CALL FOR PAPERS | Translational Research in Acute Lung Injury and Pulmonary Fibrosis

A preterm pig model of lung immaturity and spontaneous infant respiratory distress syndrome

Frank Caminita,1 Marie van der Merwe,2 Brittany Hance,2 Ramesh Krishnan,3 Sarah Miller,4 Karyl Buddington,5 and Randal K. Buddington2

1Draeger Medical, Telford, Pennsylvania; 2Department of Health and Sport Science, University of Memphis, Memphis, Tennessee; 3Division of Neonatology, Department of Pediatrics, University of Tennessee Health Science Center, Memphis, Tennessee; 4Loewenburg School of Nursing, University of Memphis, Memphis, Tennessee; and 5Director of Animal Care, University of Memphis, Memphis, Tennessee

Submitted 26 June 2014; accepted in final form 7 November 2014

Caminita F, van der Merwe M, Hance B, Krishnan R, Miller S, Buddington K, Buddington RK. A preterm pig model of lung immaturity and spontaneous infant respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 308: L118–L129, 2015. First published November 14, 2014; doi:10.1152/ajplung.00173.2014.—Respiratory distress syndrome (RDS) and bronchopulmonary dysplasia remain the leading causes of preterm infant morbidity, mortality, and lifelong disability. Research to improve outcomes requires translational large animal models for RDS. Preterm pigs delivered by caesarian section at gestation days (GD) 98, 100, 102, and 104 were provided 24 h of neonatal intensive care, monitoring (pulse oximetry, blood gases, serum biomarkers, radiography), and nutritional support, with or without intubation and mechanical ventilation (MV; pressure control ventilation with volume guarantee). Spontaneous development of RDS and mortality without MV are inversely related with GD at delivery and correspond with inadequacy of tidal volume and gas exchange. GD 98 and 100 pigs have consolidated lungs, immature alveolar architecture, and minimal surfactant protein-B expression, and MV is essential at GD 98. Although GD 102 pigs had some alveoli lined by pneumocytes and surfactant was released in response and MV is essential at GD 98. Although GD 102 pigs had some alveoli lined by pneumocytes and surfactant was released in response to MV, blood gases and radiography revealed limited recruitment 1–2 h after delivery, and mortality at 24 h was 66% (35/53) with supplemental oxygen provided by a mask and 69% (9/13) with bubble continuous positive airway pressure (8–9 cmH2O). The lungs at GD 104 had higher densities of thin-walled alveoli that secreted surfactant, and MV was not essential. Between GD 98 and 102, preterm pigs have ventilation inadequacies and risks of RDS that mimic those of preterm infants born during the saccular phase of lung development, are compatible with standards of neonatal intensive care, and are alternative to fetal nonhuman primates and lambs.

Despite advances in clinical care, respiratory disease syndrome (RDS) remains the single most common cause of morbidity and mortality in the first month of life after preterm birth (72). The more severe bronchopulmonary dysplasia (BPD) that afflicts preterm infants, particularly those that require ventilation support and suffer other complications (35), has lifelong consequences (36). RDS and BPD are clinical syndromes of chronic respiratory insufficiency that without intervention lead to hypoxemic respiratory failure and death. The risk and severity of RDS and BPD among the 12% of infants that are born preterm (<37 wk of gestation; see Ref. 12) are inversely related with gestational age at birth (3, 4), with the risk decreasing 31% with each additional week of gestation (47). The combined increases in the number of preterm births and the percentage of infants surviving delivery earlier in gestation are resulting in more infants with immature lung development who are susceptible to adverse sequelae that can persist throughout life (24). However, improvements in outcomes have been limited (7).

Ethical considerations restrict using neonates as experimental subjects, making animal models essential for evaluating ventilation support strategies after preterm birth. Newborn small animal models (e.g., mice, rats, guinea pigs, rabbits) are not compatible with neonatal intensive care unit (NICU) equipment and protocols, and chronic studies are restricted to term or near-term newborns with lungs that are sufficiently mature for extraterrine life. Additionally, the narrow-diameter endotracheal (ET) tubes or 22- to 23-gauge needles that are required for small animal models have high resistance, complicating clinical translation and interpretations of the relationship between pressure and volume of ventilation. Two large animal models, the preterm baboon and fetal lamb, dominate studies of the lung immaturity, the responses to mechanical ventilation (MV), and the safety and efficacy of existing and experimental interventions, such as surfactant administration and prenatal steroids (2). Baboons and other nonhuman primates are valuable models for lung development and responses to MV (2). However, the limited access to baboon colonies, high costs, and the need for specialized facilities and care has restricted their use as models (71). Lambs also have limitations as a translational model. The low ex utero survival during the saccular phase of lung development requires the fetus to remain connected to the placenta, and the studies are acute (most are <6 h) and do not include the dramatic changes in pulmonary hemodynamics when the umbilical cord is clamped (66). When lambs are used for chronic ex utero studies, they are harvested at later stages of gestation that are relevant to preterm infants delivered at 32 wk and later (22). Moreover,
the long necks of lambs (2) create an anatomic dead space that is larger than in infants and pigs and limits the assessment of noninvasive ventilation, such as continuous positive airway pressure (CPAP).

