



Published in final edited form as:

Pediatrics. 2010 November ; 126(5): . doi:10.1542/peds.2009-3445.

Isolation of tracheal aspirate mesenchymal stromal cells predicts bronchopulmonary dysplasia

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Abstract

Background—We have isolated mesenchymal stromal cells (MSCs) from tracheal aspirates of premature infants with respiratory distress. Under the influence of transforming growth factor- β , MSCs differentiate into α -smooth muscle actin-expressing myofibroblasts. Myofibroblasts are increased in the lungs of patients with bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurely-born infants.

Objective—We tested whether isolation of MSCs from tracheal aspirates of premature infants with respiratory distress during the first week of life correlates with BPD.

Patients and Methods—Eighty-four infants born at gestational age <33 weeks and requiring mechanical ventilation were studied. Aspirates were collected during suctioning and centrifuged. Cell pellets were resuspended in culture medium and plated. Adherent cells were grown to confluence.

Results—MSCs were isolated from the tracheal aspirates of 56 infants; 28 infants showed no MSCs. There was no statistical difference in gestational age or birth weight between the ‘MSC’ and ‘no MSC’ groups. In the MSC group, 12 died and 25 developed BPD, as defined by a requirement for supplemental O₂ at 36 wks post-menstrual age. In the ‘no MSC’ group, 6 infants died and 1 developed BPD. Accounting for the potential influences of gender, birth weight, gestational age, number of tracheal aspirate samples taken and the duration of endotracheal intubation (up to 7 days), isolation of MSCs increased the adjusted odds ratio of BPD over 21-fold (95% confidence intervals: 1.82, 265.85).

Conclusions—Isolation of tracheal aspirate MSCs predicts the development of BPD, which suggests that MSCs play an important role in the pathogenesis of this disease.

Keywords

chronic lung disease; lipofibroblast; myofibroblast

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INTRODUCTION

With improvements in neonatal care, the survival of very premature infants has increased. However, improvements in survival have been accompanied by a corresponding increase in the incidence of bronchopulmonary dysplasia (BPD), a fibrotic lung disease requiring supplemental oxygen for months or years¹. Survivors of BPD have abnormal lung function even as young adults^{2,3}, making BPD one of the leading causes of pediatric lung disease.

Before the surfactant era, airway remodeling and parenchymal fibrosis were prominent histologic findings in BPD. More recently, the lungs of infants dying from BPD have shown less fibrosis and more uniform inflation. However, there are larger and fewer alveoli, as well as poorly formed secondary crests, indicating an interference with septation^{4,5}. Further, alveolar septa are thickened with collagen and elongated cells resembling fibroblasts⁶. A study of infants with respiratory distress syndrome (RDS) showed the appearance of α -smooth muscle actin-positive myofibroblasts in the alveolar septa four days after birth⁷. Myofibroblasts featured intense immunoreactivity for transforming growth factor (TGF)- β , a stimulus for myofibroblastic differentiation. TGF- β overexpression in neonatal mouse lungs results in structural changes of BPD including abnormal alveolar structure and vascular development⁸. TGF- β also induces proliferation of α -smooth muscle actin-positive cells within the septal walls. Together, these results indicate that BPD may result in part from the abnormal differentiation or proliferation of alveolar mesenchymal progenitor cells to myofibroblasts.

We have shown that tracheal aspirates from week-old premature infants undergoing mechanical ventilation for RDS contain fibroblast-like cells with colony-forming potential, surface markers and differentiation potential typically found in mesenchymal stem cells⁹. Recent work suggests that there is a hierarchy of multipotent mesenchymal stromal cells ranging from true self-renewing stem cells with multilineage differentiation capacity to those with more restricted differentiation potential, until a state of complete restriction to the fibroblast is reached¹⁰. Since we have not thoroughly tested the clonogenicity or self-renewal of neonatal lung mesenchymal cells, we now refer to them as mesenchymal stromal cells (MSCs), which have a more restricted differentiation potential. Based on their potential to differentiate into myofibroblasts¹¹, we tested whether isolation of MSCs from tracheal aspirates of premature infants with respiratory distress during the first week of life would correlate with development of BPD.

