nebulized administration of surfactant (Curosurf) identified one eligible randomized trial by Berggren *et al.* of 32 premature infants.[59] Infants with respiratory distress syndrome requiring continuous positive airway pressure were randomized to receive Curosurf or to serve as controls receiving no nebulized medication. No benefits of nebulized surfactant were identified.[60] Dargaville and colleagues have investigated the effectiveness of minimally invasive surfactant therapy (MIST) that delivers surfactant using a small catheter inserted into the trachea under direct laryngoscopy. The study included 61 infants < 32 weeks GA and compared infants receiving MIST with historical controls. MIST was associated

with fewer days of oxygen therapy, but no significant difference in the incidence of BPD was found.[61] Adequately powered multicenter studies investigating non-invasive methods of surfactant delivery are required to clarify potential short- and long-term benefits.

New Ventilation Strategies

Ventilator-induced lung injury is known to play a significant role in the development of BPD. New techniques targeted at reducing the injury created by positive pressure are under investigation. Sustained inflation (SI) is the use of a prolonged inspiratory pressure to establish functional residual capacity in the lung. SI has been investigated as a lung protective strategy starting immediately after birth. In a cohort of preterm infants SI followed by positive end expiratory pressure (PEEP) was compared to standard delivery room management without SI. SI followed by PEEP was associated with a lower occurrence of BPD in survivors.[62] Evidence from animal studies has supported the role of SI in cardiorespiratory transition. However, a recent study in fetal sheep demonstrated that SI prior to ventilation did not reduce acute-phase and pro-inflammatory responses.[63] Additional studies in preterm infants are required and RCTs comparing SI followed by NCPAP in the delivery room with NCPAP alone[64] and with intermittent positive pressure ventilation are ongoing.[65]

Non-invasive ventilation strategies to reduce lung injury and the incidence of BPD have been adopted, but many extremely preterm infants still require intubation and ventilation. Non-invasive high frequency ventilation (HFV) has been

proposed as a gentler strategy to improve carbon dioxide clearance and minimize the need for intubation and ventilation. A small pilot study investigating the effectiveness of non-invasive HFV in stable preterm infants demonstrated a significant reduction in pCO2 following 2 hours of non-invasive HFV.[66] Very limited evidence is currently available for non-invasive HFV and the impact it may have on BPD is unknown. Studies investigating the effectiveness and tolerance of non-invasive HFV in neonates are planned or ongoing.[67, 68]

Conclusion

The multifactorial nature of BPD makes the search for therapies and even the assessment of the effectiveness of current therapies very challenging. Strategies to prevent or treat BPD remain limited and the incidence of BPD is at best stable. Caffeine and vitamin A are currently available therapies that provide modest reductions in the incidence of BPD. Similarly, non-invasive ventilatory support with selective surfactant therapy and careful oxygen delivery, confer limited benefits. Dexamethasone is the only identified effective therapy for BPD and may be an appropriate option for selected ventilator-dependent infants. However, the side effect profile significantly limits its use and makes it an unacceptable option for many patients. Physicians and researchers are continuing to refine the use of current preventative and therapeutic strategies, but new effective therapies for BPD are greatly needed.

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Chapter 3:

The Potential of Mesenchymal Stromal Cell-Based Therapies for Bronchopulmonary Dysplasia

Stem cell research and literature has expanded at a rapid rate over the past decade with a variety of cell types undergoing exploration for therapeutic benefit. Stem cell-based therapies have demonstrated benefit in adult diseases and are now actively under investigation as a potential therapy for BPD.[1] BPD is one of the most common and serious complications of extreme premature birth. Survivors of preterm birth with BPD are at increased risk for long-term neurodevelopmental and pulmonary morbidity. Innovative strategies are needed, and stem cell-based therapies represent a promising and novel approach to treating BPD.

Understanding Stem Cells

Stem cells are undifferentiated cells that have the capacity to differentiate into a variety of cell types. They are capable of self-renewal, signifying that they are able to divide multiple times while maintaining an undifferentiated state.[2] Stem cells can be generally divided into embryonic and adult stem cells, or classified according to potency or differentiation potential. Embryonic stems cells are exclusively derived from the fertilized egg or blastocyst, while all other stems cells are termed adult stem cells and can be derived from a variety of tissues including the umbilical cord, bone marrow, liver and retina.[3] In mammals, adult stem cells are involved in the regeneration and/or repair of tissue.

Stem cells classified by differential potential are divided into four general categories: totipotent, pluripotent, multipotent and unipotent (Table 3-1). Unipotent stem cells differ from non-stem cells in that they have the ability to regenerate.[2, 3]

Table 3-1. Classification of stem cells by differential potential.[2, 3]

Cell Type	**Differentiation Potential**
Totipotent	Capable of forming mesoderm, endoderm, ectoderm, germ cells and extra-embryonic tissues. *Example*: Zygote
Pluripotent	Capable of forming mesoderm, endoderm, ectoderm and germ cells. *Example*: Embryonic stem cells from inner blastocyst
Multipotent	Capable of forming multiple related cell types. *Example*: Hematopoietic stem cells
Unipotent	Capable of forming only one defined cell type. *Example*: Spermatogonial stem cells

Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are multipotent adult cells that have the capability of differentiating into multiple mesenchymal cell types and possess significant ability for expansion. The International Society for Cellular Therapy identifies the minimal criteria to define a human multipotent MSC as:

- plastic-adherent in standard culture conditions
- ≥ 95% of the population must express surface antigens CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR.
- *in-vitro* must differentiate to osteoblasts, adipocytes and chondroblasts [4]

In addition to the above minimal criteria the International Society for Cellular Therapy has recently proposed an immunological characterization for multipotent MSCs for clinical use. Goals of this characterization are in part to quantify the functional potency of MSCs and to create reproducible and comparable data from various sites. The addition of immunologic characterization may aid in validating the use of MSCs for human diseases.[5]

The International Society for Cellular Therapy has also recommended that the above-defined cells be referred to as mesenchymal stromal cells as opposed to mesenchymal stem cells. The purpose of this nomenclature is to recognize that the population of cells commonly isolated may be heterogeneous in nature and not all cells clearly demonstrate the ability to differentiate into multiple specific cell types *in-vivo* and posses the long-term self-renewal expected of a stem cell. Although it is agreed upon that a true mesenchymal stem cell does exist it is preferred that the

term stem be exclusively used for the proportion of cells that meet the criteria for a stem cell.[6]

MSCs have surfaced as a promising therapeutic modality in the field of regenerative medicine. MSCs are recognized as a particularly appealing cell type for therapeutic interventions as they can be isolated from convenient locations, are immunoprivileged and possess innate immunomodulatory/anti-inflammatory properties. They can be isolated from a variety of locations including bone marrow, adipose, placenta and the easily obtained umbilical cord and umbilical cord blood.[7] MSCs isolated from different sites may not be equal in all respects as they show phenotypic heterogeneity varying with site of origin and isolation/culture techniques used.[8]

MSCs are known to be hypo-immunogenic in humans as they do not express co-stimulatory molecules, have low level expression of class I human leukocyte antigens (HLA) and no expression of HLA class II antigens. When considering transplantation of MSCs in humans this may be of significant benefit as there is the potential to use unmatched donor MSCs.[7] *In-vitro* and animal studies have indicated that the expression of HLA class II antigens and the ability of MSCs to activate the immune response may vary with the degree of inflammation present in an anatomic region.[9, 10] However, caution must be used when applying results of preclinical trials, as variations in MSCs are present between species. Mouse MSCs are known to express HLA class II antigens, which may alter the immunogenicity observed in murine models of disease.[7] The use of autologous cells is often thought to be preferential as immunogenicity concerns are avoided. However, the autologous cells

obtained may be negatively influence by the disease state being treated, resulting in suboptimal cells. Additionally, the time required to culture cells for clinical use precludes the use of autologous cells for urgent therapies.[7] If unmatched hypo-immunogenic MSCs can be used this may be the preferred therapeutic source.

MSCs are known to be immunomodulatory in nature and can inhibit the proliferation, activation and cytokine release of T cells, B cells, natural killer cells and dendritic cells.[7] Dendritic cells are central to the processes of tolerance and immunity. MSCs have been shown to interfere with the maturation and differentiation of dendritic cells resulting in reduced ability to activate T cells and altered cytokine profile that favors the production of anti-inflammatory cytokines such as interleukin-10. The immunomodulatory properties of MSCs have prompted numerous studies investigating the therapeutic potential of these cells in managing autoimmune diseases and graft-versus-host disease in transplant patients.[10]

MSCs have been investigated and used therapeutically for over a decade[7] and are commonly used in clinical trials involving adults. They have demonstrated safety in these trials leading to the recent approval in Canada of MSCs for the treatment of graft-versus-host disease in children.[1] Despite demonstrated safety it is recognized that the use of cell-based therapies is not without its potential adverse effects and long-term risks. Specifically, the risk of tumorigenicity with MSC-based therapies is of concern. Theoretically, tumor formation in MSC treated patients could originate from malignant transformation of the MSCs or secondary to an immunosuppressive effect of the MSC-based therapy. Researchers have reported tumor formation in isolated MSCs, but the formation of such tumors has been linked to contamination of

the MSC culture with malignant cells. Currently, in patients treated with MSCs there is no knowledge of confirmed tumor formation originating from the administered MSCs. Additionally, tumor formation has not been noted in animal models using MSC-based therapies. Another potential side effect relates to the influence of MSC therapy on coagulation. Early results suggest that MSCs are capable of inhibiting systemic intravascular coagulation in animal models of DIC. The effect of MSC therapy on coagulation requires further investigation in the preclinical setting and will need to be monitored in future clinical trials.[8]

