Sex differences in cardiorespiratory transition and surfactant composition following preterm birth in sheep

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It is now widely recognized that, following preterm birth, males have a greater risk of death and illness compared with females (14, 39, 40, 52), although the difference in mortality between males and females has declined in recent years (6, 13). Most studies indicate that this increased risk in male preterm infants is a result of a greater incidence of respiratory insufficiency than females (14, 52). The greater incidence of respiratory distress syndrome (RDS) in male preterm infants compared with females (39, 48), suggests less mature lungs in males compared with females of the same gestational age. Although the “male disadvantage” following preterm birth is thought to be related to relative immaturity of pulmonary surfactant production (34), many questions remain. Foremost among these is whether there are structural and physiological differences in the developing lungs between sexes and whether there are different physiological responses to preterm birth that could contribute to the increased risk of respiratory illness and death in males born before term. It is also unclear whether males differ physiologically from females before preterm birth.

Previous studies have shown higher lecithin/sphingomyelin (L/S) ratio and higher concentrations of saturated phosphatidylcholine in amniotic fluid during human pregnancy with female fetuses, indicating a greater degree of lung maturity (in terms of surfactant composition) in females (15, 45). Similar findings have been reported in lung lavage and amniotic fluid samples from the rabbit (28), mouse (26), and rat (1). Interestingly, data from fetal monkeys (46) and the developing chick (27) have shown a reversal of the sex difference in pulmonary surfactant production, with males being advanced relative to females.

Surfactant therapy in preterm infants has been successful in decreasing the rate of death and RDS in both sexes; however, male neonates continue to show a higher incidence of respiratory morbidity (34). Male infants require more doses of surfactant, significantly longer mechanical support, and a greater proportion are oxygen dependent at 36 wk postmenstrual age (40). Findings such as these suggest that there may be sex differences in factors other than pulmonary surfactant that could contribute to the “male disadvantage.” Thus the respiratory mechanisms contributing to the increased morbidity in preterm male infants requires further investigation.

We have recently demonstrated the male disadvantage following preterm birth in sheep (12); male lambs born at 133 days of gestational age (term approx. 147 dGA) had a 44% survival rate, compared with 76% in females. In common with observations in human preterm infants, male preterm lambs appeared more likely to experience respiratory insufficiency than females. Thus our objective was to identify the basis for the male disadvantage in respiratory adaptation during the transition from fetal to postnatal life following preterm birth, using our ovine model of preterm birth. As well as examining lung architecture we have compared the composition of surfactant phospholipids and the gene expression of surfactant proteins in the lungs of preterm males and females.

METHODS

Experimental procedure. All procedures were approved by the Monash University Animal Ethics Committee. Date-mated pregnant crossbred ewes (Border-Leicester × Merino, n = 21) carrying singleton fetuses underwent aseptic surgery at ~125 days after mating for the chronic implantation of fetal catheters later used to obtain physiological data before and after preterm birth; term is ~147 days. Polyvinyl catheters were implanted into a fetal carotid artery and jugular vein and into the trachea and amniotic sac for sampling fetal blood, fetal lung liquid, and amniotic fluid. After a fetal thoracotomy,
an intrapleural balloon (approx. 0.5 ml) was inserted into the intrapleural space for monitoring breathing after birth. A loose-fitting vascular occluder (OC16, In Vivo Metric, Healdsburg, CA) was positioned around the umbilical cord and secured to the abdominal wall; this was used at delivery to briefly occlude the umbilical cord thereby preventing anesthetic agents administered to the ewe during cesarean section from entering the fetal circulation. Catheters were exteriorized and the ewes allowed to recover from surgery. Ampicillin sodium (Aspen Pharmcare, Australia) was administered into the amniotic sac (800 mg/4 ml) and fetus (200 mg/ml iv) for 3 days after surgery. To monitor fetal arterial pressure, the arterial and amniotic catheters were connected to pressure transducers (“DTX Plus” transducer, Becton Dickinson), and amniotic pressure was subtracted from arterial pressure; data were recorded digitally (PowerLab8/30 using Chart for Windows software, version 5.01, ADInstruments). Fetal arterial pressure and heart rate (HR) were recorded for 1 h on 131 and 132 days of gestation (dGA); fetal HR was derived from the arterial pressure waveform. After arterial pressure was recorded at 131 dGA, betamethasone (5.7 mg im, Celestone Chronodose, Schering-Plough) was administered to the ewe, as the majority of preterm infants are exposed to exogenous corticosteroids before birth (31). Fetal arterial blood was sampled each day following surgery for analysis of blood gases (ABL800, Radiometer, Denmark) and other markers of fetal physiological status.