In light of these limitations of baboons and lambs, we evaluated preterm pigs as a large animal model that is clinically relevant for infants delivered at 30 wk and earlier. Preterm pigs are compatible with chronic intensive care using NICU equipment and protocols (22), pregnant sows are readily and widely available, and the large litters permit comparisons of ventilation support strategies using genetically defined siblings of known gestational ages. Moreover, preterm and neonatal pigs are already recognized as models for human infants (9, 44, 56), and the relationship between gestational age at delivery and requirements for intensive care has been partly established (22) with multiple litters of preterm pigs delivered at gestational days (GD) 91, 94, 97, 100, 104, and 111. Our objective was to identify a gestational age suitable for investigating the pathophysiology of spontaneous RDS and for evaluating chronic ventilation support strategies. We used single litters of pigs delivered at GD 98 (delivery before GD 97 results in 100% mortality in <40 min, even with MV; see Ref. 22), 100, 102, and 104 to verify the relationship between gestational age and the intensity of critical care required for the first 24 h of ex utero life. An additional eight litters of preterm pigs were delivered at GD 102 as a model for preterm infants that are surfactant deficient, are at risk of RDS, and only with intense critical care and MV have a high likelihood of survival.

MATERIALS AND METHODS

Preterm pigs were harvested at GD 98 (14 pigs from 1 litter), 100 (9 pigs from 1 litter), 102 (107 pigs from 9 litters), and 104 (10 pigs from 1 litter) from sows of a defined and consistent proprietary genetic lineage developed from several strains of pigs. The sows were artificially inseminated by similarly genetically defined boar semen, thereby reducing genetic variance. Gestation length for sows of the genetic line used is 114 to 115 days. All procedures associated with the procurement of sows, the harvest of fetal pigs by caesarian section, and postnatal care and sampling were approved by the Institutional Animal Care and Use Committees of the University of Tennessee Health Sciences Center (site of caesarian sections) and the University of Memphis (site for critical care of preterm pigs). Antenatal steroid therapy was not provided to enhance lung development, since one of the objectives was to evaluate normal pulmonary characteristics and responses after preterm birth.

Delivery and immediate postnatal care of preterm pigs. The preterm pigs were harvested following established methods (56). Briefly, the sows were sedated (5 mg/kg im Telazol) and placed on gas anesthesia (isoflurane). A sterile surgical field was prepared along the anesthetic (lidocaine) was injected along the incision site. The uterus was exposed, and individual pigs were removed through an incision. Blood in the umbilical cord was squeezed into the newborn pig to maximize blood volume before the cord was clamped and cut. Each newborn pig was dried using a prewarmed towel and held vertically with the head down to drain fluids before the mouth, nose, and upper airway were cleared of secretions using vacuum aspiration.

Once a preterm pig started spontaneous ventilation it was placed in a warmed incubator (40–42°C) with supplemental oxygen. The pigs were initially (first 2–3 h after delivery) group housed and placed side by side. This reinforces spontaneous breathing, whereas individually housed preterm pigs often cease breathing (unpublished observations). Gentle chest compression and pulling of the tongue were used to resuscitate pigs that did not begin to spontaneously ventilate after delivery. If this proved ineffective a respiratory stimulant [Dopram (doxapram hydrochloride; 0.1 ml)] was administered either sublingually or by accessing a vessel in the umbilical stump.

After the litter was delivered (10–20 min, depending on litter size) the sow was killed, and a portion of a lower lung lobe was collected and served as control tissue while the pigs were transported to an intensive care facility in a warmed chest with supplemental oxygen. The supplemental oxygen was provided to GD 102 and earlier pigs using a mask placed over the snout. Upon arrival, the pigs were weighed and placed on bags of water in incubators maintained at 38–39°C for thermoregulation. Within 3 h after delivery, an umbilical vessel catheter (3.5 Fr.; Utah Medical Products, Midvale, UT) was inserted in one of the two umbilical arteries, advanced 15–20 cm/kg, and secured in the umbilicus using sutures and to the skin using tape, staples, and superglue (ethyl cyanoacrylate). Even GD 98 pigs are active, and unless the umbilical artery catheter (UAC) is adequately secured it can be removed by movement of the extremities. The placement of the UAC allowed for provision of parenteral nutrition (PN), administration of medications, and for removal of arterial blood for gas analysis. Once the UAC was placed and secured, PN was initiated (4 ml·kg⁻¹·h⁻¹) using an all-in-one solution formulated by a pharmacy (People’s Pharmacy, Memphis, TN) with (per L) 116 g glucose, 60.5 g of amino acids (Travasol; Baxter Healthcare, Deerfield, IL), and 31.3 g lipid (Intralipid; Baxter Healthcare), with electrolytes, vitamins, and minerals (3.33 ml Infuvite Pediatric and 4.4 ml Multitrace elements; Baxter Healthcare). Within 30 min after the start of PN, the pigs received 5 ml/kg of plasma prepared from maternal blood collected aseptically during the surgery in heparinized Vacutainer tubes from a uterine vein. Fetal pigs do not acquire maternal immunoglobulins in utero, and the provision of maternal plasma partially compensates for the deprivation of colostrum, which normally provides passive immunity (56). Cephalexin (50 mg/kg; Glaxo Wellcome) was administered one time via the UAC for prophylaxis against infections. Lactated Ringer solution was provided to maintain fluid homeostasis (0–4 ml/h) based on tissue perfusion.

