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Early View

Research letter

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NASAL AIRWAY EPITHELIAL REPAIR AFTER VERY PRETERM BIRTH

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INTRODUCTION: Preterm birth rates are increasing and now account for more than 11% of global births. Simultaneously, advances in neonatal care have led to increased survival of lower gestation neonates. A complication of preterm birth, and the biggest determinant of survival, is lung and airway immaturity. After preterm birth, the immature respiratory system is exposed to pro-inflammatory stimuli like injury from resuscitation and oxygen toxicity. The airway epithelium, the physical barrier between insults and the airways, is particularly vulnerable to injury. If epithelial barrier integrity cannot be restored rapidly following damage (i.e. via aberrant repair), the respiratory system is left unprotected, increasing the risk of infection, inflammation and tissue damage. Altered epithelial repair may play an important role in the ongoing respiratory health problems experienced by preterm survivors including severe respiratory infections throughout early life, or low and declining lung function (1-3). Deficits are further exacerbated in those with bronchopulmonary dysplasia (BPD). The mechanisms contributing to ongoing respiratory problems are currently unknown, though likely begin in early life. Until now, understanding the role of the preterm epithelial barrier has been limited by a lack of appropriate cellular models. Our study aimed to assess the reparative capacity of the airway epithelium in survivors of preterm birth and its association with early life outcomes; with the hypothesis that preterm airway epithelial cells have an abnormal repair mechanism.

METHODS:

Study population: Primary nasal epithelial cells (NEC) were collected from infant survivors of very preterm birth (<32 weeks gestation; n=32, median (range) = 1.4 (1.2-1.5) years of age/1.2 (1.0-1.3) years corrected postnatal age). BPD was defined as: the requirement for at least 28 days supplemental O_2. Perinatal data and lung function outcomes were collected as part of the Preterm Infant Function and Clinical Outcomes (PIFCO) cohort study (Child and Adolescence Health Service, Perth, Western Australia; Ethics #2014083EP). Term born

infant's samples (n=6, median 2.5 (2.0-2.7) years) were provided through the Western Australian Epithelial Research Program (WAERP), (St John of God Subiaco Hospital; Ethics #901.1421). Written informed consent was provided by all parents.

Sampling and culture: NEC were collected from the nasal turbinate(s) as previously described (4, 5). Cellular morphology was assessed via light microscopy, and epithelial cell lineage confirmed by PCR and immunofluorescence (4). Confluent cultures were wounded using an Essen WoundMaker, and repair assessed over 72-hours using image-tracking software (IncuCyte ZOOM®, Essen Bioscience) (4). All experiments were completed between passage 2 and 3 to ensure consistency.

Statistics: Perinatal factors including; gestation, birthweight z-score, duration of respiratory support, BPD and antenatal/postnatal steroids were correlated with extent of wound closure (Spearman's Rho or point-biserial as appropriate). Mann-Whitney U test was used to assess statistical differences in wound closure rates (SPSS: v26). P values of <0.05 were considered statistically significant.

RESULTS: Successful cultures were established from 6 term (6 brushed, 100% culture success) and 22 preterm infants (32 brushed, 69% culture success). All cultures exhibited a typical cobblestone epithelial morphology (Fig. 1A & B). Epithelial lineage was confirmed at both gene and protein levels (term n=6, preterm n=8) (Fig. 1C & D-G); established cells solely expressed epithelial-specific markers cytokeratin 5 & 19, with no significant differences; but not the mesenchymal-specific marker, vimentin. Perinatal or anthropometric

factors including gestational age, chorioamnionitis and respiratory support were not associated with culture success (data not shown).

NEC cultures from infants born preterm had significantly impaired reparative capacity ($p<0.05$; Fig. 1L & M), while those from term infants completely repaired, typically by 60 hours (Fig. 1L). Further assessment identified a spectrum of repair responses in the preterm group (Fig. 1M). Interestingly, NEC from only four preterm participants fully healed by 72 hours, with cells from all remaining participants failing to completely repair (n=18). Of these, over half (10/18) had wound closure below 50% (Fig. 1L & M). Wound repair capacity did not differ between preterm infants with or without BPD, nor correlate with gestation, birthweight z-score, duration of oxygen or respiratory support. However, decreased repair was associated with the administration of antenatal steroids >24 hours prior to delivery (Fig. 1N).

DISCUSSION: This study is the first to identify functional defects in the reparative capacity in NEC of preterm infants. Almost all preterm infants in this cohort exhibited defective NEC repair in their second year of life, suggesting that not only is a defect present, it is retained beyond the neonatal period. Defective repair has previously been suggested by Been *et al*. (6) who showed that bronchoalveolar lavage fluid (BALf) collected from preterm infants post-birth elicited defective wound repair when added to immortalized commercially available alveolar type II cells. Our findings advance the field further, showing that the effect is intrinsic to the airway epithelium and improving the clinical relevance by using primary NEC obtained from preterm infants.