**Delivery of lambs.** Eleven female and 10 male lambs were delivered at 133 dGA, ~14 days before term; this age was chosen as it is the earliest at which independent survival is possible in lambs (12). Immediately before delivery, the umbilical cord occluder was inflated briefly while thiopentone sodium (25 ml; 50 mg/ml iv; Pentothal, Boehringer Ingelheim) was administered to the ewe. With the ewe in a supine position, the lamb was quickly delivered, together with its catheters, by cesarean section and the umbilical cord ligated. The lamb was dried, weighed, and placed under a heat source to maintain rectal temperature between 38°C and 39°C. After delivery of the lamb, the ewe was euthanized using pentobarbitone sodium (325 mg/ml iv). Lambs were monitored for 4 h while unanesthetized and breathing spontaneously; they were not intubated or mechanically ventilated. Supplemental oxygen was administered via a loose-fitting face mask as required to maintain arterial O₂ saturation (SaO₂) above 80%. Arterial blood was sampled at 5 min after birth and every 15 min thereafter. Intravenous saline of 3 ml/kg iv was administered every 15 min for the first hour, while 3 ml of 5% glucose solution was administered intravenously 5 min after delivery and every 15 min thereafter if plasma glucose levels fell below 5 mmol/l. During the 4-h postnatal monitoring period, arterial pressure, HR, intrapleural pressure, and rectal temperature were continuously recorded. If arterial pH fell below 7.0 and arterial partial pressure of CO₂ (PaCO₂) became higher than 100 mmHg in two consecutive 15-min periods, the lamb was intubated, ventilated, and no longer used in this study. At the end of the 4-h study period, static lung compliance was measured as detailed below.

**Static lung compliance.** At the end of the 4-h postnatal study period, lambs were anesthetized with thiopentone sodium (50 mg iv; Pentothal) and intubated with a cuffed endotracheal tube (4 mm diameter) connected to an infant ventilator (Dräger Babylog 800+, DrägerMedical International, Lubeck, Germany). Lambs were allowed to breathe 100% O₂ for 3 min, after which the endotracheal tube was clamped for 3 min to deplete the lungs. The lambs were then euthanized by an overdose of pentobarbitone sodium (325 mg/ml iv). The chest wall was opened along the sternum and a pressure-volume curve created by inflating the lungs with air to a maximal inflation pressure of 40 cmH₂O and then systematically removing the necessary volume required to maintain luminal pressures of 30, 20, 10, and 0 cmH₂O. Static lung compliance was calculated using the luminal volume difference between 10 and 0 cmH₂O of luminal pressure.

** Necropsy.** After the measurement of static lung compliance, the lungs were removed and weighed. The left bronchus was ligated, and the left and right lungs were separated. Small portions of the left lung (avoiding major airways and blood vessels) were snap-frozen in liquid nitrogen and stored at −80°C for molecular analysis. The upper lobe of the right lung was isolated and cannulated for the collection of bronchoalveolar lavage fluid (BALF). To collect BALF, saline was infused into the lung lobe via the bronchial cannula until it was maximally expanded and then the saline was withdrawn; this was performed three times, and the final sample was collected as BALF. The BALF was centrifuged at 250 g (Heraeus Multifuge 86-R Cen trifuge, Thermo Electron) for 7 min to separate the cellular component. The supernatant was collected and stored at −80°C for surfac tant phospholipid analysis and determination of protein concentration. The right lung was then fixed via the trachea using 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) at a distending pressure of 30 cmH₂O for histological analysis. Other major organs including the heart, liver, spleen, kidneys, and adrenal glands were collected and weighed.

**Estimation of lung volume.** The volume of the right lung was measured using the Cavalieri method (24). Briefly, grids of 5 mm × 5 mm were positioned over lung sections of 5 mm thickness, and the number of points overlapping the tissue was counted. Lung volume was then estimated by multiplying the sum of the points overlapping the lung tissue by the area of each grid (25 mm²) and thickness of the lung slice (5 mm).

**Histological preparation of lung tissue.** Randomly selected sections from the upper, middle, and lower right lung lobes were processed and embedded in paraffin for histological analysis. Sections (5 μm thick) were stained and then examined using a light microscope. Color images were captured using a digital camera (SPOT Insight 4Meg Fire Wire Color Mosaic 14.2, Diagnostic Instruments) linked to image analysis software (Image Pro Plus, version 6.0). All measurements were made on coded slides by the same observer (N. Ishak) who was blinded to the experimental groups. For each morphometric parameter, we obtained data from a total of 15 randomly chosen fields of view per animal: 5 fields were used from each of the upper, middle, and lower lobes of the lung.

For electron microscopy analysis of the blood-air barrier and lamellar bodies in type II alveolar epithelial cells, randomly selected tissue blocks (approx. 2 mm³) from the upper, middle, and lower right lung lobes were postfixed in 4% glutaraldehyde at room temperature for 2 h and then overnight at 4°C. The next day, the sections were washed in 0.1 mol/l cacodylate buffer and incubated in 1% osmium tetroxide (in cacodylate buffer) for 2 min in a microwave tissue processor at 80 W under vacuum followed by 2 min incubation at room temperature; the osmium tetroxide incubations were conducted four times. Sections were dehydrated, infiltrated with epoxy resin (Procare 812, ProSciTech) in a microwave tissue processor at 250 W under vacuum, and then embedded in epoxy resin at 60°C for 2 days. Coded ultrathin sections (70–90 nm) were cut using a diamond knife, mounted on 200-mesh copper grids, and stained with saturated uranyl acetate and lead citrate. Sections were viewed using a Hitachi H800 transmission electron microscope fitted with a digital image acquisition system. All measurements were made using coded slides by the same observer (G. Maritz) who was blinded to the experimental groups; micrographs of final magnification of ×5,000 were used.