The pigs were individually housed after the UAC was placed, and nutrition and ventilation support were initiated. Thereafter, heart rate, oxygen saturation, and perfusion status were monitored using pulse oximetry (Radical 7, Radical 8, or Radical 5; Massimo). Hematology and blood chemistries were measured at 1- to 2-h intervals using the HM2 and VetScan platforms and Comprehensive Diagnostic rotors (Abaxis, Union City, CA). Skin color was assessed hourly as an indicator of general health status. After intravascular access was established, Dopram was administered via the UAC to pigs that ceased spontaneous breathing. Subsequently, a single loading dose of caffeine was administered (10 mg/kg) as a respiratory stimulant. Pigs that became active during the study period and required sedation received ketamine to effect.

Ventilation protocols. Exogenous surfactant was not administered to the pigs, since this would interfere with measurement of endogenous surfactant production. A single GD 98 pig was intubated immediately after the airway was cleared, and ventilation was started using a transport ventilator with 100% oxygen delivered at a peak inspiratory pressure (PIP) of 15 cmH₂O, a positive end-expiratory pressure (PEEP) of 5 cmH₂O with a respiratory rate of 60 breaths/min. The remaining GD 98 pigs were provided 100% oxygen using a mask. Two pigs of average body weight from each of the litters of GD 100, 102, and 104 pigs were randomly assigned to MV and were intubated after arrival at the intensive care facility (1–2 h after delivery) using ET tubes (2.5 or 3 Fr.). Initially, uncuffed ET tubes (Malinckrodt) were used following NICU protocols for preterm infants. However, with some pigs, uncuffed plastic ET tubes can result in variable and sometimes excessive leakage (>80%) that exceeded the leaks commonly seen in human infants. When this occurred, cuffed rubber ET tubes (2.5 Fr.; Jorgensen Laboratories, Loveland, CO) were used to reduce leakage to <10%. After placement, the ET tubes were connected to neonatal ventilators (Drager Babylog VN500) using Pres-
Innovative Methodology

L120

PRETERM PIG AS A MODEL FOR NEONATAL LUNG DISEASE

sure Control Ventilation with Volume Guarantee in the Assist Control mode (PC-AC-VG), a targeted tidal volume of 5 ml/kg, and a PEEP at 4 cmH₂O. The minimum PIP needed to target the tidal volume was automatically adjusted by the ventilator. The initial respiratory rate was set at 40 breaths/min and the fraction of inspired air as oxygen (FIO₂) was set at 40%. Arterial blood gas analyses were performed (iSTAT and CG4 cartridges; Abaxis) at the start of MV and every 4 h thereafter. The initial settings were adjusted, if necessary, based on the results, and if changes were required follow up analyses were made every 30 min. The FIO₂ was adjusted based on blood gas analysis of PaO₂ in combination with pulse oximetry with a targeted SpO₂ range of 92–95%; SpO₂ was directly related with changes in FIO₂. In the absence of spontaneous breathing in the AC-VG mode, the set ventilator rate was reduced from 40 to 30 breaths/min. This did not cause a significant decrease in PaO₂, but the resulting moderate hypercapnia caused by the increase in carbon dioxide to 40–50 mmHg is considered appropriate for preterm infants (57). Pigs that developed metabolic acidosis (pH <7.2) were given a bicarbonate solution (10 mmol) via the UAC. Ventilation support was provided for 24 h.

The remaining GD 100 and 104 pigs and some of the GD 102 pigs (n = 53) were used as controls and had an open mask placed over the snout to provide 100% oxygen at 2–3 l/min (NC group). The mask did not form a seal and therefore did not increase airway pressure. The supplemental oxygen was provided throughout the 24-h period for GD 100 and 102 pigs but for only 8 h to the GD 104 control pigs. The remaining GD 102 pigs (n = 13) were provided bubble continuous positive airway pressure (bCPAP) for 24 h to assess efficacy in light of evidence for improved outcomes (37). This was accomplished using masks with a rubber membrane with a hole that formed a seal around the snout. A mixture of 40% O₂ and 60% room air was provided at a rate of 5–6 l/min. An outflow tube from the mask was submerged 6 cm in water to create a positive airway pressure. Spontaneous breathing could be inhibited by submerging the tube 10 cm or deeper. Pigs that developed symptoms of RDS (increased rate and work of breathing, heart rate <100 for at least 10 min, pallor and poor tissue perfusion, nonresponsive, persistent pH <7.2 and PaO₂ <80, and increased pCO₂ and lactate) were not transitioned to MV, were killed and necropsied, and tissues were collected.

Tissue harvesting and processing. After 24 h the surviving pigs were killed (1 ml/kg iv Euthasol; Virbac, Fort Worth, TX). Digital radiography was used to assess lung inflation of the GD 102 pigs before death. Necropsies were performed immediately after death, whether due to RDS, dying, or unknown causes to visually assess the lungs and collect tissues. After the heart and lungs were removed en bloc, bronchoalveolar lavage fluid (BALF) was collected from the left lung by inserting an ET tube and instilling 2 ml of normal saline. The mixture of saline and lung exudate was aspirated, placed in a cryogenic tube, flash-frozen in liquid nitrogen, and stored at −70°C until analyzed for phosphatidylcholine (PC) and fibroblast growth factor. Spontaneous breathing could be inhibited by submerging the tube 10 cm or deeper. Pigs that developed symptoms of RDS (increased rate and work of breathing, heart rate <100 for at least 10 min, pallor and poor tissue perfusion, nonresponsive, persistent pH <7.2 and PaO₂ <80, and increased pCO₂ and lactate) were not transitioned to MV, were killed and necropsied, and tissues were collected.