PATIENTS AND METHODS

Patients

We examined tracheal aspirates from 77 infants admitted to the University of Michigan C.S. Mott Children's Hospital Holden Newborn Intensive Care Unit between July 2005 and August 2009. We also examined tracheal aspirates from 7 infants admitted to the St. Joseph Mercy Hospital between January 2007 and June 2008. Entry criteria included gestational age at birth ≥ 33 wk, mechanical ventilation for respiratory distress, and age ≥ 7 d. Infants with acute sepsis during the first week of life were excluded. Chorioamnionitis and necrotizing enterocolitis were diagnosed on clinical grounds. Informed consent was obtained. This study was approved by both institutions' Investigational Review Boards.

Tracheal aspirate collection and cell culture

Endotracheal tube suctioning was performed as needed to maintain tube patency. The need for suctioning was determined by the nurse and respiratory therapist. Specimens were centrifuged ($1200 \times g$ for 5 min at 15°C) and supernatants stored at -80°C . Cell pellets were

resuspended in 2 ml Dulbecco's Modified Essential Medium with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 500 µg/ml Fungizone. Cell culture medium was refreshed daily for three days and three times a week thereafter. Adherent cells were incubated at 37°C in 5% CO₂. MSC colony forming units were identified by eye under light microscopy.

We examined a total of 142 tracheal aspirates from 84 infants. 37 babies had one sample taken, 37 babies had two samples taken, 9 babies had three samples taken, and 1 baby had four samples taken. Some subjects had at least one sample from which MSCs were isolated and at least one sample from which they were not. These babies were classified as positive for MSCs.

Measurement of latent TGF-β1 in the tracheal aspirates

TGF-β1 levels were measured by ELISA (R&D Systems, Minneapolis, MN). Latent TGF-β1 was activated to immunoreactive TGF-β1 by acidification with 1 N HCl (10 min in room temperature). Samples were neutralized with 1.2 N NaOH/0.5 M HEPES. To control for sample dilution, TGF-β levels were normalized for the secretory component of IgA (scIgA)¹².

Statistical analysis

Group mean data are expressed as mean±SD, except for days on mechanical ventilation and supplemental O₂, which were described as median (interquartile range). Demographic and clinical data were compared by unpaired t-test, Mann-Whitney test or Fisher's exact test, as required. The diagnosis of BPD was the primary outcome variable. BPD was defined as the need for supplemental O₂ to maintain an acceptable O₂ saturation by pulse oximeter at 36 wks post-menstrual age. Logistic regression was used to estimate the relative odds of BPD as a function of MSCs, gender, gestational age, birth weight, number of tracheal aspirate samples taken, and days of endotracheal intubation (up to seven), with simultaneous mutual adjustment of these odds for each other, specified *a priori*. The last two variables were included in the analysis to test whether multiple sampling and a longer duration of endotracheal intubation (thereby allowing more opportunities for suctioning) could explain a higher yield of MSCs in infants developing BPD, if present. Days of mechanical ventilation and supplemental O₂ were considered secondary outcome variables. The fraction of patients yielding MSCs was correlated with gestational age at birth using weighted linear regression, with each point weighted by the number of infants at each age. Similarly, the fraction of samples yielding MSCs was correlated with postnatal age, with each point weighted by the number of samples at each age. A significance level of 0.05 was used for all hypothesis tests.