**Morphometric analysis of the lungs.** Paraffin-embedded sections of lung tissue were stained with hematoxylin and eosin. Measurements were made by superimposing test grids over projected images of the lung. Tissue and air-space fractions, mean linear intercept, septal crest density and septal thickness were determined by point counting methods (2, 35). Analyses were performed at a final magnification of ×200. The harmonic mean thickness (T_h) of the blood-air barrier was measured as previously described (6); T_h is the distance that respiratory gases must travel across the blood-air barrier. The volume density of the lamellar bodies in type II alveolar epithelial cells was determined by point counting (13).

**Elastin and collagen analysis.** Paraffin-embedded sections of lung tissue were stained for elastin using the Hart’s resorcin-fuchsin stain and counterstained with tartarazine (0.25%) in saturated picric acid. For the collagen analysis, paraffin-embedded sections were stained
using the Gordon and Sweet reticular fiber stain, which stains collagen types I and III; the sections were counterstained with eosin. Analyses were performed at a final magnification of ×1,000. For each field of view, the area of tissue stained for either elastin or collagen was expressed as a percentage of the total area of tissue.

Gene expression analysis. Relative surfactant protein (SP)-A, SP-B, SP-C, and SP-D mRNA levels in lung tissue were measured using quantitative real-time PCR (qPCR), as previously described (37). Briefly, total RNA was extracted (RNaseasy Midi kit, Qiagen), treated with DNase (Qiagen), and then reverse-transcribed into cDNA (Maloney’s murine leukemia virus reverse transcriptase, RNase H Minus, Point Mutant Kit, Promega, Madison, WI). qPCR was performed using a SYBR green detection method and a Stratagene Mx3000P detection system (Agilent Technologies) under previously described reaction conditions (37). Dissociation curves were performed for each qPCR experiment to ensure that a single PCR product had been amplified per primer set. Each sample was measured in triplicate, and a negative control sample that did not contain template cDNA was included in each run. The relative mRNA levels of each gene for each animal were normalized to the mRNA levels of the housekeeping gene ribosomal protein S29 for that animal, using the ΔΔCₐ method (where Cₛ is cycle threshold). Values were expressed relative to the mean mRNA levels in female lambs.

Surfactant phospholipid analysis. To assess the effects of sex on surfactant phospholipid composition before birth, amniotic fluid was analyzed at 131 and 133 dGA, as previously described (38). Amniotic fluid was collected via the amniotic sac catheter; samples were stored at −80°C until analyzed. We also assessed the phospholipid composition of BALF obtained at necropsy at 133 dGA.

Briefly, phospholipids were extracted from the amniotic fluid samples and BALF supernatant (10 μl) with 2:1 chloroform-methanol (200 μl) following the addition of internal standards (100 pmol each of phosphatidylglycerol (PG) 17:0/17:0, phosphatidylserine (PS) 17:0/17:0, phosphatidylcholine (PC) 13:0/13:0, and phosphatidylethanolamine (PE) 17:0/17:0 (Avanti Polar Lipids)). Prior liquid chromatographic separation was performed on a 1.8-μm, 50% 2.1 mm C18 column (Zorbax) at 300 μl/min using gradient conditions previously described (38). Individual lipid species were quantified using scheduled multiple reactions monitoring in positive-ion mode. Multiple-reaction monitoring experiments were based on product ion of 184 mass-to-charge ratio (m/z) of [phosphocholine]+ for PC and neutral loss of 189 Da for PG, 141 Da for PE, 185 Da for PS, and 277 Da for PI. Each ion pair was monitored for 10–50 ms with a resolution of 0.7 atomic mass units at half-peak height and averaged from continuous scans over the elution period. Lipid concentrations were calculated by relation of the peak area of each species to the peak area of the corresponding internal standard (17). Total lipids of each class were calculated by summation of the number of carbon atoms in the fatty acid chains esterified at the sn-1 and sn-2 positions, respectively, and x and y are the number of double bonds in the fatty acid chains.

Protein concentration in BALF. BALF samples were centrifuged at 9,280 g at 4°C (model 5415R, Eppendorf) before a protein assay was performed (Bio-Rad). The supernatant was diluted with 0.9% saline (1:4), and 10 μl of the solution werepipetted into a 96-well plate. Dye reagent (200 μl, 1:5 dilution in distilled deionized water; Bio-Rad cat. no. 500-0006) was added to the samples in the wells. Absorbance of the samples and bovine serum albumin standards was measured at 595 nm using a plate reader (FLUOstar Omega, BMG Labtech). Soluble protein concentration was calculated from the standard curve.

Cortisol assay. Plasma cortisol concentrations were measured in samples of fetal arterial blood collected at 131 and 133 dGA using an established method (7).