Biochemical and molecular markers of lung maturation. A portion of the BALF collected from GD 100 and 102 pigs was used to measure PC by a colorimetric assay (Cayman Chemical, Ann Arbor, MI). Another portion of the BALF collected from GD 100 pigs was used for measurement of keratinocyte growth factor 7 (KGF7) using a commercial assay kit (USCN Life Science, Houston, TX).

Western blotting was used to detect SP-A and SP-B, using lysates prepared from frozen tissue harvested from the lower left lung. β-Actin was used as a loading control. Although ELISA kits for SP-B are commercially available, Western analysis was used because of greater specificity. The lung tissue was homogenized in 1 ml of cold RIPA buffer (no. BP-115D; Boston Bioproducts, Ashland, MA) supplemented with 5 mM EDTA and Halt Protease Inhibitor Cocktail (Thermo-Scientific, Rockville, IL). The homogenate was centrifuged (10,000 g; 20 min; 4°C), and protein content of the supernatant (cell lysate) was measured. Lysate protein (50 μg) was resolved on 4–15% (for SP-A) or 12% (for SP-B) SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with anti-SP-A primary antibody (AB3420; Millipore, Billerica, MA) at a dilution of 1:2,000 in 5% bovine serum albumin (BSA), anti-SP-B primary antibody (HP 7002; Hycult Biotech, Plymouth Meeting, PA) at 1:100 in 5% BSA, and anti-β-actin primary antibody (49678; Cell Signaling Technology, Danvers, MA). The blots were washed and incubated with a secondary donkey anti-rabbit antibody conjugated with horse-radish peroxidase (1:10,000) in 5% boiling grade milk. After secondary antibody hybridization, the blots were washed and developed using SuperSignal West Pico ECL reagents (34080; Thermo-Scientific). Band intensity was quantified using a Chemiluminescent imager (Fotodyne, Hartland, WI) and ImageJ software (Software program; National Institutes of Health).

Statistical analysis. Differences between stages of gestation and treatments (controls vs. MV) were detected using t-tests with P < 0.05 as the critical value of significance.

RESULTS

The fetal pigs harvested between 85 and 92% of term had body weights ranging from 356 to 1,263 g (Table 1). The fetuses, even those harvested at GD 98, exhibited spontaneous ventilation within 2–3 min after delivery.

Health and gross pathology of control pigs. Fetuses delivered at GD 98 suffered significant respiratory distress beginning almost immediately after birth, and only the one pig provided MV survived past 3 h after delivery. Of the seven control pigs harvested at GD 100 and provided supplemental oxygen via a mask, four died of symptoms characteristic of RDS within 3 h after delivery. The remaining three that survived for 24 h with supplemental oxygen had lungs characterized by extensive atelectasis.

Survival of GD 102 pigs provided bCPAP (31%) was similar to that of the control pigs (34%). When the control and bCPAP GD 102 pigs were pooled, survival rates varied among the seven litters, which can be partly attributed to differences in average birth weight (Fig. 1). Specifically, litters with lower average birth weights experienced higher 24 h morbidity and mortality.

The GD 100 and 102 control pigs that were provided only supplemental oxygen initially did well based on skin color, blood gases, and pulse oximetry. However, the respiration rate

Table 1. Average body weights of preterm pigs harvested at different days of gestation

<table>
<thead>
<tr>
<th>Age</th>
<th>Body Wt, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD 98 (14, 1)</td>
<td>743 ± 206</td>
</tr>
<tr>
<td>GD 100 (9, 1)</td>
<td>858 ± 50</td>
</tr>
<tr>
<td>GD 102 (102, 9)</td>
<td>843 ± 19</td>
</tr>
<tr>
<td>GD 104 (10, 1)</td>
<td>805 ± 45</td>
</tr>
</tbody>
</table>

GD, age in gestational days. Values in parentheses are no. of individual pigs and the no. of litters, respectively.
started to increase in 70–80% of the pigs as early as 2 h after delivery and as late as 12 h. This coincided with a decline in gas exchange (based on pulse oximetry and blood gases) that was visually evident by the skin color changing from pink to gray. These pigs became moribund and had to be killed, or death rapidly ensued. The provision of bCPAP did not reduce the incidence of respiratory distress. The GD 100, 102, and 104 control pigs that did survive for 24 h appeared healthy based on skin color, pulse oximetry, and blood gases.

The pigs delivered at GD 104 did not require intense ventilation support, did not show symptoms of respiratory distress, and thrived with supplemental oxygen provided for only the first 4–8 h after delivery with room air thereafter. Of the eight GD 104 pigs that were used as controls (supplemental oxygen for 8 h), one died within 24 h of delivery from unknown causes.