Based on our initial outcomes data⁹, we estimated the number of patients needed to find a significant difference in the proportion of infants developing BPD between the MSC and non-MSC groups. Assuming a first group proportion of 0.75, second group proportion of 0.14 and a ratio of sample sizes of 0.81, the sample size calculation to achieve a 90% power for detecting a difference in the proportion of infants developing BPD was 17 in the MSC group and 13 in the no MSC group¹³. Therefore, the current study was sufficiently powered to accept or reject the null hypothesis that the proportion of infants developing BPD would be no different in subjects who did or did not have MSCs isolated from the tracheal aspirate fluid, provided that a difference of the magnitude specified above actually exists.

RESULTS

We examined tracheal aspirates from 84 infants. MSCs were isolated from 56 infants (Figure 1). Colony forming units developed 7-14 days after tracheal aspirate plating. MSC isolation negatively correlated with gestational age at birth ($p < 0.001$, Figure 2a). There was also a significant positive correlation between postnatal age and MSC yield ($p < 0.001$, Figure 2b).

We compared the demographics and outcomes of infants from whom tracheal aspirate MSCs were isolated ($n=56$) to those from whom MSCs were not isolated ($n=28$) (Table 1). There were no differences in the groups with regard to gender, incidence of necrotizing enterocolitis or incidence of late-onset sepsis. Birth weight and gestational age at birth tended to be lower, the number of surfactant doses tended to be higher, and the history of maternal chorioamnionitis higher in the MSC group. In the MSC group, 12 patients died (10 before 36 wks post-menstrual age) and 5 were lost to follow up (for example, they were transferred to another hospital prior to 36 wks post-menstrual age). Of the 42 patients evaluated at 36 wks post-menstrual age, 25 developed BPD. In the non-MS group, 6 patients died before 36 wks post-menstrual age and 2 were lost to follow up. Of the 20 patients evaluated at 36 wks post-conceptual age, only 1 developed BPD ($p < 0.001$). Considering the two secondary outcome variables, the MSC group showed greater days of mechanical ventilation and supplemental O_2 administration (both $p < 0.001$). Sepsis and necrotizing enterocolitis were the most common causes of death (Table 2).

We reasoned that the observed difference in BPD prevalence between the “no MSC” and “MSC” groups could be due disparities in sample collection between infants who ultimately develop BPD and those who do not. For example, patients developing BPD were intubated for a longer period of time than patients without BPD, allowing a longer period for sample collection within the first week of life (Table 3). Accordingly, patients with BPD had more samples collected than patients who did not develop BPD. Also, average postnatal age at the time of the first tracheal aspirate collection was higher in the BPD group. Since MSC yield increased with postnatal age, these factors would tend to increase the likelihood of isolating MSCs from the tracheal aspirates of patients developing BPD.

To determine the independent predictive value of MSC isolation for the development of BPD, we performed multivariate logistic regression analysis. This analysis took into account the factors of gender, gestational age, birthweight, number of samples collected and days of endotracheal intubation (up to 7). Patients dying or lost to follow up prior to 36 wks post-menstrual age were eliminated from this analysis, leaving 42 in the MSC group and 20 in the non-MS group. While univariate analysis showed that MSC isolation, gestational age, birthweight, and days of endotracheal intubation each affected the development of BPD (each $p < 0.001$), only birth weight and MSC isolation were independent predictors of BPD (Table 4), with MSC isolation being by far the strongest predictor. If MSCs were present, the odds of BPD as defined here increased nearly 22 times, adjusting for the other risk factors (adjusted odds ratio = 21.98, 95% confidence interval = 1.818, 265.854). Birth weight was a negative predictor of BPD (adjusted odds ratio = 0.561, 95% confidence interval = 0.331, 0.950). A one hundred-gram increase in birth weight decreased the odds of BPD by 43.9%. The number of samples collected or days of endotracheal intubation did not increase the adjusted odds of BPD development.