Statistical analyses. Physiological data were analyzed over three different time periods. First, the prenatal data were analyzed between 128 dGA and 133 dGA. The postnatal data were separately analyzed over 1) the first hour after delivery and 2) the next 3 h, because physiological responses differed between these periods. Blood chemistry data, cortisol concentrations, surfactant phospholipid composition in amniotic fluid, and pressure-volume curves were all analyzed using a one-way repeated measures ANOVA, with sex (Pₛ) and time (Pₜ) as factors. Where appropriate, the Greenhouse-Geisser correction was used. Significant differences detected by ANOVA were further analyzed by the Fisher least significant difference (LSD) post hoc test. Data relating to the requirement for supplemental oxygen were analyzed by a log rank (Mantel-Cox) test. We used the Student’s unpaired t-test to examine sex-related differences in body weights and dimensions, organ weights, surfactant phospholipid composition in BALF, relative SP mRNA levels, static lung compliance, protein concentration in BALF, and lung architecture data. All statistical tests were performed using IBM SPSS Statistics, Version 19 for Windows (IBM, Armonk, NY). The level of significance was taken at P < 0.05; data are presented as means ± SE.

RESULTS

Prenatal physiology. Between 128 and 133 dGA there were no significant differences between male and female fetuses in arterial partial pressure of O₂ (PₐO₂) (overall mean 20.0 ± 0.9 mmHg), SₐO₂ (57.0 ± 2.6%), PₐCO₂ (50.0 ± 0.6 mmHg), mean arterial pressure (MAP, 44.3 ± 0.4 mmHg), HR (162 ± 3 beats/min), and blood concentrations of glucose (1.00 ± 0.03 mmol/l) and lactate (1.83 ± 0.08 mmol/l) (Figs. 1 and 2). However, there was a strong trend for male fetuses to have a lower arterial pH than females (7.358 ± 0.009 vs. 7.380 ± 0.005, Pₛ = 0.052; Fig. 1D).

Although the overall sex (treatment) effect for plasma cortisol concentrations at 131 and 133 dGA was not significant (Pₛ = 0.582), there was a significant interaction between sex and time (Pₛ×T = 0.023, Fig. 3). In female fetuses, plasma cortisol concentration significantly increased more than three-fold from 9.2 ± 2.9 ng/ml at 131 dGA, immediately before betamethasone administration, to 29.9 ± 5.8 ng/ml at 133 dGA. In contrast, plasma cortisol concentration in male fetuses was similar at 131 dGA and 133 dGA (13.1 ± 4.6 vs. 19.0 ± 6.4 ng/ml).

Postnatal physiology from 0 to 1 h. During the first hour following delivery there were significant differences between males and females in time-related changes in arterial PₐCO₂, pH, glucose and lactate concentrations, and MAP (Figs. 1 and 2). There was no significant difference between males and females in PₐO₂ after birth (Fig. 1A). Although lambs received supplemental oxygen to maintain SₐO₂ above 80%, SₐO₂ tended to be lower in male lambs (Pₛ = 0.076) during the first hour after birth; this was most apparent at 5 min after birth when SₐO₂ in males was 20.5 ± 3.6% and in females was 44.8 ± 7.9%. Females reached the target of 80% SₐO₂ at 15 min, whereas the males took 30 min (Fig. 1B). The requirement for supplemental oxygen during the 4 h after delivery was significantly greater in males than in females (Fig. 4).

In both males and females the maximal PₐCO₂ occurred at 15 min after delivery (Fig. 1C), when values were significantly greater in males than females (98.7 ± 4.3 vs. 80.9 ± 5.6...
mmHg). \( \text{PaCO}_2 \) remained significantly higher in males than in females from 15 to 60 min after delivery (Fig. 1C); at 60 min, \( \text{PaCO}_2 \) in males (91.6 ± 12.3 mmHg) was 44% higher than in females (63.0 ± 6.0 mmHg).

Arterial pH in both males and females reached minimal values at 15 min after delivery (Fig. 1D); the minimal pH was significantly lower in males than females (6.927 ± 0.038 vs. 7.088 ± 0.041). At each time point between 15 and 60 min...

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**Fig. 1.** Blood gas measurements before and after birth. Values for arterial partial pressure of O\(_2\) (\( \text{PaO}_2 \), A), arterial O\(_2\) saturation (\( \text{SaO}_2 \), B), arterial partial pressure of CO\(_2\) (\( \text{PaCO}_2 \), C), and arterial pH (D) on gestational days 128–133 and for up to 240 min following cesarean-section at 133 days of gestational age in male (●) and female (○) fetuses and lambs. Note different time scales before and after delivery. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery. \( P \) values: \( P_S \) is between factor variable (sex), \( P_T \) is repeated factor variable (time), and \( P_S\times T \) is the interaction term. Data are shown as means ± SE. *\( P < 0.05 \), males vs. female.
after birth, males had a significantly lower arterial pH than females (Fig. 1D; $P_S < 0.05$).