Health and gross pathology of pigs provided MV. Even with immediate intubation and use of MV, the GD 98 pigs did not survive for >8 h, and at death the lungs had regions with obvious signs of atelectasis and trauma. After 24 h the lungs of the GD 100 pigs allocated to MV (n = 2) had regions that remained consolidated with other areas that appeared to have damage consistent with ventilator-induced lung injury (VILI).

All 41 of the GD 102 pigs that were placed on MV survived for 24 h. Metabolic acidosis (blood pH <7.2) was common among the ventilated pigs, and many developed bradycardia and reduced tissue perfusion before death. Regions of atelectasis were evident after 24 h of MV, with variation in the extent.

The two GD 104 pigs that were placed on MV survived for 24 h and maintained acceptable blood gases, and a large proportion of the lungs was recruited and appeared normal.

Radiographs of GD 102 pigs. The lungs of newborn (<2 h) GD 102 pigs before assignment to controls were fluid filled and unexpanded (Fig. 2, A and B). The lungs of control pigs that survived 24 h remained consolidated (Fig. 2, C–F). Radiographs of GD 102 pigs after 24 h of MV revealed partial recruitment of the lungs, which was consistent with gross pathology (Fig. 2, G and H).

Qualitative histology. The following description of qualitative histology was made using sections prepared from the center of the right lung. There was histological evidence of incomplete ventilation at all ages. The magnitude of consolidation after 24 h decreased with increasing gestational age at birth.

The lungs of pigs harvested at GD 98 and 100 that were allowed to spontaneously breathe remained extensively consolidated, with only limited regions with open spaces. This was obvious at low magnification (Fig. 3). At higher magnification, the alveolar architecture did not exhibit the thin walls typical of the normal adult lung, and the small proportion of alveoli that were present was not uniformly open. Despite the high cellularity, there were no signs of inflammatory cell infiltration, even in pigs that died with symptoms consistent with RDS.

After 24 h of spontaneous breathing, the lungs of GD 102 pigs were more open than those of similar-aged GD 100 pigs, and a lower proportion of the lungs appeared consolidated (Fig. 3). There were still some areas that remained consolidated, with distinct differences in openness seen between adjacent lobes and even within lobules. The thin-walled alveoli seen in GD 102 pigs were more abundant than at GD 100 and were lined by cells that appeared to be pneumocytes. Still, the paucity of thin alveolar walls of control GD 102 pigs and the low alveolar patency were consistent with surfactant deficiency. Cellularity of the lungs of 24-h-old control GD 102 pigs was high compared with adult lungs, but again without evidence of inflammatory cell infiltration.

The lungs of control GD 104 pigs after 24 h of spontaneous breathing had a greater proportion of openness with higher densities of thin-walled alveoli compared with GD 102 pigs. The lungs were largely open, but not completely, and abundant thin-walled alveoli were evident. None of the spontaneously breathing GD 104 pigs had the fibrinous arrangement of hyaline membranes indicative of air compartment edema, although some of the air spaces had evidence of proteinaceous deposits.

The responses to MV varied across ages (Fig. 4). The lungs of GD 98 pigs provided MV for 8 h remained consolidated. Providing 24 h of MV to the GD 100 pigs recruited portions of the lungs, with the conducting airways uniformly open, from the bronchi to the alveolar ducts. However, there were regional and lobule differences in the extent of recruitment based on degree of openness. Moreover, despite the use of MV, microatelectasis remained extensive, although not as much as seen in the 24 h control GD 102 pigs. Based on the histology, a large fraction of the gas exchange by GD 102 pigs would be taking place in the conducting regions rather than the alveoli. Moreover, there was evidence of alveolar damage based on apparent fusion of alveoli (Fig. 4F). The lungs of GD 104 pigs after 24 h of MV were extensively recruited, although regions of atelectasis remained evident. At higher magnification, there was evidence of a greater proportion of thin-walled alveoli and less microatelectasis compared with GD 102 pigs provided MV.

Biochemical and molecular markers of lung maturation. Lung tissues collected from preterm pigs harvested at GD 98, 100, 102, and 104 demonstrated an age-dependent increase in the expression of SP-B (Fig. 5A). Moreover, pigs delivered at GD 102 and subject to MV for 24 h showed a dramatic increase in SP-B expression, whereas actin levels were unchanged (Fig.
SP-A was not highly expressed by any of the preterm pigs but was detected in lysates prepared from the sows, and there was also no change in expression with MV (data not shown).

PPC concentrations increased between GD 100 and 102 (Fig. 6). Moreover, providing ventilation support to GD 100 preterm pigs accelerated secretion of PC in BALF, resulting in PC concentrations comparable to those of GD 102 pigs.

KGF7 was detected in BALF collected from GD 100 pigs that died within 1 h after delivery \( (n=4; \text{Fig. 7}) \). Concentrations 24 h later were higher in BALF from pigs provided MV \( (n=2) \) and slightly but not significantly higher for pigs receiving supplemental oxygen by a mask \( (n=3) \).