Given the role of TGF- β in the fibroproliferative response, we measured latent TGF- β in tracheal aspirates from 28 infants, 13 of whom went on to develop BPD. There was no difference in latent TGF- β between groups (no BPD, 223 ± 133 pg/ml, 0.24 ± 0.13 pg/ng scIgA; BPD, 255 ± 183 pg/ml, 0.30 ± 0.26 pg/ng scIgA; mean \pm SD). On the other hand,

normalized TGF- β levels were higher in patients with MSCs (cell-negative, 162 ± 113 pg/ml, 0.129 ± 0.096 pg/ng scIgA; cell-positive, 263 ± 163 pg/ml, 0.317 ± 0.209 pg/ng scIgA, $p=0.038$), consistent with our finding that MSCs produce TGF- β ¹¹.

DISCUSSION

The precise mechanisms responsible for BPD remain unclear. Hyperoxia, mechanical ventilation and maternal chorioamnionitis have been implicated as inciting factors. Interleukin (IL)-8, IL-1 β and tumor necrosis factor- α are elevated in tracheal aspirates and bronchoalveolar lavage fluid of preterm infants who develop BPD ¹⁴⁻¹⁶ and IL-1 β overexpression disrupts postnatal alveolar septation in the mouse ¹⁷, suggesting that inflammation plays a role. Expression of vascular endothelial growth factor (VEGF) is decreased in infants dying with BPD ⁶ and VEGF promotes lung angiogenesis and prevents alveolar damage in hyperoxia-exposed rats ^{18, 19}, evidence that angiogenesis participates in the alveolarization process. Infants with RDS and BPD show the appearance of α -smooth muscle actin- and TGF- β -positive myofibroblasts in the alveolar septa ⁷, and tracheal aspirate TGF- β 1 levels are increased in preterm infants who develop BPD ²⁰. TGF- β overexpression in neonatal mouse lungs results in structural changes of BPD including proliferation of α -actin-positive myofibroblasts within the alveolar septal walls, abnormal alveolar structure and vascular development ⁸. Together, these results indicate that BPD may result in part from the abnormal differentiation of alveolar mesenchymal progenitor cells to myofibroblasts, under the influence of TGF- β .

We have shown that tracheal aspirates from premature infants with RDS contain fibroblast-like cells with colony-forming potential, surface markers and differentiation potential found in MSCs ⁹. We also observed that MSCs express mRNAs encoding contractile and extracellular matrix proteins, consistent with a myofibroblast progenitor phenotype. Treatment of MSCs with TGF- β induced myofibroblastic differentiation ¹¹. We therefore reasoned that MSCs are more likely to be isolated from the tracheal aspirates of premature infants who develop BPD than those who do not. We found that, if MSCs were present, the adjusted odds of BPD increased nearly 22 times. Further, in the “MSC group,” 25 of 42 patients evaluated at 36 wks post-menstrual age developed BPD. In contrast, in the “no MSC group,” only 1 of 20 patients developed BPD.

The high predictive value of MSC isolation for the development of BPD suggests that these cells play a significant physiologic role in the development of chronic lung disease. As noted above, MSCs could serve as precursors for myofibroblasts, the number of which are increased in BPD ⁷. The myofibroblast is the key effector cell in fibrogenesis ^{21, 22}. Myofibroblasts are therefore likely to contribute to alveolar fibrosis and abnormal alveolar development. On the other hand, it is also conceivable that MSCs play a positive role. During development, alveolar myofibroblasts are required for the formation of secondary septi ^{23, 24}. Cultured MSCs may also be differentiated to lipofibroblasts ⁹, which provide type II cells with neutral lipid for surfactant synthesis ²⁵.

On the basis of the high odds ratio and the binary nature of this measurement, isolation of MSCs from the tracheal aspirate may also represent a promising biomarker for the development of chronic lung disease. However, it may take up to two weeks for colonies of MSCs to appear on plastic culture dishes, which may diminish the utility of this marker.