The immunomodulatory and anti-inflammatory properties of MSCs identify them as a potentially beneficial therapy for multiple disease processes. Stem cell research initially postulated that cell-cell interactions were essential in creating the therapeutic benefits observed with MSC-based therapies. Recent studies utilizing MSC-conditioned media have demonstrated that the healing properties of MSC are primarily conferred by paracrine mechanisms.[11]

Conditioned Media

Conditioned media is a medium that contains proteins, exosomes and other bioactive molecules that have been secreted by cells cultured in the media for a period of time. It may also be referred to as cell-free culture supernatant or cultured media. Secreted proteins play an essential role in intercellular communication over short distances via the interstitial space or longer distances upon entering the bloodstream or cerebrospinal fluid.[12] These paracrine mediators are known to be critical regulators in the processes of cell growth, differentiation, repair and

angiogenesis.[13] Bioactive molecules are released by cells *in vivo* or *in vitro* through classical or non-classical secretion methods. The classical secretion pathway involves translocation of a synthesized protein into the lumen of the endoplasmic reticulum (ER), transport through the Golgi complex, and release by exocytosis.[12] This classical pathway requires the protein to contain an ER signaling sequence. Proteins secreted by the non-classical pathway lack this signaling sequence and are released by multiple different mechanisms including direct transport through the plasma membrane, shedding of plasma membrane microvesicles, and exocytosis of lysosomes/exosomes.[13]

The term secretome is used to refer to the intricate milieu of molecules that are released from living cells, including those present in conditioned media. The components of a secretome can include serum proteins, hormones, enzymes, cytokines, chemokines, growth factors and angiogenic factors.[12, 14] Approximately 10% of the human genome is dedicated to encoding components of the secretome. Interest in characterizing the secretome of stem/progenitor cells, including mesenchymal stromal cells, has increased exponentially with the hopes of acquiring insight into the mechanisms of their anti-inflammatory, immunomodulatory, reparative and angiogenic properties. Additionally, the realization that conditioned media confers benefits previously thought to be mediated only by a living cell has spurred the search for novel therapies originating in the secretome.[14]

The conditioned media from human umbilical cord MSCs is not extensively characterized. Studies have demonstrated that MSCs obtained from different origins demonstrate variation in their potential for differentiation/proliferation and anti-

inflammatory or reparative properties. Increased knowledge of differing MSC secretome profiles would allow for selection of preferred MSCs for distinct therapeutic purposes. Human MSCs isolated from the Wharton's jelly of the umbilical cord are easily obtained and demonstrate greater cell proliferation compared with MSCs isolated from bone marrow. Similarly, the conditioned media obtained from culturing MSCs of different tissue origin demonstrates different proportions and types of bioactive molecules. Conditioned media of human MSCs isolated from the Wharton's jelly of the umbilical cord contains higher concentrations of chemokines, pro-inflammatory proteins, and growth factors compared to MSC conditioned media from adipose tissue or bone marrow (Table 3-2).[15] The secretion profile of MSCs isolated from the Wharton's jelly of the umbilical cord suggests therapeutic benefits including cell proliferation/survival and anti-inflammatory, anti-fibrotic and pro-angiogenic effects. Low levels of extracellular matrix components and matrix metalloproteinases indicate Wharton's jelly MSCs are less suited to cartilage, bone and skin regeneration therapies.[15, 16]

Table 3-2. Bioactive molecules identified in the conditioned media of human umbilical cord MSCs isolated from Wharton's jelly and quantitative comparison to conditioned media of MSCs isolated from adipose tissue (AT) or bone marrow (BM).[15-17]

Molecule identified in conditioned media of Wharton's jelly MSCs	Quantity in conditioned media from other MSC sources:		Ref
	AT	BM	
Chemokines:			
Eotaxin	↑	Similar	14
Interferon inducible protein-10	↓	↓	14
Monocyte chemoattractant protein (MCP)-1	↓	↓	14
Macrophage inflammatory protein (MIP)-1β	None	None	14
RANTES	↓	↓	14
Anti-inflammatory cytokines:			
Interleukin (IL)-1 receptor antagonist	↓	↓	14
Interferon (IFN)-α	None	None	14
Pro-inflammatory cytokines:			
IL-6	↓	↓	14
IL-7	↑	Similar	14
IL-8	↓	↓	14
IL-12	↑	Similar	14

Growth Factors:			
Granulocyte colony stimulating factor	None	None	14
Hepatocyte growth factor (HGF)	↓	↓	14,15
Transforming growth factor (TGF)-β1	Similar	Similar	14,15
TGF-β2	↓	↓	14,15
Platelet-derived growth factor (PDGF)-AA	↓	↓	14,15
PDGF-AB	---	Similar	15
PDGF receptor-α (Ra)	---	Similar	15
PDGF receptor-β (Rb)	---	Similar	15
Placental growth factor	↑	Similar	14,15
Angiogenic Factors:			
Angiogenin	↑	↓	14
Angiopoietin-1	Similar	Similar	14
Endostatin	↓	↓	14
Acidic fibroblast growth factor	↑	Similar	14,15
Thrombospondin-2	↓	↓	14
Vascular endothelial growth factor (VEGF)	↑	↑	14,15
VEGF-D	---	Similar	15
Extracellular matrix components:			
Collagen I and III	↑	Similar	14
Elastin	Similar	Similar	14

Matrix metalloproteinases:			
Matrix metalloproteinase (MMP)-1	↑	↓	14
MMP-3	↑	Similar	14
Prostaglandin E2	↑	↑	16

MSCs: mesenchymal stromal cells; AT: adipose tissue; BM: bone marrow; Ref: references; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; IL: interleukin; IFN: interferon; HGF: hepatocyte growth factor; TGF: transforming growth factor; PDGF: platelet derived growth factor; VEGF: vascular endothelial growth factor; MMP: matrix metalloproteinase

The Potential of MSC-Based Therapies for BPD

Pre-clinical studies

There is increasing animal and human evidence to support the therapeutic potential of stem/progenitor cells for numerous diseases processes including BPD. Evidence suggests that depletion or dysfunction of these cell populations in the developing lung may contribute to the pathogenesis of BPD.[18, 19] The identified alterations in resident stem/progenitor cell populations indicate that replacement of these cells could provide therapeutic benefits in treating BPD. Of the various stem/progenitor cell therapies, MSCs have been the most frequently studied in relation to BPD.[20] As described, MSCs are particularly appealing for treating neonatal diseases, as they have innate immunomodulatory and regenerative abilities,[21] have demonstrated safety in other populations,[22] and are able to be isolated from umbilical cord blood or Wharton's jelly.[20]

In experimental models of BPD administration of bone marrow-derived MSCs intratracheally, intravenously, or intraperitoneally attenuated lung inflammation, alveolar growth arrest and lung vascular damage.[18, 23-25] In the hyperoxia animal model of BPD, intratracheal delivery of human umbilical cord blood-derived MSCs attenuated hyperoxia induced lung injury and demonstrated a dose dependent effect.[26] Low engraftment rates of MSCs in the lung suggest that the therapeutic benefits are mediated by a paracrine mechanism.[18, 27] These observations are supported by experiments demonstrating that the conditioned media from MSC cultures attenuates lung injury.[18, 28]

In the hyperoxia model of BPD, intravenous mouse bone marrow-derived MSC conditioned media prevented pulmonary hypertension, lung vascular remodeling and alveolar injury.[29] In a hyperoxia model of established BPD, intravenous mouse bone marrow derived-MSC conditioned media reversed pulmonary hypertension, parenchymal fibrosis, normalized lung function and reduced alveolar injury.[11] Intraperitoneal human cord blood-derived MSC conditioned media prevented hyperoxia-induced lung injury, improved lung angiogenesis and demonstrated improved exercise capacity and safety in rats assessed 6 months after treatment.[30] In some studies, benefits conferred by the MSC conditioned media were greater than benefits conferred by the MSCs themselves.[29]

Clinical studies

MSC based therapies for BPD recently crossed the barrier from preclinical to clinical trials with the first published study using MSCs as therapy for BPD in

neonates. Chang *et al* completed a clinical trial investigating the feasibility and safety of MSCs as a therapy for BPD. This open-label phase I dose escalation trial of human umbilical cord blood-derived MSCs included nine premature infants born between 23 and 29 weeks gestational age. Infants 5-14 days of age were eligible for inclusion if they were requiring ventilation with a rate > 12 breaths/minute and supplemental oxygen > 25%. Infants were treated with a single intratracheal allogenic transplantation of human umbilical cord blood MSCs obtained from term infants and used within 24 hours of manufacturing. The first three infants received a low-dose of 1×10^7 cells/kg, no dose-limiting toxicity occurred; therefore, the next six infants received a high-dose of 2×10^7 cells/kg. Dose-limiting toxicity was defined as death within 6 hours of MSC therapy or anaphylactic shock related to the MSC therapy.[31]

Adverse outcomes of the treatment group were compared with a cohort of historical case matched infants. The transplantation procedure was well tolerated by all patients. Six infants subsequently developed serious adverse events (SAEs) that were not attributed to the MSC therapy. The SAEs consisted of PDA requiring ligation, pneumothorax related to PDA ligation, necrotizing enterocolitis requiring surgery, periventricular leukomalacia and retinopathy of prematurity ≥ stage 3. There were no significant differences in the frequency of SAEs between MSC treated infants and the historical controls, with the exception of a reduction in the severity of BPD in treated infants. Interestingly, the group of infants treated with high-dose MSCs appeared to have a longer duration of ventilation, although not statistically significant, when compared to the low-dose group.