In females, plasma glucose concentration increased from a prenatal level (90 min before birth) of 1.0 ± 0.1 to 3.4 ± 0.6 mmol/l at 15 min after birth and remained at this level until 240 min (Fig. 2A). During the first hour after birth there was a significantly greater increase in plasma glucose levels in males than females. In males, plasma glucose concentrations in-
creased from a prenatal value of 1.0 ± 0.1 mmol/l to reach a maximum value of 5.5 ± 0.8 mmol/l at 30 min after birth; values remained significantly higher than in females at 45 and 60 min.

Plasma lactate concentration in males increased from 1.8 ± 0.2 mmol/l before birth, to a maximum of 7.5 ± 0.8 mmol/l at 15 min after delivery; between 15 and 60 min after delivery, values in males were significantly higher than in females (Fig. 2B).

Within the first hour after birth, males had a significantly higher MAP than females (Fig. 2C). MAP in males increased from a prenatal value of 46.3 ± 2.9 mmHg (at 132 dGA) to 66.4 ± 4.5 mmHg 15 min after birth and then fell to 59.3 ± 3.0 mmHg at 60 min. MAP in females increased from 45.7 ± 0.9 mmHg at 132 dGA to 59.4 ± 3.4 mmHg at 15 min after birth and then fell to 53.4 ± 1.2 mmHg at 60 min. Fifteen minutes after birth, HR was 240 ± 13 beats/min in males and 215 ± 9 beats/min in females; HR remained significantly higher than fetal values at 90 min after birth (Fig. 2D). The postnatal changes in HR were not different between males and females.

After the end of the first hour of the monitoring period, two male lambs (20%) were euthanized as their arterial pH was below 7.0 and PaCO\textsubscript{2} was above 100 mmHg for two consecutive 15 min periods; no female lambs were euthanized.

Postnatal physiology from 1 to 4 h. From 90 to 240 min after birth, males tended to have a lower pH than females at each time point ($P_s = 0.065$, Fig. 1D). There were no significant differences in PaO\textsubscript{2}, SaO\textsubscript{2}, PaCO\textsubscript{2}, plasma glucose and lactate concentrations, MAP, or HR between males and females between 75 to 240 min after birth (Figs. 1 and 2).

Static lung compliance. Lungs of male lambs needed a significantly lower volume of air to reach a luminal pressure of 40 cmH\textsubscript{2}O compared with females (58 ± 8 vs. 91 ± 10 ml/kg body wt, Fig. 5A). In males, the volume of air remaining in the lungs when luminal pressure had fallen to 30, 20, and 10 cmH\textsubscript{2}O was also significantly lower than those in females. Static lung compliance in males was significantly lower than in females (3.26 ± 0.69 vs. 5.21 ± 0.37 ml·cmH\textsubscript{2}O\textsuperscript{−1}·kg\textsuperscript{−1}, Fig. 5B).

Surfactant phospholipid composition. In amniotic fluid, the relative proportions of the major phospholipid classes (PC, PE, PG, PI, and PS) were not different between male and female fetuses at 131 or 133 dGA (Fig. 6, A–E). In BALF collected at 4 h after delivery, the proportion of PE was significantly lower in males than in females (Fig. 6A), whereas the proportion of PC was significantly higher in males (Fig. 6B). The PC-to-PE ratio in BALF in males was 11:1, which was significantly lower than the PC-to-PE ratio in females of 15:1 ($P < 0.05$). BALF of males contained a significantly lower proportion of the PC molecular species 32:0 and a significantly greater proportion of the PC molecular species 34:2 and 36:2 (Fig. 6F) than in females. There were no other significant differences in...
the proportions of molecular species in the other phospholipid classes (Fig. 6, G–J).

SP gene expression. The gene expression of SP-A, -B, -C, and -D in lung tissue was similar in male and female lambs (Fig. 7).

Protein concentration of BALF. In BALF collected at necropsy, males had a significantly higher concentration of total protein compared with females (3.04 ± 0.50 vs. 1.84 ± 0.25 mg/ml, Fig. 8).

Body and organ weights. The body weights of male and female lambs at necropsy were similar (3.39 ± 0.18 vs. 3.15 ± 0.21 kg). Likewise, the crown-to-rump length (48.7 ± 0.8 vs. 49.3 ± 1.5 cm), thoracic girth (34.1 ± 0.7 vs. 32.8 ± 1.2 cm), and ponderal index (2.92 ± 0.10 vs. 2.73 ± 0.13 g/cm³ × 100)
were similar in males and females. The absolute and relative (to body weight) dry and wet weights of the lung were not different between males and females (Table 1). In addition, the absolute and relative (to body weight) weights of the heart, liver, kidneys, adrenals, and spleen were not different between males and females (data not shown).

**Lung morphology.** Lung weights (wet and dry) and right lung volume were not different between males and females (Table 1). The tissue fraction in the lungs of males (35.8 ± 2.2%) tended to be greater than in females (30.9 ± 0.7%; \( P = 0.06 \); Table 1). There were no significant differences between males and females in the mean linear intercept and the proportion of lung tissue occupied by septal crests, elastin, and collagen; similarly, the septal thickness, the blood-air barrier thickness, and the percentage area of type II alveolar epithelial cells occupied by lamellar bodies were not different between males and females (Table 1).