We defined BPD as the need for supplemental O₂ to maintain an acceptable O₂ saturation by pulse oximeter at 36 wks post-menstrual age. This definition includes infants with both “moderate” and “severe” BPD ¹. Premature infants requiring supplemental O₂ at 36 wks post-menstrual age have a significantly greater need for pulmonary medications and

incidence of rehospitalization at 18-22 months' corrected age than infants not requiring supplemental O₂ ²⁶. However, we acknowledge that, with increasing survival of very low birth weight, the duration of O₂ therapy, including treatment with supplemental O₂ at 36 wks post-menstrual age, may no longer identify all infants with chronic lung disease. For example, one study found that, while the 36 wk cutoff was more accurate in predicting poor pulmonary outcomes compared to earlier post-menstrual ages, the overall accuracy was only 63% ²⁷.

It is possible that other variables account for the higher prevalence of BPD in the MSC group. Birth weight and gestational age tended to be lower, and the incidence of a history of maternal chorioamnionitis higher, in the MSC group. Low birth weight and chorioamnionitis have each been previously identified as risk factors for the development of BPD ²⁸. There was also a negative correlation between gestational age and MSC isolation. It is therefore possible that the observed relationship between MSC isolation and BPD might simply reflect the known association between low birth weight and chronic lung disease. However, multivariate logistic regression analysis showed that, while there was an association between birth weight and development of BPD, isolation of MSCs was a much stronger predictor of poor pulmonary outcome. In addition, patients developing BPD were intubated for a longer period of time than patients not developing BPD, allowing a longer period of time for sample collection. Accordingly, patients with BPD had more samples collected than patients without BPD. In addition, the average postnatal age of the first tracheal aspirate collection was significantly higher in the BPD group, and MSC yield increased with postnatal age. Together, these data suggest the possibility that infants who did not develop BPD, who were more likely to be extubated earlier in the course of their disease, had a lower yield of MSCs simply because they were sampled at earlier time points and sampled fewer times than those who developed BPD. Consistent with this, the only patient in the "non-MSc" group who developed BPD was sampled on day 1 of life, when the yield of MSCs was low. To test this, we included the number of samples per subject and the number of days which we had access to tracheal aspirate samples (days the subject was endotracheally intubated, up to seven) in our multivariate logistic regression analysis. While inclusion of these variables decreased the predictive value of MSC isolation for the development of BPD (odds ratio decreased from 31.50 to 21.98, data not shown), they were not independent predictors of BPD. Thus, the independent association of MSC isolation with the development of BPD most likely reflects the degree of injury in the lungs of these patients, rather than simply a sampling bias.

Organs carry their own population of pluripotent MSCs in a perivascular compartment ²⁹. MSCs have been noted to arise from both the large and small arteries ^{30, 31} as well as capillaries ²⁹. Together with the finding that tracheal aspirates sample more proximally in the tracheobronchial tree than bronchoalveolar lavage ¹², we speculate that MSCs are derived from the perivascular tissue of pulmonary and bronchial arteries.

One potential limitation of our study concerns the wide confidence interval for the MSC odds ratio. This reflects the fact that there was very little variance in infants from whom no MSCs were isolated; only one infant in this group developed BPD. Another limitation concerns our choice of BPD as the primary outcome variable. Because death is a competing outcome for BPD, some investigators have selected BPD or death, rather than BPD alone, as a primary outcome variable. Had we employed BPD or death rather than BPD alone as a primary outcome variable, the difference between "MSC" and "no MSC" groups would still have been highly significant.

CONCLUSIONS

Isolation of tracheal aspirate MSCs predicts the development of BPD. MSCs may therefore play an important role in the pathogenesis of BPD. MSC may also represent a biomarker for adverse pulmonary outcomes in prematurely-born infants.

Acknowledgments

The authors thank Brady T. West, M.A. and the University of Michigan Center for Statistical Consultation and Research for their assistance with statistical analysis.

Supported by NIH grant HL90134 (M.B.H.)