The primary objective of this study was to examine the safety and feasibility of MSC therapy for BPD in high-risk premature neonates; conclusions about the effect of this therapy on the severity of BPD cannot be made.[31] Although the stated study population included premature neonates at high-risk of BPD, the ventilation criteria specified could be considered conservative in many neonatal intensive care units, potentially reflecting a lower-risk population. This likely would not alter the primary safety/feasibility outcome but may skew the risk-benefit ratio of an investigational therapy in premature neonates.

The same investigators are proceeding with a phase II randomized, double-blinded, multicenter, controlled trial using an intratracheal transplantation of human umbilical cord blood-derived MSCs at the low-dose (1×10^7 cells/kg) for treatment of BPD in premature infants. A sample size of seventy infants is targeted for the primary outcome of moderate to severe BPD or mortality at 36 weeks CGA.[32] Long-term follow-up studies of infants enrolled in both the phase I and phase II trials are planned. Infants in the phase I trial will be followed to 21 +/- 3 months CGA, while infants in the phase II trial will be followed to 60 months CGA.[33, 34]

Conclusion

There is a growing body of evidence from pre-clinical and *in-vitro* studies to suggest that MSC-based therapies may be effective in preventing BPD. The first clinical trials of MSC therapy for BPD are in their infancy. While results of these trials are anxiously awaited, ongoing research is required to elucidate the mechanism by which MSCs act. Accumulating evidence supports the paracrine

mechanism of MSC-based therapies. MSC conditioned media, or its components, represent an attractive therapy as they obviate the need to deliver live cells to susceptible preterm neonates. Additional, pre-clinical research is needed to support the paracrine theory and the potential future therapeutic role of MSC conditioned media or it's components.

Testing of new therapies is particularly challenging in the vulnerable preterm population. Appropriately, many clinicians and regulating agencies are hesitant to permit clinical trials of novel therapies in neonates prior to rigorous and extensive pre-clinical testing. This creates a need for clinically relevant, cost effective, animals models of BPD through which new therapies and the mechanisms by which they act can be investigated.

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Chapter 4:

Establishment of a Clinically Relevant Mouse Model of Bronchopulmonary Dysplasia

The prevalence and continued paucity of effective therapies for BPD have spurred extensive research pertaining to BPD pathogenesis, prevention, and treatment. BPD research is primarily conducted using animal models, as pathologic specimens from infants with BPD are infrequently obtained; in part due to improved survival of premature infants. Numerous animal models of BPD have been established to simulate hallmarks of the disease.[1] Our objective was to develop a more clinically relevant model of BPD in neonatal mice that incorporates the multifactorial nature of the disease.

Established animal models of BPD

Small animal models

Rodent models of BPD are particularly common, in part due to relatively low cost, short/reliable gestational duration (18 days), easy breeding, availability of strains, and availability of immunologic reagents.[2] Mice are well suited for investigation of respiratory diseases affecting preterm infants as they have structurally immature lungs at the time of birth. The newborn mouse lung is in the early saccular stage of lung development, lacking true alveoli and possessing only primary saccules.[3] Similarly, infants born between 24 and 28 weeks gestational age are typically in the early saccular stage of lung development.[4] Postnatal mouse lung development including septation, rapid alveolarization and angiogenesis occur primarily between 4 and 14 days of postnatal life.[3] Despite structural similarities to the human preterm lung, mice born at term do not require respiratory assistance secondary to the presence of mature surfactant and an intact respiratory drive.[5]

Many of the common rodent models employ excess oxygen and/or mechanical stretch to create the characteristic BPD findings of alveolar simplification and disrupted vascular development.[1]

Hyperoxia models

High-inspired oxygen concentrations were recognized as a key factor in the development of BPD as early as Northway's original BPD publication in 1967.[6] This knowledge has been exploited to create the most commonly encountered animal model of BPD: the hyperoxia model. Exposure of mice to 100% oxygen for 7 days after birth creates phases of pulmonary injury including pulmonary edema/hemorrhage and reactive mitochondrial changes. If animals survive the initial injury, they progressed to chronic injury and repair, characterized by increased collagen deposition and fibroblast proliferation.[7] Studies performed on newborn rats demonstrated that in addition to lung injury hyperoxia induces architectural lung changes. Rats exposed to > 95% oxygen for 7 days after birth develop abnormally enlarged air spaces and abnormal capillary structure reminiscent of BPD, and these alterations persists to 40 days of age.[8] Continued experiments have demonstrated that even moderate hyperoxia, at 65% inspired oxygen, results in alveolar simplification in newborn mice.[9] The hyperoxia model of BPD is an attractive model as it is simple to execute and standardize, creates reproducible results, and provides the opportunity for long-term follow-up experiments.[1] However, the concentrations of oxygen used are often in excess of

that used in clinical practice and the model incorporates only one of the identified risk factors for BPD.[1, 10]

Mechanical ventilation models

Mechanical ventilation is also recognized as a primary risk factor for the development of BPD in preterm infants and has been incorporated into small animal models. Neonatal mice mechanically ventilated for 24 hours with 40% oxygen demonstrate impaired alveolar septation creating structural defects similar to those observed in BPD. Molecular analysis also revealed reduced lung expression of proteins and genes essential to normal lung development.[11] Mechanical ventilation of immature mice for 24 hours without associated hyperoxia also inhibits alveolar septation and angiogenesis, confirming the independent role of mechanical stretch in experimental lung injury.[12] Models incorporating mechanical ventilation of neonatal rodents can be technically challenging and time limited.

Inflammation models

Pre and postnatal inflammation in preterm infants contributes to the pathogenesis of BPD. Prenatal inflammation/infection in the form of chorioamnionitis complicates up to 70% of preterm deliveries, and represents a risk factor for the development of BPD.[13] Postnatal infection/inflammation, including sepsis and necrotizing enterocolitis, have a clear association with the development of BPD.[14] Lipopolysaccharide (LPS) is a cell wall component in gram-negative bacteria and recognized as a potent inducer of the immune system.[15] In fetal mouse

lung explants, LPS has exhibited the ability to inhibit formation of new saccular airways.[16] Postnatal intratracheal administration of LPS to 6-day-old rats produced lung injury as demonstrated by significant inflammatory cell recruitment, reduced lung volume and reduced alveolar surface area.[17]

Most small animal models of BPD involve the exposure of an animal to one, or possibly two factors, implicated in the pathogenesis of BPD.[10] This is a significant limitation of the most commonly used hyperoxia model of BPD. The multifactorial nature of BPD and the current clinical scenarios encountered in preterm infants are difficult to replicate, particularly in small animal models.[10]

Large animal models

The non-human primate model, utilizing premature baboons, is the most authentic animal model of BPD.[10] The baboon model, developed by Coalson and colleagues,[18] has been updated and modified to incorporate many of the key factors influencing the development of BPD in neonates.[19] Premature baboons are delivered at two-thirds term gestation (corresponds to approximately 27 weeks gestation in humans) by cesarean section, after administration of antenatal glucocorticoids. The baboons are delivered sufficiently preterm to require respiratory support for survival and receive standard therapies including exogenous surfactant, appropriate oxygenation, mechanical ventilation utilizing volume-sparing strategies, placement of arterial and venous lines, systemic antibiotics, parenteral nutrition, and active management of PDA and hypotension. The lungs of immature baboons surviving beyond 1month of age demonstrate histopathologic

features reminiscent of BPD, including alveolar and vascular hypoplasia.[18] Research employing this realistic model of BPD is more suitable for extrapolation to human infants with BPD. The ethical considerations, prolonged duration, and substantial cost involved in this model limit its wide spread utilization.[10]

Creating a clinically relevant mouse model of BPD

The small animal model we developed incorporates the multifactorial nature of BPD, providing the opportunity to investigate potential BPD therapies at a fraction of the cost of large animal models. Three critical contributing factors to the pathogenesis of BPD are inflammation, mechanical ventilation and supplemental oxygen. These factors were integrated into a single *in-vivo* experimental model of BPD in neonatal mice.

Neonatal C57BL/6 mice (Charles River Laboratories) received intraperitoneal LPS and approximately 48 hours later, at postnatal day 9-10, underwent mechanical ventilation for 8 hours with 40% oxygen. The postnatal age of 9-10 days was chosen to ensure adequate weight and maturity of mouse pups, allowing them to tolerate the combined stressors of inflammation/mechanical ventilation and permit the technically challenging surgery. Additionally, mouse pups at this age are in the alveolar stage of lung development, allowing assessment of the effect of this model on alveolarization. The 52 pups successfully completing 8 hours of ventilation weighed 3.44 – 6.19 g at the time of ventilation (Table 4-1) and 65% were male.