**DISCUSSION**

A major finding of our study was that the lungs of male preterm lambs were less compliant than lungs of females. In the absence of significant sex-related differences in lung morphology, our data suggest that the cause of the lower static lung compliance in male preterm lambs was altered surfactant phospholipid composition, coupled with elevated protein content of BALF, which could impair surfactant function. It is likely that the poorer physiological adaptation to preterm birth in male preterm lambs, especially during the first hour after birth, and the higher mortality rates observed in our previous study (12) are a result of low lung compliance.

![Fig. 7. Surfactant protein (SP) gene expression.](BASIS OF PRETERM MALE DISADVANTAGE)

**Fig. 7.** Surfactant protein (SP) gene expression. Relative SP-A (A), SP-B (B), SP-C (C), and SP-D (D) mRNA levels were determined by quantitative real-time PCR in lung tissue of female (open bars) and male (closed bars) lambs collected at necropsy. Data are shown as means ± SE.

**Table 1. Lung weight, volume, and morphology in male and female lambs**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
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</thead>
<tbody>
<tr>
<td>Wet lung Wt, g</td>
<td>116.5 ± 7.6</td>
<td>106.6 ± 12.1</td>
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<tr>
<td>Wet lung Wt/BW, g/kg</td>
<td>34.4 ± 1.6</td>
<td>31.8 ± 2.8</td>
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<tr>
<td>Dry lung Wt, g</td>
<td>15.6 ± 1.1</td>
<td>14.3 ± 1.7</td>
</tr>
<tr>
<td>Dry lung Wt/BW, g/kg</td>
<td>4.6 ± 0.2</td>
<td>4.4 ± 0.5</td>
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<tr>
<td>Dry/Wet lung Wt</td>
<td>0.13 ± 0.00</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Right lung volume, cm³</td>
<td>101.5 ± 5.4</td>
<td>104.3 ± 8.6</td>
</tr>
<tr>
<td>Tissue space, %</td>
<td>35.8 ± 2.2</td>
<td>30.9 ± 0.7</td>
</tr>
<tr>
<td>Air space, %</td>
<td>64.2 ± 2.2</td>
<td>69.1 ± 0.7</td>
</tr>
<tr>
<td>Mean linear intercept, μm</td>
<td>102.6 ± 5.1</td>
<td>107.7 ± 2.1</td>
</tr>
<tr>
<td>Elastin deposition, %</td>
<td>5.0 ± 0.3</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>Collagen deposition, %</td>
<td>20.2 ± 1.2</td>
<td>19.1 ± 0.6</td>
</tr>
<tr>
<td>Septal crest density, %</td>
<td>3.0 ± 0.2</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Septal thickness, μm</td>
<td>3.7 ± 0.3</td>
<td>3.6 ± 0.2</td>
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<tr>
<td>Blood-air barrier thickness, μm</td>
<td>0.23 ± 0.01</td>
<td>0.26 ± 0.02</td>
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<tr>
<td>Lamellar bodies in type II cells, %</td>
<td>20.6 ± 2.2</td>
<td>20.7 ± 2.2</td>
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</table>

Data represented as means ± SE. Lambs were delivered by cesarean-section at 133 days of gestational age, and the lungs were collected at necropsy, following 4 h of spontaneous breathing. Wt, weight, BW, body weight.
**Pulmonary surfactant composition.** Although there was no evidence of differences in surfactant phospholipid composition between sexes before birth, the BALF of postnatal males had a 6% lower proportion of PC, the major component of surfactant phospholipids, than females. Within the PC class of phospholipids, the proportion of PC32:0, commonly known as dipalmitoylphosphatidylcholine (DPPC), was 10% lower in the BALF of males than females; DPPC is the only surface active component of lung surfactant capable of lowering surface tension to near zero levels (49). Whether a reduction of 10% in DPPC is sufficient to significantly impair lung compliance is currently unknown; however, the slope of the deflation limb of the pressure-volume curve suggests that surfactant function is impaired in males compared with females.

The higher proportions of the plasma PCs PC34:2 and PC36:2 in BALF from males are suggestive of greater plasma exudation in the alveoli, which is likely caused by higher vascular permeability in the lungs of males. Such an increase in permeability may account for the 65% increase in the concentration of total protein in the BALF observed in male lambs compared with females. As pulmonary surfactant function can be inhibited by plasma proteins (20, 50), the presence of plasma proteins in the alveolar space is likely to reduce the effectiveness of DPPC (and other phospholipids) in lowering surface tension within the lungs. Higher pulmonary vascular permeability in perterm males may be an innate characteristic that could compromise lung function.

