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Unveiling surfactant protein-A dynamics in human fetal lung development: histological and immunohistochemical insights from Myanmar

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ABSTRACT

Background: Surfactant protein-A (SP-A) is the most prevalent protein in the pulmonary surfactant system and it is expressed in Type II alveolar epithelial cells.

Materials and methods: We evaluated SP-A expression in 92 fetal human lungs at various gestational ages in Myanmar (Burma) using hematoxylin and eosin staining and immunohistochemical assays.

Results: We detected tubular structures in the fetal lungs during the canalicular stage of development at gestational weeks 22–25. Bronchioles were detected between 26–27 and 28–33 weeks, when primitive alveoli were evident. At 34–40 weeks, clusters of alveolar sacs opened from the alveolar ducts during the saccular development stage. At 40–44 weeks, extremely thin

alveolar walls resembled sections of adult lungs. Type II cells secreting surfactant were undetectable at 22–25 weeks, but became detectable from 26 weeks, and the abundance of Type II cells increased after 28 weeks. Surfactant spread throughout the alveoli at 34 weeks. Because the positivity index of these cells significantly correlated with a gestational age of 26–33 weeks, we established a formula to estimate gestational age.

Conclusions: Our findings improve understanding of fetal lung development and maturity, and provide valuable insights into the diagnosis and management of respiratory disorders among premature infants in Myanmar.

Keywords: surfactant protein-A, human fetal lung, histology, immunohistochemistry, Myanmar

INTRODUCTION

Pulmonary surfactants are important components of the alveolar lining in mammalian lungs [22] and are synthesized and stored in alveolar Type II cells before being secreted into the alveolar space, where they stabilize alveoli by lowering surface tension [3]. Biological maturation of the fetal lung normally occurs only during late pregnancy. Fetal lung development has attracted considerable interest because it can indicate the likelihood of fetal survival. Surfactant protein-A (SP-A) plays an important role in the development and function of alveolar Type II pneumocytes in human fetal lungs. It is a major component of pulmonary surfactants that comprise a complex of phospholipids and proteins that reduce surface tension at the air-liquid interface, facilitating gas exchange and is crucial for maintaining lung homeostasis and preventing respiratory distress. Type II pneumocytes start expressing SP-A at about 29 weeks of gestation, and this increases until about 39 weeks, then remains constant throughout adulthood. This sequential distribution of cells containing SP-A during pulmonary development is important for lung maturation and the postnatal transition from fetal to neonatal lung function. Therefore, preterm infants can be at risk of infant respiratory distress syndrome (IRDS) due to a surfactant deficiency. Surfactant-replacement therapy is a fundamental part of treatment for this condition [1, 14, 16, 19, 21, 24, 27–29, 32]. The incidence of IRDS increases as gestational age decreases [2, 37]. Continued intrauterine life must be weighed against premature delivery in high-risk pregnancies. The ability to detect surfactant levels in such situations could help to evaluate the risk of IRDS. Moreover, IRDS is a leading cause of death among preterm infants, and its prevalence is higher in

developing countries, such as Bangladesh and Myanmar [11, 18]. The risk of IRDS depends on gestational age, being > 50% at < 28 weeks and 5% from 37 weeks [11].

Fetal development begins with forming a zygote that undergoes cleavage and becomes a blastocyst, which implants in the uterine lining around the second week of gestation. By the end of the first trimester, the development of the major organs, such as the heart, lungs, brain, and spinal cord, is established. During the second trimester, the fetus develops more, and the organ systems become more functional. In the last trimester, the fetus experiences substantial increases in both weight and length with significant maturation of the organs to ensure the viability of the newborn [5, 6, 32]. Significant morphological changes in fetal lungs during gestation allow respiration and gas exchange at birth, progressing through five developmental stages from embryonic to alveolar. These stages involve processes such as branching morphogenesis during the embryonic stage (4–7 weeks), the differentiation of airway structures with cartilage, smooth muscle, and mucous glands in the pseudo glandular stage (5–17 weeks), the formation of gas-exchanging portions of the airway during the canalicular stage (16–26 weeks), the septation of air sacs and increased surfactant production during the saccular stage (24–36 weeks), and continued alveolar formation during the alveolar stage (36 weeks to term). These stages highlight the dynamic complexity of human lung development, underscoring ongoing research interest and the crucial role of surfactants in facilitating appropriate lung maturation and function [29, 32].