Anesthesia and analgesia

This experimental model includes the humane induction of anesthesia with ketamine (Bimeda-MTC Animal Health Inc.) and xylazine (Bayer Animal Health). Ketamine is a dissociative anesthetic agent that provides analgesia, but may be associated with muscle rigidity,[20] tachycardia and increased blood pressure. The detrimental side effects of ketamine are temporized by the addition of xylazine.[20] Xylazine is an α_2-agonist that provides both analgesia, sedation[21] and muscle relaxation, but is associated with bradycardia and hypotension. The combination of ketamine and xylazine is frequently used to induce anesthesia in rodent studies as it reliably creates analgesia, sedation and relaxation.[22] Hypothermia[21] and bradycardia are recognized potential side effects of this anesthetic regime.[21, 22]

Mouse pups received an intraperitoneal injection of the ketamine/xylazine solution prior to surgery at a dose of: ketamine 60 micrograms (mcg)/g and xylazine 12 mcg/g. Once spontaneous movement ceased, animals were placed supine with their limbs and head gently secured to a 4 x 4 gauze pad using tape. Prior to initiating surgery, response to a painful stimulus was assessed by grasping of the neck skin with straight forceps, additional anesthesia was provided as required. Pups were continuously monitored throughout the 8 hours of ventilation and response to tactile stimulation was assessed every 30 minutes to indicate depth of anesthesia (Appendix 1). Additional doses of ketamine/xylazine solution (ratio 1 mcg: 0.2 mcg) were administered as required for agitation (spontaneous movement) or exaggerate response to tactile stimulus. During initial experiments top up anesthetic solution was provided at 10 mcg/g ketamine, but due to the high

number of injections required to maintain adequate anesthesia the top up dose was gradually increased to 15 mcg/g and subsequently 20-25 mcg/g ketamine. This increase in top up anesthetic reduced the number of injections required by each mouse from an average of 7.1 injections per mouse to 6 injections per mouse to 5.6 injections per mouse respectively. On average pups required a total dose of 152.7 mcg/g of ketamine to induce and maintain anesthesia for the duration of ventilation (Table 4-1).

Table 4-1. Weight and anesthetic parameters of all mouse pups ventilated for 8 hours with 40% oxygen after receiving intraperitoneal LPS.

	Minimum	**Maximum**	**Average**
Animal weight	3.44 g	6.19 g	4.92 g
Time from Initial Anesthetic to Start of Ventilation (minutes)	5	30	11.1
Total Anesthetic Dose (mcg/g)	109	229	152.7
Total # of Anesthetic Injections	4	10	6.2

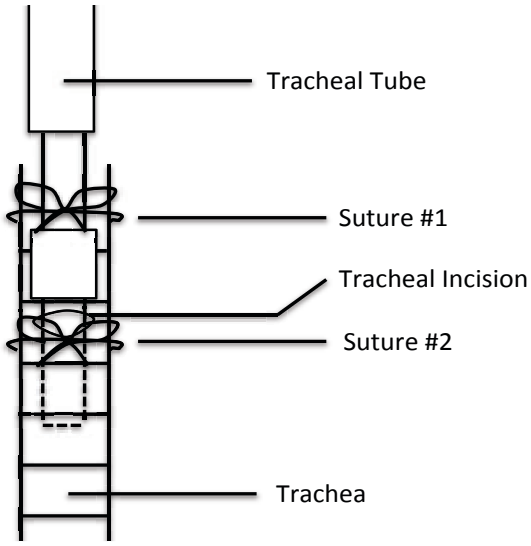
Inflammation

An inflammatory response was induced with a single intraperitoneal injection of LPS (*Escherichia coli* 055:B5, Sigma-Aldrich) at a dose of 4 mcg/g on postnatal day 7-8, corresponding to approximately 48 hours prior to the initiation of mechanical ventilation.

Surgery

Under a dissecting microscope a surgical tracheostomy was created and a custom made tracheal tube was inserted. In detail, using scissors a midline neck incision was initiated and extended dorsally at approximately a 45-degree angle bilaterally and joined at the base to create a triangle. This triangular section of skin was excised to permit adequate exposure. Soft tissues were bluntly dissected/divided to expose the trachea. Muscle tissue surround the trachea was divided with scissors and the trachea was isolated using blunt dissection. Two silk suture threads were threaded underneath the trachea. A small opening was made between cartilage rings of the trachea using a 26-gauge needle. The tracheal tube was inserted and secured with two sutures, one cephalic and one dorsal to the tracheal opening (Figure 4-1). Custom tracheal tubes were made using 24-gauge IV catheters (BD Insyte-W) and tubing.

Figure 4-1. Diagram of custom tracheal tube inserted and secured after surgical tracheostomy.

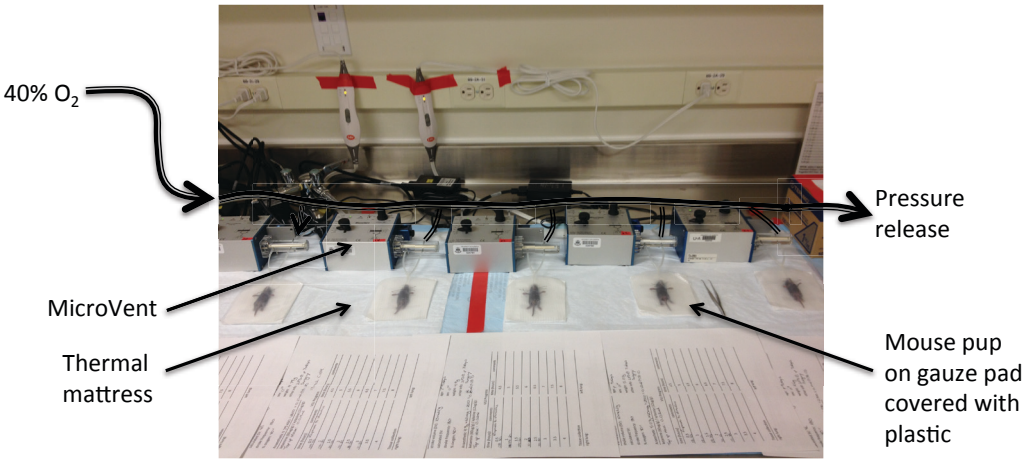


Ventilation

After tracheostomy anesthetized pups received mechanical ventilation for 8 hours at a rate of 180 breaths/minute and a tidal volume of 10 microliters/g using a small animal ventilator (MicroVent Model 848; Hugo Sachs Elektronik - Harvard Apparatus). In mice, these ventilator settings have previously demonstrated the ability to maintain a blood pH and CO_2 within the physiologic range.[11] The ventilator uses a valve-less piston pump to deliver the set volume at the specified rate and has reduced tubing requirements to minimize tidal volume error to < 3 microliters.[23] Positive end expiratory pressure was maintained at atmospheric pressure as studies performed by Bland *et al*. suggest end expiratory pressures > 1 cm H_2O create impaired venous return leading to cyanosis and death.[11] Equipment

for five pups was available; therefore up to 5 pups were able to be ventilated simultaneously (Figure 4-2).

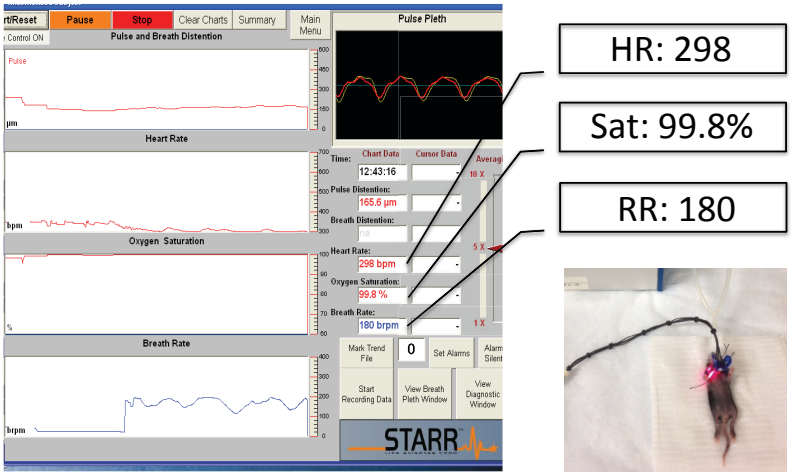
Figure 4-2. Diagram/photo depicting 5 mouse pups undergoing mechanical ventilation with 40% oxygen.



Oxygen

Pups received 40% oxygen for the duration of mechanical ventilation. Modest hyperoxia was chosen to reflect the current cautious approach to supplemental oxygen employed in the clinical setting. Additionally, studies have shown mechanical ventilation with 40% oxygen reproducibly creates lung injury in neonatal mice.[11] The oxygen saturation of a single mouse was assessed using a small animal pulse oximeter with an extra-small throat sensor (MouseOx Plus, STARR Lifescience Corp). After 8 hours of mechanical ventilation with 40% oxygen the pup demonstrated a saturation of 99.8% (Figure 4-3). Oxygen saturations of additional pups were not assessed due to interference of the saturation probe with the surgical site and tracheal tube.

Figure 4-3. Neonatal mouse pup after 8 hours of mechanical ventilation with 40% oxygen maintains oxygen saturation of 99.8%.



Temperature management

Temperature was monitored using a mercury thermometer placed directly adjacent to pups during ventilation. Pups were placed on thermal mattresses and heat was adjusted to maintain ambient temperature between 30-35 °C. During ventilation mice pups were covered with a sheet of parafilm to limit heat and water losses.

Conclusion

Our clinically relevant mouse model of BPD was developed by superimposing three factors – inflammation, mechanical ventilation, supplemental oxygen – on the structurally immature neonatal mouse lung. This experimental model is well suited to the investigation of new therapies for BPD as it is relatively inexpensive

compared to large animal models, uses available equipment, integrates established BPD models and incorporates the multifactorial nature of BPD.