**DISCUSSION**

Preterm pigs reproduce the patterns of pulmonary development, surfactant expression and composition of preterm infants (26), and RDS histopathology (15). Similar to preterm infants, the responses of preterm pigs to extrauterine life and the incidence, onset, and severity of RDS are related with gestational age. RDS develops spontaneously; involves one or both lungs; is consistent with decreased compliance and residual lung capacity; is associated with gross, radiographic, and histopathological evidence of extensive atelectasis; and results in compromised blood gases and bradycardia. Even though exogenous surfactant therapy immediately after preterm birth might have improved outcomes and reduced RDS among the pigs (22), doing so would have compromised evaluating postnatal secretion of endogenous secretion. Furthermore, routine administration of surfactant is not universal for newborn infants delivered at \( \geq 28 \) wk of gestation. The ability to provide chronic ex utero intensive care to preterm pigs harvested at gestational ages that are relevant to infants delivered before \( 32 \) wk will enhance efforts to improve ventilation support strategies and outcomes beyond the current incremental changes (7).

**Lung development of humans and pigs and risk of RDS**

Lung growth in pigs is matched to body size, and between 40 and 96% of gestation the lungs average 3.26% of body weight (40). Based on sonographic measurements of lung volumes (10, 55, 62) and assuming a lung mass of 1 g/cm³, the lungs of human fetuses average 2.5% of body weight from 20 to 36 wk of gestation. This is similar to the 2.8% of body weight represented by the lungs of preterm infants born at 28–30 wk (20).
Human lung development on a cellular and tissue basis is separated into well-characterized phases (1, 30, 64). During the pseudoglandular phase from 6 to 16 wk of gestation the airways begin to develop and extend to terminal bronchioles. Because extrauterine life is presently not possible because of a lack of adequate functional surface area for gas exchange, we did not attempt to harvest preterm pigs during this phase of lung development.

The next phase of human lung development (canicular or acinar; weeks 16–24) is when alveolar ducts form and become lined by type II cells capable of surfactant synthesis that can differentiate into type I cells associated with gas exchange. Only after saccules lined with type 1 cells capable of gas exchange develop during the final weeks of the canalicular phase does extrauterine survival become possible, but, because of the limited capacities for gas exchange, MV is essential immediately after delivery. The inability to resuscitate GD 91 and 94 pigs (22) is consistent with inadequate densities of saccules for ventilation.

Because the saccules and the associated capillaries continue to proliferate during the saccular phase (weeks 24–34), the risk of RDS and the need for intensive ventilation support decline. The GD 98, 100, 102, and 104 pigs span this critical period of development. The gross anatomy and histopathology of the lungs, gelatinous skin, underdeveloped eyes, and other anatomical and clinical features of newborn GD 98 (present study) and GD 97 (22) pigs, along with the significant mortality even with immediate MV using high PIP (25–30 cmH2O) at 60 beats/min and FIO2 up to 100%, mimics human infants delivered at 24 wk that require MV, exogenous surfactant administration, and intensive care, yet still have only about a 50% likelihood of survival (61). The improved lung functions and 8 h survival of GD 97 pigs after administration of antenatal steroids to the sow (22) are also consistent with delivery early in the saccular phase.

The GD 100 pigs, like 28- to 30-wk infants, are at an intermediate stage of the saccular phase when alveoli are beginning to develop, capacities for gas exchange are improving, and the risk of RDS and need for MV are decreasing. Although only 31% of the control GD 102 pigs developed RDS and died before 24 h, the lungs of the survivors had extensive regions of atelectasis. The variation in the time of onset, incidence, and severity of RDS among the GD 102 litters is similar to the “honeymoon” period during which many 26- to 28-wk infants spontaneously breathe, and blood gases remain within an acceptable range with noninvasive ventilation support before some develop pulmonary distress and require MV for survival (61). It is uncertain if MV would have eventually been required for the control GD 100 pigs that had lower arterial pO2, increased pCO2, and extensive regions of pulmonary atelectasis 24 h after delivery.

The GD 102 pigs, like 28- to 30-wk infants, are at an intermediate stage of the saccular phase when alveoli are beginning to develop, capacities for gas exchange are improving, and the risk of RDS and need for MV are decreasing. Although only 31% of the control GD 102 pigs developed RDS and died before 24 h, the lungs of the survivors had extensive regions of atelectasis. The variation in the time of onset, incidence, and severity of RDS among the GD 102 litters...
suggests birth weight and litter are confounding variables that must be included in statistical models. Other factors to be considered include litter size and the parity, nutrition, and health status of the sow. Gender may also contribute, with males differing from females with respect to surfactant composition and function (29) and an increased risk of RDS (4).

Not until about week 32 when distinct alveoli become abundant does the risk of RDS decline markedly (61). The even more advanced lung maturity and >80% survival of the GD 104 pigs with minimal ventilation support (present study, 22) and >90% survival rates for the GD 105 pigs used as a model for NEC (9, 56) are consistent with preterm infants born at the transition from the saccular phase to the alveolar phase who have a <20% RDS incidence (61). Despite the lack of RDS, the areas of atelectasis that remained at 24 h and other indicators of physiological immaturity (22) suggest this gestational age is relevant to 32- to 34-wk infants that generally require minimal ventilation support.