Previous studies have used the L/S ratio as an index of lung maturity. In our study, we could not calculate the L/S ratio as we did not measure sphingomyelin; however, lecithin is largely composed of PCs, and the terms are often used interchangeably. PE is similar to sphingomyelin in that both are considered to be membrane lipids present in pulmonary surfactant, likely from the ejection of some of the membrane of lamellar bodies during exocytosis of surfactant into the liquid lining the alveoli (49). The PC-to-PE ratio in the BALF of males was 11:1, which was significantly lower than the PC-to-PE ratio in females of 15:1. As in studies in humans and rabbits (15, 28, 45) our findings support the notion that the maturity of the pulmonary surfactant system of males is retarded compared with that of females. Indeed, sex differences in the differentiation and stimulation of surfactant synthesis in type II cells are considered to be a result of sex differences in the function of pulmonary fibroblasts, which can be influenced by androgens (42, 44) and glucocorticoids (11, 35, 36, 43).

We attempted to correlate the levels of surfactant phospholipids, PC-to-PE ratio, and BALF protein levels against static lung compliance and found no significant relationship between these variables. This suggests that the lower lung compliance of males is not solely due to altered surfactant production and/or function. Indeed, it has been suggested that the underlying mechanisms for sex differences in infant respiratory function are not likely due to any one particular pathway, and that the mechanism is likely to be multifactorial (10).

**Lung architecture.** Previous studies have shown differences in lung architecture between male and female children (42) and adult rodents (41). As with developing rodents (41) we found no significant differences between male and female preterm lambs. However, there was a strong trend for lungs of males to have a higher tissue fraction than females, which is consistent with a lower lung compliance in males. Septal crest density, which is an indicator of alveolarization, was not different between the sexes, and this is consistent with the similar content of elastin observed. Thicknesses of the interalveolar septa and of the blood-air barrier were also not different between the sexes. Interestingly the areal density of surfactant-containing lamellar bodies in type II alveolar epithelial cells was not different between males and females. Because there were no sex-related differences observed in lung morphology, it is likely that the observed differences in lung compliance were largely a result of differences in the properties or amount of pulmonary surfactant.

**Fetal hormones and lung maturation.** The dramatic increase in plasma cortisol concentrations from 131 to 133 dGA in female fetuses may have contributed to the better physiological transition at birth observed in females. The reason for the lack of increased cortisol in males is unclear, but our findings are compatible with those from human fetuses following exposure to betamethasone (43). The absence of a cortisol response in males could be due to immaturity of the hypothalamic-pituitary-adrenal (HPA) axis (19, 25), a reduction in expression of fetal lung 11β-hydroxysteroid dehydrogenase-1 (41), which converts cortisone to cortisol, or alterations in the activity of placental 11β-hydroxysteroid dehydrogenase-2, which converts cortisol to cortisone (11).

In our study, we administered a single, small dose of betamethasone (5.7 mg) before birth to ensure survival of our preterm lambs without the need for intensive care with ventilatory support (12); this dose is considerably smaller than the clinical dose given to women (11–24 mg, on 2 successive days) who are at risk of preterm delivery (31). Since betamethasone administration has been shown to lower plasma cortisol levels (4, 9), we cannot rule out the possibility that betamethasone differentially affected endogenous cortisol levels. Both dexamethasone and betamethasone have been shown to induce maturation of surfactant production in the lung (5), but we cannot be certain if the small dose of betamethasone we used in our study was more effective in maturing the lungs of females compared with males. In support of this, a previous study (51) has shown that a single intramuscular injection of betamethasone (0.5 mg/kg) 48 h before delivery at 128 dGA improved lung compliance in fetal sheep, with females showing a significantly greater improvement than males. Studies in humans have also suggested that the pulmonary maturational response to exogenous glucocorticoids is greater in females than males (4), providing further evidence that males have a developmental delay in lung maturation. However, the number and the binding affinity of glucocorticoid receptors (GR) to glucocorticoids in the fetal sheep lung were not different between sexes at 124–125 dGA (22), and no sex differences were found in the number of GR or their binding affinity in the lungs of rabbits (16) and rats (3) at late gestation. Instead, it has been suggested that differential regulation of GRs between males and females may contribute to the sexual dimorphism (34). Interestingly, a recent meta-analysis showed that antenatal betamethasone administration was associated with a significant decrease in the rate of RDS in males (RR 0.29; 95% CI 0.15 to 0.57), whereas the use of dexamethasone was not (RR 0.78; 95% CI 0.57 to 1.07) (32). Conversely, dexamethasone use was beneficial in decreasing RDS in females (RR 0.51; 95% CI 0.32 to 0.81) but betamethasone was not.

Sex hormones are also considered to play a role in lung maturation, apparently to the disadvantage of males. Testosterone and 5α-dihydrotestosterone have been shown to delay male lung
maturation and surfactant production in human lung explants (42) and in animal models (27, 29). 5-Dehydroepiandrosterone delays both the spontaneous and dexamethasone-stimulated increases in saturated PC synthesis in lung tissue in vitro (38) and thus may contribute to the altered surfactant production seen in our study. In contrast to the effects of androgens, estrogens have been shown to accelerate lung maturation and stimulate surfactant production in the fetal rabbit and rat (18, 21); however, the contribution of estrogens to sex differences in lung development is questionable as there are no studies to our knowledge that show a difference in estradiol levels in male and female fetuses. Differences in sex hormone metabolism may also play a role in sex-related differences in lung development (34). Knowledge of the molecular basis for the male delay in lung maturation is increasing and candidate genes under the control of androgens, such as those genes controlling transforming growth factor-β and the Wnt pathway, have been identified (8, 44).