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Chapter 5:

The Paracrine Effect of Mesenchymal Stromal Cells on Multifactorial Lung Injury in Neonatal Mice

Mechanical ventilation and supplemental oxygen are life saving therapies frequently required by infants born extremely premature. These interventions, although necessary for survival, injure the immature lung of preterm neonates and contribute substantially to the development of BPD.[1] BPD is the most common complication of prematurity and may confer life long consequences for graduates of neonatal intensive care units.[2] Neonates with BPD may remain oxygen dependent for many months,[2] have increased rates of readmission to hospital, increased respiratory symptoms and increased need for respiratory medications.[3] The effect BPD has on pulmonary aging remains an area of investigation. Significant concern exists regarding the potential increased risk, or early onset, of adult pulmonary diseases in BPD survivors.[2]

MSCs represent an attractive source of cell-based therapies for BPD, as they are immune-privileged cells possessing anti-inflammatory, immunomodulatory, and reparative capabilities.[4] Systemic or intratracheal delivery of MSCs attenuate lung inflammation, alveolar growth arrest and lung vascular damage in hyperoxia models of BPD.[5-8] Conditioned media from MSCs also confers beneficial effects, supporting the hypothesis that MSCs therapeutic benefits are mediated via a paracrine mechanism.[5, 9] MSCs multifactorial, advantageous effects appear an ideal therapeutic match for the truly multifactorial pathogenesis of BPD.

We hypothesized that MSC conditioned media would attenuate the development of lung injury in a multifactorial animal model of BPD. To test this hypothesis we developed a clinically relevant, small animal, multifactorial model of BPD by combining inflammation, supplemental oxygen and mechanical ventilation

in neonatal mice. We assessed the ability of this model to create the "hallmark" histopathologic findings of BPD, including alveolar simplification and capillary vessel rarefaction, in neonatal mice. As our primary objective, we investigated the ability of human umbilical cord-derived MSC conditioned media to prevent lung injury in this clinically more relevant mouse model of BPD.

Methods

The ability of human umbilical cord-derived MSC conditioned media to accelerate wound healing was confirmed *in vitro* and the ability to prevent lung injury was examined *in vivo*. The Institutional Animal Care and Use Committee at the University of Alberta approved all procedures (Protocol #: Aup00000341).

Preparation of MSC conditioned media

Human umbilical cord-derived MSCs were isolated from 6 umbilical cords of either sex and characterized according to the criteria specified by the International Society for Cellular Therapy, [10] by previous lab member Marius Möbius. To obtain conditioned media, MSCs were grown to approximately 90% confluency then incubated for 24 hours in serum-free alpha Minimum Essential Medium (aMEM, Sigma-Aldrich) at 37 °C in 5% O_2 and 5% CO_2. Media was then collected and centrifuged for 30 min at 2500 x g to pellet any solid debris. To further concentrate this primary conditioned media, the supernatant was transferred into ultra centrifugal filters (Amicon Ultra-15, Millipore) with a nominal molecular weight

limit of 3 kDa and centrifuged at 4000 x g for 40 minutes. The concentrated conditioned media was harvested and stored at -80°C.

In-Vitro Experiment:

Wound healing assay

The reparative benefits of soluble factors present in MSC conditioned media was tested using an *in vitro* wound healing assay. Commercially available rat lung alveolar type II epithelial cells (RLE 6TN, ATCC) were seeded into a plastic 24-well cell culture plate, at a concentration of 60,000 cells per well, with Ham's F12 nutrient mixture (Gibco Life Technologies) + 10% fetal bovine serum + 1% penicillin-streptomycin-fungizone. At 48 hours, cells reached 95% confluency and the cell monolayer was scraped with a p200 pipette tip. Wells were washed with PBS to remove detached cells and medium was replaced with 500 microliters of human umbilical cord-derived MSC conditioned media or Ham's F12 nutrient mixture (serum free). The surface area of the wound was recorded over time and the percentage wound closure was calculated using OpenLab Software (Version 5, Quorum Technologies Inc.).

In Vivo Experiments*:*

Experimental design

This study included 4 separate experimental groups of C57BL/6 mouse pups at postnatal day 9-10, weighing 4.96g ± 0.68g (mean ± standard deviation). The 4 groups investigated were: control, lung injury, treatment and placebo (Appendix 2).

All pups in the lung injury, treatment and placebo groups received an intraperitoneal injection of LPS (4 mcg/g) approximately 48 hours prior to surgical tracheostomy under anesthesia with ketamine (60 mcg/g) and xylazine (12mcg/g), as previously described (Chapter 4), and were then mechanically ventilated for 8 hours with 40% oxygen at a rate of 180 breaths/minute with a tidal volume of 10 microliters/g. While under anesthesia, and immediately prior to surgical tracheostomy and mechanical ventilation, pups in the treatment group received a single intratracheal injection of MSC conditioned media at a dose of 3 microliters/g. In an identical fashion and dose, pups in the placebo group received a placebo injection of aMEM (vehicle) to control for factors attributing to administration of intratracheal fluid and components present in conditioned media vehicle. On completion of ventilation, pups were euthanized with intraperitoneal pentobarbital sodium and lungs were subsequently resected. Unventilated pups that did not receive LPS served as controls and lungs were harvested for comparison at the same time as littermates. Controls were distributed throughout the 15 litters used for these experiments.

Distribution experiments

Distribution experiments were conducted to assess the distribution of intratracheally administered MSC conditioned media in the mouse lung. Under anesthesia, 8 mouse pups (C57BL/6) at postnatal day 9-10, received an intratracheal injection of MSC conditioned media in a 1:1 ratio with fluorescent dye (CellBrite Cytoplasmic Membrane Dye, Biotium Inc., 30023) at a total volume of 3

microliters/g. Following injection, a surgical tracheostomy was created and pups were mechanically ventilated for 30 minutes with 40% oxygen at a rate of 180 breaths/minute and tidal volume of 10 microliters/g. The mechanical ventilation duration of 30 minutes was chosen after preliminary studies at 30 minutes, 1 hour, 2 hours and 4 hours revealed that visualization of dye was optimal after 30 minutes. On completion of 30 minutes of mechanical ventilation, pups were euthanized and distribution of the MSC conditioned media/fluorescent dye was visualized using In-vivo Imaging System FX PRO (Bruker Molecular Imaging).

Inflammatory cytokines and oxidative stress

Right lung tissue was snap frozen in liquid nitrogen and stored at -80°C for measurement of inflammatory cytokines and oxidative stress. Frozen lung samples were homogenized in 0.5% Tween-20/PBS + protease inhibitor cocktail and centrifuged. Macrophage Inflammatory Protein – 2 (MIP-2, R&D Systems, SMM200) and Monocyte Chemoattractant Protein – 1 (MCP-1, R&D Systems, SMJE00) were quantified using enzyme-linked immunosorbent assay (ELISA). The antioxidant capability of frozen lung samples was measured using the total antioxidant capacity kit (TAC, Cell Biolabs Inc., STA-360) as an indicator of oxidative stress. Free radicals are commonly neutralized by antioxidants using a single electron transfer mechanism. The TAC ELISA kit quantifies the ability of an antioxidant to reduce a compound by single electron transfer, namely reducing copper (II) to copper (I). The assay procedures were performed according to manufacturer instructions.

Lung morphometry

Lungs were inflated and fixed *in situ* via the trachea with a zinc formalin solution at a constant pressure of 20 cm H_2O. The trachea was ligated and lungs were placed in zinc formalin solution overnight. The follow day excess tissue was dissected away and lungs were transferred to a 70% ethanol solution. Lungs were paraffin embedded and cut into 5 micrometer thick serial sections and stained with hematoxylin and eosin. Alveolar structures of the left lung were quantified using the mean linear intercept (MLI) as an estimate of alveolar diameter. The MLI was determined using a motorized microscope stage (Leica Microsystems) and OpenLab software. Briefly, at 400X magnification, in a minimum of 240 fields of view per lung, a 155.34 micrometer line was superimposed on each field of view and the number of alveolar septae crossing this line were counted. Multiplying the length of the line by the number of fields of view and dividing this product by the total number of intersections calculated the MLI for each animal.

Immunohistochemistry

Vascular quantification was performed using immunohistochemistry to identify vessels in fixed sections of lung. Paraffin sections from the left lung were stained for von Willebrand Factor (vWF) (1:200, Dako, A0082). Briefly, sections were deparaffinized in an ethanol series. Antigen retrieval was performed by incubating slides in heated sodium citrate buffer (10 mM pH 6.0) for 20 minutes, followed by blocking of endogenous peroxidase activity in 3% H_2O_2/PBS for 20 minutes. Non-specific binding was blocked by incubating in Tris-HCl/NaCl/BSA

buffer overnight at 4°C. After overnight incubation, sections were incubated with vWF antibody (primary antibody) diluted in Dako antibody diluent (Dako, S0809) for 45 minutes at room temperature. Subsequently, sections were rinsed with PBS and incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:1000, Invitrogen, B2770) diluted in Dako antibody diluent for 1 hour at room temperature. Antibody signal was amplified by incubation with streptavidin-HRP complex (1:1000 in Dako diluent; Invitrogen, S911) for 30 minutes at room temperature and visualized with 3,3′-Diaminobenzidine (DAB) staining. Tissue sections were counterstained Mayer's hematoxylin, dehydrated in an ethanol series and coverslipped.

Lung sections were analyzed by light microscopy using a Leica microscope. Photomicrographs were taken randomly at 400x magnification. vWF-positive vessels were counted in fifteen representative high power fields (HPF) per animal, by a blinded observer, using OpenLab software. The number of vessels per HPF was subsequently averaged for each animal.