Fig. 4. Histology of the right lung harvested from GD 98 pigs after 4 h of mechanical ventilation using a transport ventilator (A and B) and from GD 100 (C and D), 102 (E and F), and 104 (G and H) pigs after 24 h of mechanical ventilation using Pressure Control Ventilation with Volume Guarantee in the Assist Control mode of Draeger VN500 Babylog Pediatric Ventilators. Photos in the left column were taken using a ×1.25 objective lens, the bars represent 1,000 μm, and photos in the right column were taken at ×10 with the bars for 100 μm.
The alveolar phase of lung development (36 wk to 18 mo after birth) involves a branching pattern of lung growth whereby the associated airways multiply and there is expansion of alveoli increasing the surface area for gas exchange. Infants delivered from 37 to 40 wk are considered to be at “term” and have an RDS incidence of <2% (61). The present study did not include gestational ages that are representative of this phase of lung development.

The biochemical markers of lung maturation provide additional evidence for the relevance of the preterm pig. Surfactant proteins are present in human amniotic fluid at 24 wk (14, 26), and levels of expression increase as gestation continues (31, 32). The presence of SP-B in lysates prepared from GD 100 and even more in GD 102 preterm pigs (present study) reveals an early and increasing expression, with a similar developmental trajectory in baboons (17, 48) and lambs (58). These findings are clinically relevant, since SP-B is responsive to glucocorticoid and caffeine therapy (23). Pulmonary surfactant is a mixture of about 10% proteins and 90% lipids, which is relatively consistent across species (11). Phospholipids and particularly PC are the dominant components of the lipid component of surfactant. The detection of PC in BALF collected from GD 100 pigs suggests phospholipid synthesis does not limit early surfactant production for the preterm pig.

Responses to bCPAP and MV. The use of CPAP avoids VILI caused by currently used MV protocols (18, 37, 63). However, the proactive use of bCPAP resulted in lung recruitment that was at best partial (present study). Moreover, the similar 24-h outcomes compared with control pigs confirm CPAP does not prevent RDS and the eventual need for MV (18). CPAP does increase the risk of pneumothorax (18) and is less effective for preterm infants who are small for gestational age (SGA) or born earlier in gestation (60).

The initiation of MV within 2 h after delivery and before onset of clinical symptoms of RDS resulted in 100% survival at 24 h for GD 100 and 102 pigs at risk of RDS. The radiographs of GD 102 pigs provided MV revealed greater recruitment of the lungs relative to the control and bCPAP pigs, areas of consolidation remained after 24 h, and there was evidence of alveolar rupture consistent with VILI. The lack of histopathological evidence of inflammation and inflammatory cell infiltration among preterm pigs with RDS and those provided MV suggests >24 h are necessary to elicit detectable levels of inflammatory molecules, even though patterns of gene expression can respond sooner.
The increased concentration of surfactant in response to MV is a novel finding and suggests the physical expansion of the lungs triggers secretion. The relationship between MV and KGF7, which regulates proliferation of pulmonary epithelial cells and expression of surfactant proteins (70), is also significant. Preterm infants with higher concentrations of KGF7 in BALF have a lower risk of acute lung injuries that can lead to BPD (19), whereas infants that develop BPD are characterized by lower expression of KGF (6). The lower concentration of KGF7 measured in the BALF from control GD 100 pigs corresponded with poorly recruited lungs, and a higher risk of RDS, with BPD a likely sequelae. The higher concentrations of SP-B and KGF7 in BALF from the MV pigs suggest the protractive use of MV may reduce the risk of RDS, although this needs to be determined.

**Lung development and studies with other species.** Investigations of fetal lung development have been dominated by seven animal models, including the pig (Table 2). The saccular phase of lung development is of clinical importance and particularly early in this phase when the risk of RDS is higher. Among small animal models, the saccular phase begins relatively early in the precocial guinea pig (75%), later in the altricial rabbit (84%), and just before birth for mice and rats. The saccular phase begins for sheep (28) and baboons (16) at ~75 and 71% of gestation, respectively. The majority of fetal lambs are used at about GD 125 and later, well after the start of the saccular phase at ~GD 108. The gestation of baboons is more variable, making it difficult to predict beforehand the stage of lung development at the time fetuses are studied. The shorter gestation of the pig compared with baboons and sheep does result in a more compressed sequence of lung development, potentially shortening the period needed to evaluate postnatal responses.

Precocial lambs and pigs have lungs that are larger and more developed compared with newborn infants and baboons that are altricial and less active (41) with a greater proportion of the alveolarization phase occurring after birth (49). Similarly, altricial mice, rats, and rabbits have lungs that are less developed at birth compared with the precocial guinea pig.

**The preterm pig as a translational model.** Because of similar pulmonary characteristics and other attributes, pigs have been developed as models for cystic fibrosis research (33, 46, 51–54, 68) and as potential sources of lungs for xenotransplants (42, 43). Preterm and newborn term pigs have been used to search for biomarkers of fetal lung maturation and risk of RDS (27), postnatal changes in pulmonary blood flow (25), for acute studies of lung injury (39, 50), and for exploring interventions for surfactant deficiency associated with RDS (69). The present and previous (22) findings establish preterm pigs delivered between GD 98 and 104 as a translational model for the almost 4% of infants born before 34 wk (12) with extreme lung immaturity that pose the greatest challenges for acute and chronic intensive care. Importantly, chronic care can be provided to preterm pigs in the saccular phase of lung development with the same ventilation, fluid, and nutrition support protocols, monitoring equipment, surgical procedures, and clinical interventions used for preterm infants in the NICU. Although not used for the present studies, additional catheters can be placed in the other umbilical artery for invasive blood pressure monitoring and in the single umbilical vein.