Abbreviations

MSC	mesenchymal stem cell
BPD	bronchopulmonary dysplasia
RDS	respiratory distress syndrome
TGF	transforming growth factor

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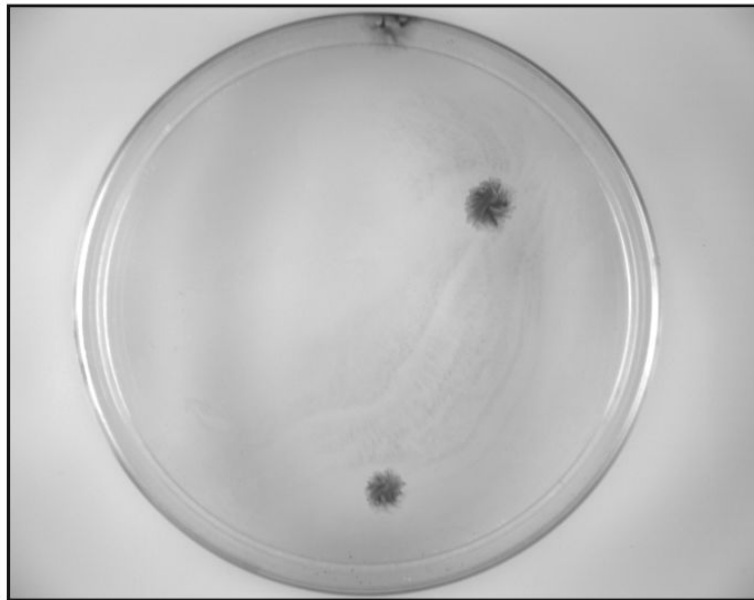


Figure 1. MSC colony forming units-fibroblast (CFU-F) develop between 7-14 days after plating of tracheal aspirates
Colonies are stained with crystal violet. Three CFU-F are shown.

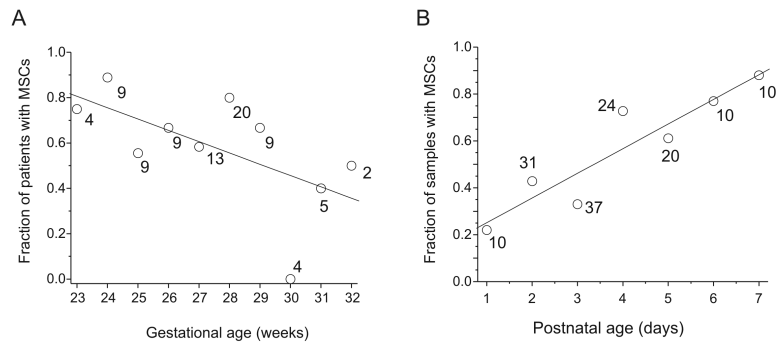


Figure 2. Relationships between the isolation of MSCs from tracheal aspirate fluid and gestational age (A) and postnatal age (B)

In A, number of infants is plotted against gestational age at birth; numbers next to each data point represent the total number of infants for that point. In B, number of samples is plotting against postnatal age; numbers next to each data point represent the total number of samples for that point.

Table 1

Comparison of demographics and clinical outcomes in babies with and without MSCs in their tracheal aspirate (mean±SD where appropriate).