Statistical analysis

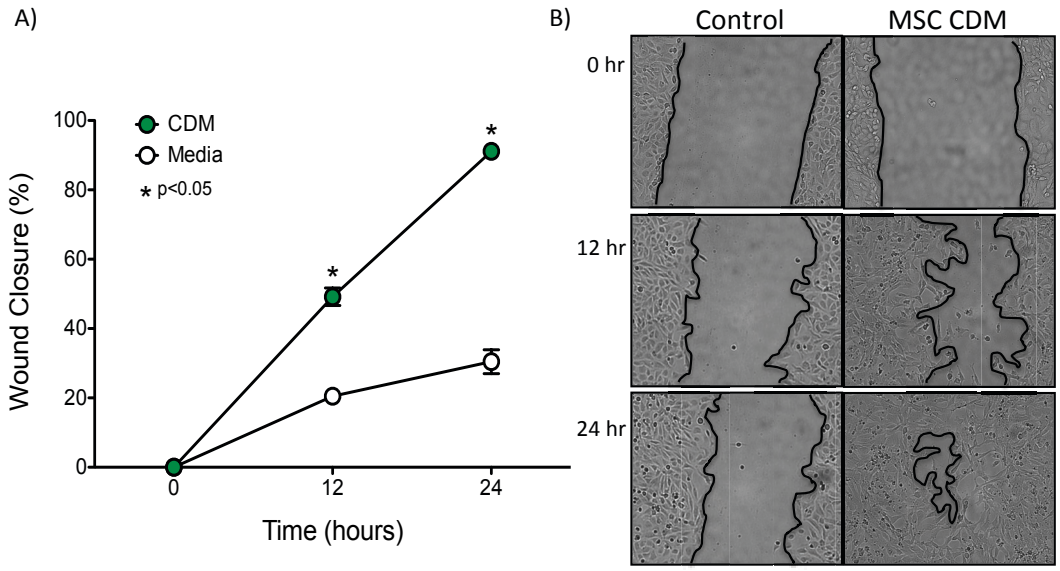
Comparisons between experimental groups were made using a one-way analysis of variance (ANOVA) with a Fisher's least significant difference post hoc test. Results are expressed as the mean ± the standard error of the mean unless otherwise specified. Data was analyzed using SPSS statistical software (IBM SPSS Statistics, Version 21) and a value of $p < 0.05$ was considered statistically significant. All group assignments were blinded prior to analysis.

Results

MSC conditioned media accelerates wound healing in vitro

To confirm the paracrine effect of MSC conditioned media *in vitro* a wound scratch assay was performed on rat lung alveolar type 2 epithelial cells (Figure 5-1). At 12 and 24 hours after the scratch, wound closure was significantly higher with MSC conditioned media compared with Ham's F12 nutrient mixture, with 49% versus 21% wound closure at 12 hours and 91% versus 30% wound closure at 24 hours ($p < 0.05$).

Figure 5-1. Human umbilical cord-derived MSC conditioned media accelerates lung epithelial cell wound healing in vitro.

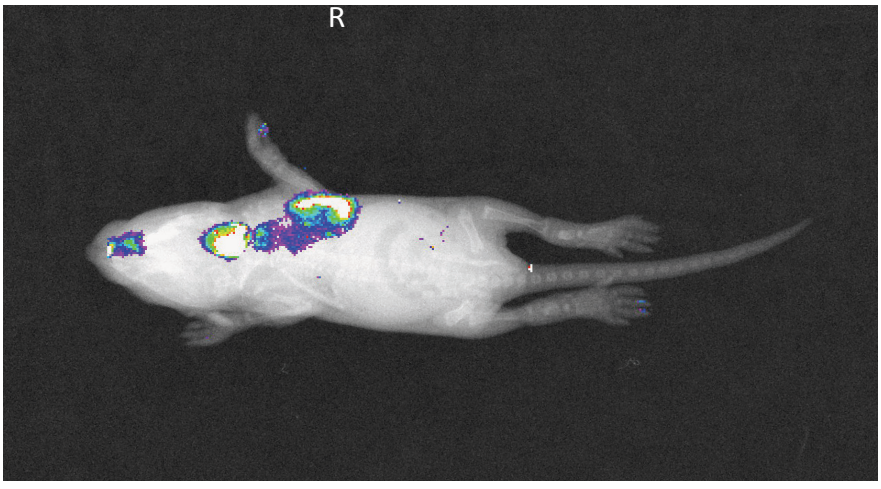


A) MSC conditioned media accelerates wound healing as demonstrated by the significantly higher percentage wound closure, at both 12 and 24 hours after initial scratch, compared with cells cultured in basic media. B) Representative photos taken at time of initial scratch, 12 hours after scratch and 24 hours after scratch. Cells incubated in MSC conditioned media visibly demonstrate accelerated healing.

Unequal distribution of MSC conditioned media

It is recognized that intratracheally administered medications may not demonstrate equal distribution within the lung, in both humans and animals. However, the intratracheal route is the most direct method of delivering medications to the lung. The distribution of intratracheally administered MSC conditioned media in the lung was assessed in 8 mouse pups and a preference for right lung deposition was identified, with this occurring in 65% of pups assessed (Figure 5-2). In 25% of pups the conditioned media was bilaterally distributed and in 12% of pups the conditioned media was delivered to the left lung. During visualization of the conditioned media/fluorescent dye, intense signal could be identified in the neck region, at the site of injection and at the snout, likely secondary to spontaneous breathing occurring during the procedure (Figure 5-2).

Figure 5-2. Intratracheally administered MSC conditioned media demonstrates preferential distribution to the right lung.

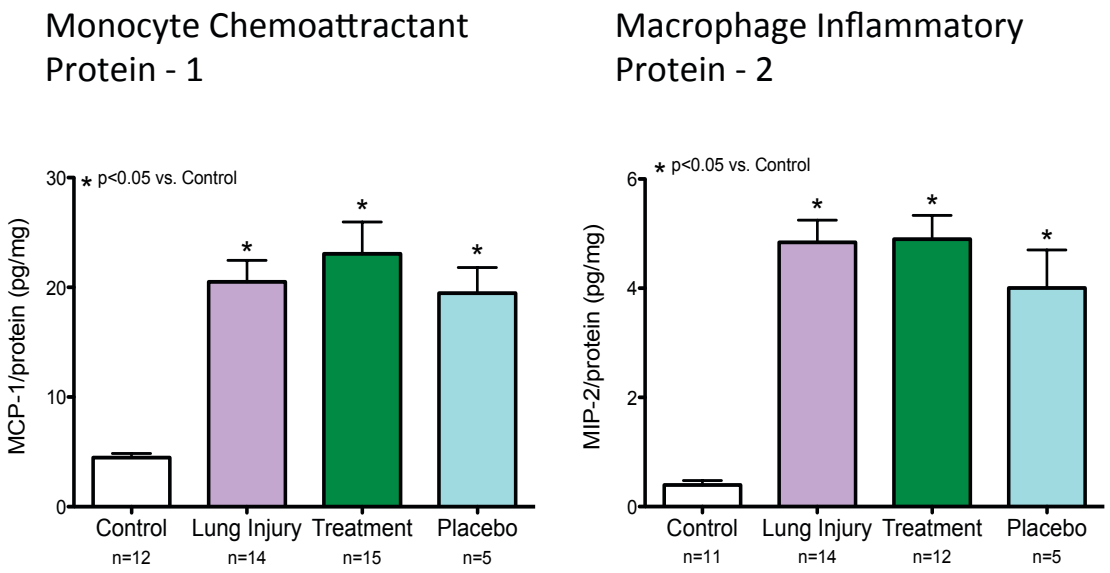


Visualization of intratracheally administered conditioned media/fluorescent dye after 30 minutes of ventilation demonstrates preferential distribution to right lung.

MSC conditioned media does not reduce quantity of inflammatory cytokines

Inflammatory cytokines MCP-1 and MIP-2 were quantified to assess the development of inflammation in the lung and the effect of MSC conditioned media on this inflammation. Levels of MCP-1 and MIP-2 were significantly elevated in the lung injury group compared with controls ($p < 0.05$), indicating the development of significant lung inflammation in this multifactorial model. Treatment with MSC conditioned media did not attenuate the development of inflammation in the lung as measured by quantification of MCP-1 and MIP-2 (Figure 5-3).

Figure 5-3. MSC conditioned media does not attenuate inflammation as measured by MCP-1 and MIP-2.

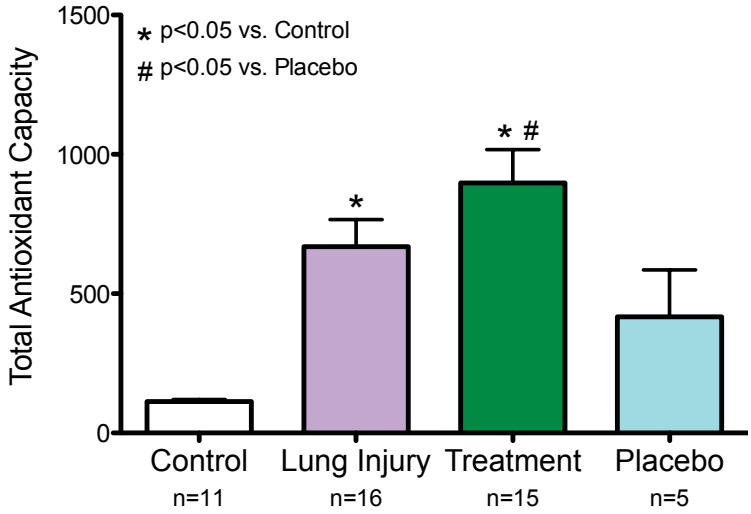


The animal model developed creates significant inflammation in ventilated, LPS exposed animals compared with controls. MSC conditioned media administered intratracheally does not reduce inflammation, as measured by MCP-1 and MIP-2, compared with untreated animals in the lung injury group.