Timed pregnancy sows of known genetic lineages are available all year from numerous suppliers. In contrast, the availability of lambs is seasonal, there are fewer suppliers, genetics are not as well defined as for pigs, and there are fewer molecular and genetic probes. The large litter sizes of sows (often >12 pigs) permit comparisons of different ventilation support strategies using siblings that share genetics, gestational length and uterine environment, and with birth weights that are similar to those of preterm infants. Moreover, most litters have one or more small pigs that represent a natural and spontaneous model for intrauterine growth restriction (IUGR). The resulting SGA pigs are likely to have compromised lung development (38), delayed surfactant protein expression (45), and be at greater risk of RDS (8), coinciding with higher morbidity for litters with lower average body weights (present study). The opportunity to directly compare SGA pigs with littermates of normal birth weight will provide insights into the consequences of being preterm and SGA. Because baboons and sheep typically have singleton births and occasional twins, many more pregnant females are needed to obtain adequate sample sizes. Furthermore, to study the consequences of being SGA, IUGR must be induced, which complicates comparisons of SGA and normal-weight siblings and introduces confounding variables.

Appropriate preterm animal models should also share with preterm infants similar patterns of development and ex utero responses for other organ systems. The preterm pig meets these criteria based on the present findings, our previous research (9, 56), and other reports for preterm pigs (22, 27, 50, 67). Notably, trajectories of development for other critical organs are shared by pigs and humans, but not lambs (22).

<p>| Table 2. Stages of lung development for humans for six commonly used animal models (assembled from various sources) and for pigs (67; current study) |</p>
<table>
<thead>
<tr>
<th>Stages of Lung Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td>Human</td>
</tr>
<tr>
<td>Weeks</td>
</tr>
<tr>
<td>Mouse</td>
</tr>
<tr>
<td>Rat</td>
</tr>
<tr>
<td>Guinea pig</td>
</tr>
<tr>
<td>Rabbit</td>
</tr>
<tr>
<td>Sheep</td>
</tr>
<tr>
<td>Baboon</td>
</tr>
<tr>
<td>Pig</td>
</tr>
</tbody>
</table>

Units are days of gestation with the percent in parentheses. PND, postnatal day. 1When saccular phase is considered to begin.
attribute enhances the translational relevance of preterm pigs for understanding how pulmonary dysfunction and MV affect development of other organs, such as the brain (65) and the relationship with multiorgan dysfunction syndrome that develops in 80% of preterm infants (13).

Investigators need to be aware of the challenges associated with using preterm pigs at GD 102 and earlier. Extensive infrastructure is required, including multiple incubators and ventilators to accommodate the large litter sizes. The intensive care facility used for this study is based on NICU capabilities and includes equipment for ventilation, provision of nutrition support, monitoring of health status, sterile surgery, and on-site diagnostics, including digital radiography, hematology, pulse oximetry, and blood gases and chemistries. The large litters require immediate and continuous 24-h attention by several individuals with training in providing intensive care, particularly respiratory therapy. This is particularly true for pigs harvested at GD 100 and earlier that require intubation and MV rapidly (<3 h after delivery) before the onset of RDS. Insertion of the ET tube in pigs is challenging, and proper placement requires practice and verification. As with preterm infants, there are unexplained mortalities that provide additional opportunities to study other disease consequences of preterm birth.

Perspectives

The transition from fetal to extraterrestrial life requires an immediate switch from placental to pulmonary gas exchange and is complicated by an increased metabolic rate (59). Infants that are born between 24 and 32 wk during the critical saccular phase have immature lungs with limited abilities for gas exchange. Unfortunately, because advances in ventilation support for these preterm infants have been incremental and limited to modifying existing protocols, RDS remains the leading cause of morbidity and mortality, and VILI remains a legitimate concern for preterm infants reliant on MV (7). Preterm pigs harvested between GD 98 and 102 are translational models for these infants and can be used to identify risk factors, to determine the mechanisms underlying the RDS disease process, and to develop improved ventilation support strategies that will reduce morbidity and improve outcomes. These studies will benefit from the sequencing of the pig genome (5) and the increasing availability of porcine-specific genetic and molecular probes and technologies.

ACKNOWLEDGMENTS

We express our appreciation to the groups headed by Gary Neimann at Upstate University in Cortland, NY, and Nader Habashi at the University of Maryland Medical Center in Baltimore for their advice, consultation, and encouragement. Louis Gatto (Cortland, NY) provided valuable histology insights. Our sincerest gratitude is extended to the numerous individuals who were responsible for providing the 24-h care of the pigs, to Draeger Medical for making the VN 500 Babylog pediatric ventilators available for these studies, and to Masimo for providing the pulse oximeters.

DISCLOSURES

The first author, Frank Caminita is employed by Draeger Medical, the company that provided the ventilators used for this study. The research reported herein can be performed using ventilators from other suppliers. None of the other authors have any conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS


REFERENCES

Innovative Methodology

L128

PRETERM PIG AS A MODEL FOR NEONATAL LUNG DISEASE


35. Landry JS, Menzies D. Mediation by 10.220.32.246 on June 21, 2017 http://ajplung.physiology.org/ Downloaded from