A correlation between defective repair and antenatal steroid administration was identified in the present study. Similarly, Been *et al.* (6) observed worse repair with the addition of BALf

of preterm infants receiving antenatal steroids. The association of antenatal exposure (in the absence of current steroid use) with dysregulated repair is intriguing and warrants further investigation into the impact of antenatal steroids on the epithelium. No additional correlations were observed between repair capacity and perinatal factors including gestation, birth weight, respiratory support or BPD diagnosis. The lack of correlation with perinatal factors suggests an underlying intrinsic mechanism may be driving the poor repair. Previous studies have shown defective repair in other chronic lung diseases such as COPD, asthma and cystic fibrosis (7-9). Our previous work has investigated underlying mechanisms of defective epithelial repair in childhood wheeze and asthma, and have found associations with impaired fibronectin-binding integrin expression (10, 11). It is possible a similar defect is driving dysregulated repair in the preterm epithelium, though alterations in cell migration and proliferation may also be contributing factor. Further research is required to identify and explore the underlying mechanisms driving poor repair in the preterm epithelium.

The clinical implications of aberrant NEC repair in preterm infants remains unknown. In other chronic respiratory diseases like asthma, the inability to maintain barrier integrity via defective repair has been associated with acute wheeze, symptom severity and increased hospital presentations (10). The dysregulated repair in preterm infants may in part explain high rates of hospitalisation, susceptibility to respiratory infection and longitudinal lung function decline observed throughout childhood. However, further investigation is needed to understand mechanistic drivers of dysregulated repair in these children. It should be noted that this study utilised nasal epithelial cells, which may not represent the lower airway. However, the unified airways hypothesis suggests upper airway cells can be used as a surrogate for the lower airway, though this remains unproven in a preterm cohort (12). We acknowledge the age difference between our cohorts, however it is unlikely this limits

interpretation given our prior work has found no age-related effects on repair capacity (9). Collectively, this study is the first to report a functional defect in NEC of infants born preterm. Only by understanding the cellular and molecular mechanisms underpinning poor respiratory outcomes after preterm birth, will it be possible to optimise the treatment and clinical management of this population.

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Statement of Ethics

This study was approved by the relevant human ethics committees (Approval #2014083EP, #901.1421), written informed consent provided by all parents.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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AUTHOR CONTRIBUTIONS: JH, DJE, TI carried out acquisition and analysis of data. JH, DJE drafted the manuscript. SJS, AK conceptualized the study, obtained funding, validated data for accuracy. All authors approved the final version.

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Demographic data for preterm infants and correlation to airway epithelial cell wound repair at 72 hours. Data presented as median (IQR) [range]. Correlation coefficients were calculated to % repair at 72 hours using either Spearman's rho correlation or point-biserial correlation as appropriate. Significant correlations ($p<0.05$) are denoted by *.

	Preterm	Correlation to % Repair at 72hours	Significance (p value)
Number of Participants (Male)	22 (17)	-0.019	0.932
Weeks of Gestation	28.3 (26.2-30.5) [24-31.7]	0.035	0.877
No. % Bronchopulmonary Dysplasia	8 (36%)	0.162	0.470
Birthweight Z-score	-0.02 (-0.8-0.45) [-1.89-1.79]	0.091	0.687
Age at Brushing (days)	508 ± 38.6 (447-583)	-0.262	0.239
Total Hours of Supplemental O_2 Required	198 (16.5-1279.5) [1-3381]	0.164	0.446
Total Hours of Respiratory Support (Any)	1223 (548.5 – 1920) [54-3082]	0.054	0.813
Total hours Mechanical Ventilation	11.5 (0.0-73.5) [0-1112]	0.007	0.974
Total hours CPAP	777 (225-1134) [54-1658]	-0.011	0.962
Total hours HHF	314.5 (0.0-719.75) [0.0-1124]	-.021	0.928
No. % Administered Surfactant	14 (64%)	0.024	0.915
No.% Received antenatal steroids >24h prior to delivery	14 (64%)	-0.442	**0.040***
No.% Received postnatal steroids	2 (9%)	-0.207	0.355
No. % Chorioamnionitis	9 (41%)	-0.205	0.360
No. % Wheeze in first year of life	13 (59%)	-0.177	0.432
No.% Respiratory hospitalisation in first year of life	9 (41%)	-0.205	0.360
No.% Atopic [a]	4 (18%)	-0.201	0.383
No.% Tobacco Smoke Exposure	6 (27%)	0.049	0.827
Shift[b] at 36 Weeks (kPa)	11.15 (9.6-12.5) [7.6-24.9]	-0.101	0.662
Shift at 1 Year (kPa)	6.70 (6.2-7.1) [5.5 – 8.9]	-0.062	0.799