MSC conditioned media does not significantly improve total antioxidant capacity

Antioxidants play an important role in scavenging free radicals and preventing oxidative damage to tissues. Total antioxidant capacity (TAC) levels were significantly elevated in the lung injury and treatment groups compared with controls ($p < 0.05$). The increased TAC level in the lung injury group likely reflects an up-regulation of innate antioxidant mechanisms, present in mouse pups, in response to the induced oxidative stress. Although TAC levels were highest in the treatment group they were not significantly higher when compared with the lung injury group (Figure 5-4).

Figure 5-4. MSC conditioned media does not significantly increase the total antioxidant capacity as measured by ELISA.

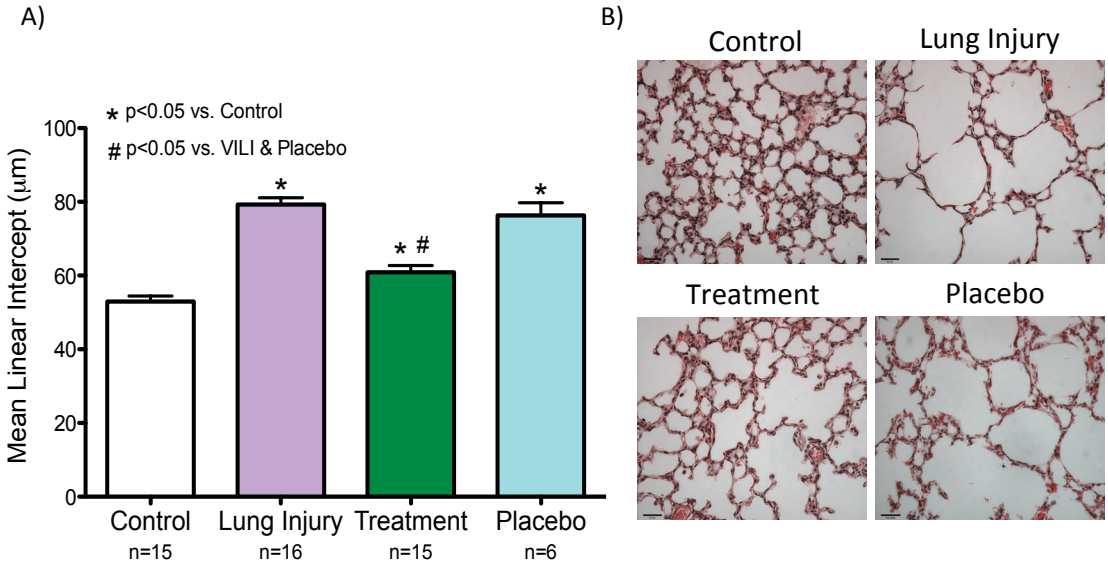


The animal model established generates oxidative stress, as demonstrated by the increased total antioxidant capacity in ventilated, LPS exposed animals compared with controls. Intratracheally administered MSC conditioned media does not significantly increased the total antioxidant capacity in treated animals, compared with untreated animals in the lung injury group.

MSC conditioned media significantly attenuates structural lung injury

The combination of inflammation, mechanical ventilation and supplemental oxygen created a histological pattern of alveolar simplification, with larger fewer alveoli, reminiscent of BPD (Figure 5-5). This is demonstrated by a significantly elevated MLI in the lung injury group compared with controls ($p < 0.05$). Intratracheal treatment with MSC conditioned media significantly attenuated structural lung injury as demonstrated by a significant reduction in the MLI compared with the untreated lung injury group ($p < 0.05$). This is reflected in the histology as smaller more numerous alveoli compared with the untreated group (Figure 5-5). Placebo treatment did not confer any benefit on structural lung injury.

Figure 5-5. MSC conditioned media attenuates structural lung injury.



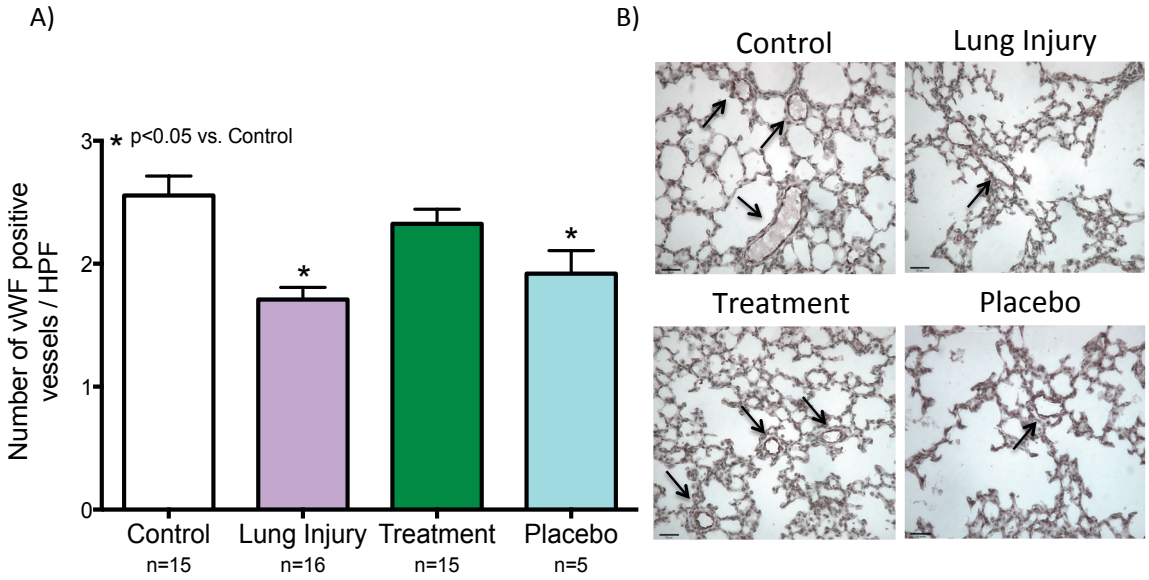
A) The animal model developed, results in significant reduction in MLI in ventilated, LPS exposed animals compared with controls. MSC conditioned media administered intratracheally attenuates the structural lung injury as demonstrated by a significant reduction in MLI compared with untreated animals in the lung injury group. B) Representative histology depicting alveolar simplification created by this multifactorial model and the attenuation of this alveolar simplification in animals receiving intratracheal MSC conditioned media.

MSC conditioned media significantly preserves lung vascular density

Rarefaction of lung capillary vessels is a histologic hallmark of BPD. This clinically relevant model of BPD produces a reduction in lung vascular density as confirmed by a reduction in the number of vessels per HPF in the lung injury group compared with the control group ($p < 0.05$; Figures 5-6). Treatment of pups with intratracheal MSC conditioned media preserves the lung vascular density such that the number of vessels per HPF in treated pups is significantly higher compared with untreated pups ($p < 0.05$). MSC conditioned media treated animals had a vessel

density comparable to that of controls. This preservation of lung vasculature was not seen in placebo treated animals (Figure 5-6).

Figure 5-6. MSC conditioned media preserves lung vascular density.



A) The animal model created, produced a significant reduction in vascular density as demonstrated by a reduction in the number of vWF positive vessels/HPF in the lung injury group. MSC conditioned media administered intratracheally preserves lung vascular density as demonstrated by the significantly greater number of vWF positive vessels/HPF in treated animals compared with the lung injury group. B) Representative histology depicting capillary rarefaction created by a more clinically relevant murine model of BPD and the preservation of vessel density in animals treated with MSC conditioned media. Arrows indicate vWF positive vessels.

Discussion

BPD is a commonly occurring pulmonary disease with lasting consequences and no effective treatments. Infants born extremely premature are particularly susceptible to the development of BPD as their immature lungs possess decreased lung compliance, underdeveloped airways and supporting structures, decreased

antioxidant capacity and surfactant deficiency.[11] The new BPD of our post-surfactant era is characterized by impaired alveolarization[12, 13] and dysregulated vasculogenesis,[14] histopathologic findings that were reproduced in the multifactorial animal model developed for this study. In comparison to many small animal models that rely on lung injury induced by a single factor,[11, 15] our model incorporates three critical factors, inflammation, supplemental oxygen and mechanical ventilation. These factors are superimposed on the neonatal mouse to generate an injury model in a structurally immature lung. Historically, some models have included animals of an age well beyond the neonatal range resulting in a model of injury occurring at later stages of lung development.[16] The model we have developed demonstrates histopathologic findings reminiscent of BPD, as well as significant pulmonary inflammation and oxidative stress indicated by increased inflammatory cytokines and up regulated antioxidant capacity.

The intratracheal administration of MSC conditioned media is a clinically applicable method of medication delivery and provides targeted delivery of paracrine factors to the lung. As experienced with other intratracheal medications, such as surfactant, the intratracheally administered conditioned media was preferentially distributed to the right lung in mice. This is a recognized potential limitation of our study, but also a limitation experienced in the clinical world. Interestingly, despite the possible reduced quantity of conditioned media reaching the left lungs, a significant improvement in alveolarization and vascular density was demonstrated based on left lung analysis. This raises questions regarding the mechanism of the beneficial effects observed. Potentially, the MSC paracrine factors

present in the conditioned media act locally at the site of delivery, but are also redistributed by the vascular network creating wide spread effects.