**Prenatal physiology.** Very few studies have documented sex differences in fetal physiology. As well as finding a strong trend for male fetuses to have a lower arterial pH than female fetuses, the present study demonstrates that female, but not male fetuses, show a rise in circulating plasma cortisol between 131 and 133 days of gestation under control conditions, which may influence lung maturation. However, in response to maternal hypoxia at 130 days of ovine gestation, the increase in circulating cortisol concentration was twice as great in male fetuses compared with females, which was attributed to a greater adrenocortical sensitivity to adrenocorticotropic hormone (ACTH) in male fetuses (17). This greater adrenal sensitivity to ACTH in the male fetus has previously been shown in male-female twin pairs (33). Whether sex differences in the sensitivity or activity of the HPA axis play a role in sex-related differences in the cardiorespiratory transition immediately postdelivery remains to be established.

**Postnatal physiology.** Our study provides novel data on physiological adaptations immediately following preterm birth in unanesthetized, spontaneously breathing preterm lambs. In both males and females, there were dramatic changes after birth including marked increases in arterial pressure and heart rate, and in blood glucose and lactate concentrations. During the first hour after delivery, males showed significantly poorer indices of physiological status, notably acidemia, hypercapnia, hyperglycemia, higher arterial pressure, and elevated plasma lactate concentrations. It seems likely that the poorer status of male lambs compared with females during the first hour was a result of restricted pulmonary gas exchange.

A major difference between sexes was evidence of CO₂ accumulation in males, and this is likely to underlie the other physiological changes observed. The inability of the male preterm lamb to eliminate CO₂ and regulate acid-base balance is likely a result of impaired gas exchange in the relatively incompliant lungs of males; poorer gas exchange in males was also indicated by their greater requirement for supplemental oxygen. Impaired gas exchange in males is unlikely to be a consequence of a reduced respiratory drive as breathing frequency and inspiratory effort between the sexes were similar (data not shown). It is possible that pulmonary perfusion was impaired in males, contributing to reduced gas exchange. Hypercapnic acidosis has been shown to interfere with the normal changes in pulmonary vasoconstriction resistance that occurs after birth (23), resulting in a generalized pulmonary vasocostriction. An increase in $\text{PaCO}_2$ can increase adrenal production of catecholamine, potentially leading to vasocostriction (47), which may explain the greater increase in MAP seen in male preterm lambs compared with females. In addition, hypercapnic acidosis has been associated with a decrease in myocardial contractility and cerebral vasodilatation (47), which may contribute to the higher incidence of morbidity in males. A limitation of our study is that we did not measure plasma catecholamine levels, cardiac output, cerebral blood flow, or pulmonary pressures. Further evidence of generalized vasocostriction is the higher plasma lactate levels of males, which could arise from poorly perfused, hypoxic tissues that are undergoing anaerobic metabolism. Because males were not hypoxemic compared with females, poor peripheral perfusion was likely responsible for the elevated lactate concentrations.

The hyperglycemia seen in male preterm lambs may be due to a stress response arising from the CO₂ retention and acidemia or could be a result of reduced glucose metabolism. Such a stress response may include an increase in catecholamine release and/or HPA axis steroids.

After the 1-h monitoring period, 2 of 10 male lambs needed to be euthanized due to poor physiological condition (i.e., severe hypercapnic acidosis), despite a targeted $\text{SaO}_2$ of above 80%; in contrast all female lambs survived for 4 h. The observed survival rate for male and female lambs during the 4-h monitoring period was similar to that previously reported for preterm lambs (12).

A recent study in sheep showed no sex-related differences in cardiopulmonary function in the 30 min after cesarean delivery (30). The findings of this study likely differ from those of the present study because the lambs were anesthetized and ventilated before and after delivery.

In conclusion, consistent with previous studies, we found that the lungs of preterm male lambs were less compliant than those of females; this difference is likely due, in part, to differences in the composition or quantity of surfactant, or in the inactivation of surfactant function by the presence of plasma proteins in the lung lumen. The lower lung compliance of males is likely responsible for most of the physiological differences between males and females during the transition at birth and may contribute to the “male disadvantage” following preterm birth.

**Perspectives and Significance**

Numerous studies in a range of species including humans show that developing males have increased mortality compared with females and are more likely to suffer adverse outcomes arising from pregnancy complications and preterm birth. The mechanisms responsible for this “male disadvantage” are poorly understood but appear to involve a complex interaction between inherent differences in genetics, hormones, and physiology. In relation to the higher incidence of respiratory insufficiency in males following preterm birth, our findings indicate that preterm males have a poorer cardiorespiratory adaptation than females of the same gestational age, and this is likely due to retarded maturation of surfactant function. A challenge for future research will be the development of differential treatment strategies for male and female preterm infants.

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