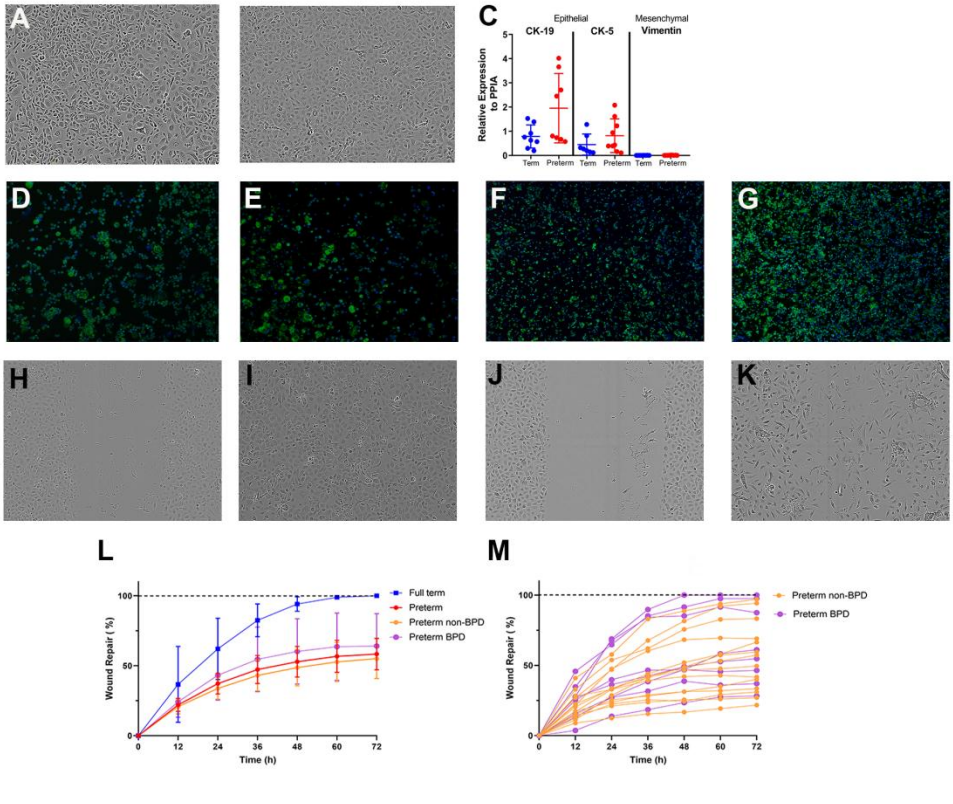
O_2 = Oxygen; CPAP =Continuous Positive Airway Pressure; HHF = Humidified High Flow

[a] Atopy defined as the presence of hayfever or eczema in the first year of life.

[b] Shift refers to the magnitude of which the oxygen-haemoglobin dissociation curve has moved along the x-axis.

FIGURE LEGEND

Fig. 1. Establishment, characterisation and functional assessment of primary airway epithelial cells from preterm infants with and without BPD and term controls. Established primary upper epithelial cell cultures of term (**A**) and preterm (**B**) born infants (10X magnification) exhibit a typical epithelial cobblestone morphology. (**C**) Gene expression of epithelial lineage markers CK-19 and CK-5 and mesenchymal cell lineage marker vimentin in term (blue, n=6) and preterm (red, n=8) primary epithelial cells. All established cultures strictly expressed epithelial lineage markers with their expression of CK-19 and CK5 and no expression of vimentin, the dashed line represents the positive control for vimentin. (**D-G**) Epithelial lineage expression was corroborated at the protein level using immunocytochemistry. Immunofluorescent staining of all cells using the nuclei stain (DAPI: blue), showed that all positively stained for CK-19, term **(D)** and preterm **(E)**, and CK-5 term **(F)** and preterm **(G)**. **(H-K)** Image of NEC repair for term (H & I) and preterm (J & K) at 0h (H & J) and 72h (I & K) (**L**) Repair rates of airway epithelial cells from term (blue, n=6), preterm (red, n=22), preterm non-BPD (orange, n=14) and preterm with BPD (purple, n=8) infants over a 72h period. Cultures from term infants completed full repair by 60h, whereas those from preterm infants were significant delayed and or failed to repair over the same period. (**M**) Repair rates of cultures from preterm non-BPD infants (orange, n=14) and preterm infants with BPD (purple, n=8). Diagnosis of BPD was not correlated with worse reparative capacity. Repair rates varied among cultures from preterm infants and no differences were observed between preterm BPD and non-BPD infants. Note C and H: Data presented as median and IQR. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$.


A
C
Epithelial
Mesenchymal
CK-19
CK-5
Vimentin
Relative Expression to PPIA
Term
Preterm
D
E
F
G
H
I
J
K
L
Full term
Preterm
Preterm non-BPD
Preterm BPD
Wound Repair (%)
Time (h)
M
Preterm non-BPD
Preterm BPD
Wound Repair (%)
Time (h)