MSC conditioned media did not significantly attenuate lung inflammation in this model, as measured by MCP-1/MIP-2. These results may reflect, in part, the presence of MCP-1 or MIP-2 in the conditioned media producing higher cytokine levels in treated animals. To investigate this theory, we attempted to quantify levels of MCP-1 and MIP-2 in the MSC conditioned media. MIP-2 was not detected while MCP-1 was confirmed to be present, but unable to be quantified secondary to immeasurable protein levels in the conditioned media. MCP-1 has previously been identified as a component of the MSC secretome.[17] Quantification of inflammatory cytokines is likely not the preferred method of assessing inflammation following conditioned media therapy. Quantification of cellular infiltration was attempted as a secondary method of assessing inflammation. However, it was noted that all animals displayed extremely low numbers of neutrophils and macrophages in the lung. Studies have documented a lower resident alveolar macrophage population and impaired cellular infiltration in neonatal rodents exposed to LPS. In rats, intratracheal LPS doses of 0.2 g/kg dry lung weight did not induce a significant increase in alveolar macrophages and only induced a modest increase in alveolar neutrophils.[18] Additionally, neonatal rats demonstrate an altered and reduced inflammatory response to mechanical ventilation compared with adult rats. It is hypothesized that the altered inflammatory response in neonatal rodents may relate to the immaturity of the immune system.[19]

We have shown that the administration of a single intratracheal dose of human umbilical cord-derived MSC conditioned media attenuates alveolar simplification and preserves lung vascular density in neonatal mice exposed to inflammation, ventilation and supplemental oxygen. Studies investigating the benefits of conditioned media have incorporated both preventative and delayed treatment approaches. Our model incorporated primarily a preventative therapeutic approach. This type of therapy would be applicable to neonates born at the extremes of prematurity who are requiring ventilatory support, as these infants are known to be at high risk of developing significant BPD. The beneficial effects of MSC conditioned media are likely the cumulative result of multiple mechanisms including anti-inflammatory, anti-oxidant and pro-angiogenic processes. This study supports previous works indicating that MSCs act via a paracrine mechanism and to our knowledge is the first study to demonstrate the therapeutic benefit of MSC conditioned media in a multifactorial, clinically relevant model of BPD.

Continued preclinical and clinical studies are required to expand our knowledge regarding the precise mechanisms of these beneficial effects and to identify the best source, dose, and timing of MSC-based therapies. As evidence supporting the beneficial therapeutic effects of MSC conditioned media builds, research investigating the components of the secretome becomes essential. The identification of key bioactive molecules present in MSC conditioned media would permit the exploitation of such molecules for the development of potential new BPD therapies. In addition to investigating the efficacy of stem cell-based therapies,

investigators must ensure a focus on creating stringent techniques and criteria for cell isolation, manufacturing and administration.

The first clinical trial investigating the effectiveness of MSCs to prevent BPD was recently approved and initiated. The results of this early clinical trial will indicate if the benefits seen in animal research translate to the clinical world. The introduction of new therapies in this particularly vulnerable population of preterm infants must be accompanied by assessment of not only short-term outcomes but also long-term respiratory and neurodevelopmental outcomes. Ideally, patients should be followed well into school age and early adulthood to allow for a comprehensive assessment. While awaiting approval of additional clinical studies, continued animal research will help establish a solid foundation for the use of MSCs, MSC conditioned media or it's components, in the prevention of BPD.

Conclusion

The combination of inflammation, mechanical ventilation and supplemental oxygen in neonatal mice creates a more clinically relevant small animal model of BPD that demonstrates lung injury and inflammation, reminiscent of clinical BPD. This multifactorial model provided the platform to investigate the *in vivo* paracrine benefits of MSC conditioned media. The intratracheal administration of human umbilical cord-derived MSC conditioned media prevented the reduction of lung vascular density and attenuated the alveolar simplification documented in untreated animals, confirming our initial hypothesis. Ultimately, the true clinical efficacy of MSC conditioned media or its identified components can only be

determined by well-designed randomized controlled trials in high-risk neonatal populations.

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Chapter 6:

Summary and Future Directions

The research comprising this Thesis endeavored to investigate the paracrine effect of MSCs on experimental lung injury and to create a clinically more relevant, multifactorial, small animal model of bronchopulmonary dysplasia (BPD). We created a clinically more relevant mouse model of BPD by exposing neonatal mice to inflammation, mechanical ventilation and supplemental oxygen. This model incorporates the multifactorial nature of BPD by combining key factors known to contribute to the development of BPD in neonates. We then tested the ability of this model to produce inflammation and histology reminiscent of BPD, including alveolar simplification and pulmonary vessel rarefaction. Our primary objective was to determine if human umbilical cord-derived MSC conditioned media would attenuate the development of lung injury in our multifactorial mouse model of BPD.

Our findings demonstrated that the combination of intraperitoneal LPS, mechanical ventilation and supplemental oxygen creates pulmonary inflammation, oxidative stress, alveolar simplification and reduced lung vascular density in neonatal mice. We have also shown that MSC conditioned media significantly attenuates alveolar simplification and preserves lung vascular density in our model. This research supports the theory that MSCs therapeutic benefits are primarily conferred in a paracrine manner. To our knowledge this is the first study to demonstrate the paracrine effect of MSCs in a mechanically ventilated newborn model.

BPD is a respiratory condition primarily affecting infants born at the extremes of prematurity. Multiple diagnostic criteria currently exist to define BPD, creating significant variability in the reported incidence of BPD and difficulties when

comparing the effectiveness of potential therapies. Exclusive use of the NIH severity based BPD definition, with the incorporation of a physiologic test for infants requiring < 30% oxygen, would reduce the observed variation in the incidence of BPD and permit comparison of potential therapies.

BPD is a multifactorial disease process that is associated with long-term health consequences including poor neurodevelopmental outcome and chronic respiratory conditions such as asthma and pulmonary hypertension. Current treatment strategies primarily attempt to reduce the post-natal injury inflicted on the premature lung. The paucity of effective therapies, with acceptable side effect profiles, has resulted in the incidence of BPD remaining unchanged or possibly increased in recent years. Stem cell-based therapies have emerged as a potential new therapeutic strategy for BPD.

We successfully developed a multifactorial small animal model of BPD; however, this model has multiple limitations. The first limitation is the relatively short ventilation duration of 8 hours. This results in the inability to assess long-term outcomes pertaining to efficacy and safety of MSC-based therapies. Additionally, the small size of the animals used and the inability to recover the mouse pups following surgery limits our endpoint assessments to histology and molecular analysis. Functional data such as pulmonary function testing could not reliably be performed on animals of the weight used in our model. Exercise tolerance would only be obtainable with a recovery model. It must also be recognized that although we sought to create a clinically more relevant model of BPD, the translation of knowledge obtained from small animal models to humans will always be limited by

species variations. Furthermore, the complexity of care in the neonatal intensive care unit is not fully captured by this model, as many interventions such as antenatal corticosteroids, surfactant therapy and sepsis were not replicated.

A portion of these limitations could be addressed by continued advancement of the model designed for this project. The creation of a clinically relevant, multifactorial, small animal model that permits recovery of the pups following ventilation would be very beneficial. This would necessitate insertion of an endotracheal tube by intubation rather than tracheostomy, followed by extubation and recovery of the animals. If possible, this type of recovery model would allow for the investigation of functional parameters and determination of the long-term benefits or side effects of MSC-based therapies. The model could also be expanded to incorporate a longer duration of ventilation to mimic the prolonged ventilation required by some neonates. Bland and colleagues have succeeded in ventilating mice for up to 24 hours.[1] An increased duration of ventilation requires the addition of a feeding regime for the pups and antibiotics to prevent potential infection. The existing model, or an advanced model, could be used to expand on our current knowledge of the effects of inflammation, ventilation and oxygen on known markers of lung growth and the developing lung itself.

Our research highlights the importance of the paracrine effect of MSCs by demonstrating that MSC conditioned media attenuates alveolar simplification and preserves lung vascular density in our experimental model of lung injury. Future therapeutic research directions include efforts to elucidate the essential components of the conditioned media, followed by testing of these components in

relevant animal models of BPD. A multitude of factors have been identified as components of the MSC secretome, but of particular future interest are exosomes. Exosomes are secreted membrane vesicles 40 to 100 nm in size containing a complex array of proteins and genetic material. The expanding quantity of research surrounding exosomes and their diverse effects make them an intriguing avenue of investigation for multifactorial diseases such as BPD.[2] Future studies could be designed to explore the therapeutic potential of MSC exosomes in multifactorial animal models of BPD.

Currently, the ideal dose and timing of MSC-based therapies is unknown and future research could investigate a possible dose response, or the benefits of prevention versus later therapy. Studies investigating the effects of MSC conditioned media or its components could be expanded to include assessment of multi-organ effects. Specifically, collection and analysis of the brain, in addition to the lung, would be of great benefit as stem cell therapies are also under investigation for neonatal neurological diseases.[3]

There is a growing body of evidence to support the use of MSCs as a therapy for BPD and the first clinical trials have been initiated. However, continued rigorous preclinical research is required to verify the safety and efficacy of MSC conditioned media in the prevention or treatment of experimental BPD before these results can be applied to vulnerable neonates.

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Appendix 1:

Data Collection/Monitoring Sheet for Mechanically Ventilated Neonatal Mice

Ventilator Induced Lung Injury in Neonatal Mice

Date:	Start time:
VILI Parameters	Mouse #:
stroke volume (SV):	age:
calculated SV:	sex:
stroke frequency:	weight:
% oxygen:	other info:

Anaesthetic

ketamine (60ug/g) / xylazine (12ug/g):

VILI Progress

time (hours)	comments	time (hours)	comments
0.5		4.5	
1		5	
1.5		5.5	
2		6	
2.5		6.5	
3		7	
3.5		7.5	
4		8	

Tissue collection

right lung:	left lung:

Appendix 2:

Experimental Design Outlining Experimental Groups and Interventions in Each Group