	MSCs	no MSCs	test statistic
<i>N</i>	56	28	
Male gender (% of total)	29 (52%)	17 (61%)	Fisher exact test, <i>p</i> = 0.49
Died (% of total)	12 (21%)	6 (21%)	Fisher exact test, <i>p</i> = 1
Lost to follow up (% of total)	5 (9%)	2 (7%)	Fisher exact test, <i>p</i> = 1
GA (wks, mean±SD)	27.2±0.3	28.1±0.5	t-test, <i>p</i> = 0.09
BW (gms, mean±SD)	1013±43	1150±89	t-test, <i>p</i> = 0.12
Surfactant doses	2.1±0.6	1.7±0.7	Mann-Whitney test, <i>p</i> = 0.072
Suspected clinical chorioamnionitis (% of total)	10 (18%)	1 (4%)	Fisher exact test, <i>p</i> = 0.09
Necrotizing enterocolitis (% of total)	7 (13%)	1 (4%)	Fisher exact test, <i>p</i> = 0.26
Culture-proven late-onset sepsis (% of total)	8 (14%)	4 (14%)	Fisher exact test, <i>p</i> = 1
BPD (% of patients evaluated at 36 wks post-menstrual age)	25 (60%)	1 (5%)	Fisher exact test, <i>p</i> < 0.001
BPD or death (% of total)	43 (77%)	7 (25%)	Fisher exact test, <i>p</i> < 0.001
Vent days, median (IQR)	22 (25)	4 (9)	Mann-Whitney test, <i>p</i> < 0.001
O ₂ days, median (IQR)	57 (110)	19 (32)	Mann-Whitney test, <i>p</i> < 0.001
O ₂ 28 days or greater (% of patients evaluated at 28 days postnatal age)	35 (81%)	10 (50%)	Fisher exact test, <i>p</i> = 0.016

Abbreviations: MSC, mesenchymal stromal cell; SD, standard deviation; *N*, number; GA, gestational age; BW, birthweight; BPD, bronchopulmonary dysplasia; vent days, days requiring mechanical ventilation; O₂ days, days requiring supplemental O₂; IQR, interquartile range.

Table 2

Mortality.

Cause of death	Age of death, day of life (weeks post-conception)
<i>MSCs isolated (n=12)</i>	
Sepsis (<i>E. coli</i>)/necrotizing enterocolitis	19 (29)
Sepsis (<i>C. albicans</i>)/necrotizing enterocolitis	17 (26)
Presumed sepsis/necrotizing enterocolitis	11 (29)
Presumed sepsis	46 (32)
Necrotizing enterocolitis	4 (24)
Sepsis (<i>S. aureus</i>)	13 (27)
Necrotizing enterocolitis	13 (30)
Necrotizing enterocolitis	9 (27)
Respiratory failure, renal failure	110 (40)
Sepsis (<i>P. aeruginosa</i>)	6 (29)
Respiratory failure, presumed sepsis	69 (37)
Necrotizing enterocolitis	12 (27)
<i>MSCs not isolated (n=6)</i>	
Necrotizing enterocolitis	21 (30)
Presumed sepsis	7 (24)
Sepsis (<i>S. aureus</i>)	7 (25)
Presumed sepsis	21 (28)
Pulmonary hemorrhage	3 (25)
Refractory hypotension, pulmonary hypertension, pulmonary interstitial emphysema, intraventricular hemorrhage	9 (27)

Table 3

Comparison of sample collection variables for babies who did not and did develop BPD (mean±SD).

	No BPD	BPD	test statistic
<i>N</i>	36	26	
Last day of endotracheal intubation during the 7-day collection period (postnatal age in days)	4.3±2.6	6.7±1.2	Mann-Whitney test, p<0.001
Number of samples	1.5±0.7	1.9±0.7	Mann-Whitney test, p=0.036
Day of first specimen collection (postnatal age in days)	2.5±1.4	3.6±1.2	Mann-Whitney test, p<0.001

Abbreviations: SD, standard deviation; *N*, number.

Table 4

Estimated logistic regression model comparing the effects of MSC isolation, gender, gestational age, birthweight (in units of 100 g), sample number, and days of endotracheal intubation (up to 7) on the development of BPD.

Variables	Odds ratio	95% C.I. for Odds Ratio		P value
		Lower	Upper	
MSC	21.983	1.818	265.854	0.015
Gender (male)	0.232	0.039	1.362	0.106
Gestational age	1.400	0.635	3.086	0.404
Birth weight	0.561	0.331	0.950	0.032
Number of samples: 2	1.294	0.238	7.041	0.766
Number of samples: 3	2.152	0.226	20.544	0.505
Last day of intubation	1.300	0.740	2.284	0.361