Human lung development has been extensively studied. A previous study used the ELISA method to demonstrate the expression of SP-A in human amniotic fluid and predict the risk of IRDS [30]. Nevertheless, to the best of our knowledge, this is the first study to gain a comprehensive understanding of lung surfactant development at various human gestational stages in Myanmar using hematoxylin and eosin (H&E) staining and immunohistochemical analysis. Our findings address a significant knowledge gap in Myanmar, where research into surfactant development at various gestational ages is scant. Our findings might contribute to improving prenatal care strategies and diagnostic tools for premature infants. Healthcare providers can better diagnose and treat respiratory distress by understanding the histological and immunohistochemical changes in fetal lungs. Our findings have crucial clinical implications for improving health outcomes and reducing morbidity and mortality rates among premature infants in Myanmar.

MATERIALS AND METHODS

Study design

This cross-sectional descriptive study investigated human fetal and neonatal lung autopsies at the North Okkalapa General and Teaching Hospital, Yangon, Myanmar.

Sample size calculation

We calculated a sample size as 92 using the formula [25]:

$n = z^2_{(1-\alpha/2)} P (1-P)/d^2$,

where n = desirable estimated sample size, α = 0.05 for 95% confidence level (CL), and z = 1.96 (standard normal deviation with 95% CL:

$n = z^2_{(1-\alpha/2)} P (1-P) /d^2$, and $n = (1.96)^2 \times 0.6 (1-0.6)/(0.1)^2 = 92$.

According to developmental history and findings related to the development of surfactants[12], the critical gestational ages are 24, 28, and 32 weeks. Therefore, we collected samples with an equal distribution at 22–24, 25–28, 29–32, and > 33 weeks.

Sampling procedure

Inclusion criteria

We analyzed lung samples derived from spontaneously aborted fetuses, stillborn, and deceased infants up to 44 weeks of gestation. The gestational age of the samples was clinically assessed by a neonatologist based on maternal ultrasound findings of the abdomen and pelvis during pregnancy or relevant gestational history.

Exclusion criteria

The exclusion criteria comprised lungs with autolysis determined by H&E staining, autopsied lungs with signs of decomposition, and death due to diseases associated with lung pathology.

Ethical considerations

The Board of Ethics and Research Committee at the University of Medicine 1, Yangon, Myanmar, approved the post-mortem collection and investigation of human fetal and neonatal lungs. Gestational age was determined based on the last menstrual period (LMP) of the mother.

Guardians of the fetuses and autopsied neonates provided written informed consent and histories after receiving a detailed description of the study.

Histology and immunohistochemical analysis

Tissue collection and preparation

Parts of the lungs were fixed in 10% formalin, then tissues were automatically processed, paraffin-embedded, sliced into 3–5 μm sections and mounted on slides following a standard procedure. The sections were stained with HE and screened for sufficient tissue quantity, normal morphology, and good tissue preservation.

We mounted 5-μm-thick sections on silane-coated slides for immunohistochemical analysis. The sections were dried overnight at 37°C and then placed at 60°C for 30 min. The sections were deparaffinized with xylene, dehydrated using 100%, 100%, 90%, and 80% toluene-ethanol and washed three to five times with distilled water for 5 min each. After heat-mediated antigen retrieval, the sections were immersed in 10 mL sodium citrate buffer (pH 6.0), at 120°C for 30 min, and washed with phosphate-buffered saline (PBS; pH 7.6) three times for 3–5 min each.

Endogenous peroxidase activity was blocked by immersing all sections in absolute methanol containing 3% H_2O_2 for 30 min at 37°C. The sections were washed thrice with PBS (pH 7.6) for 5 min each. After three washes with normal goat IgG (500 μg/mL) in PBS containing 1% bovine serum albumin, the sections were incubated with blocking antibody at room temperature for 30 min followed by an overnight incubation with 1:100-diluted anti-Human SP-A (PE10) Mouse IgG MoAb (#10375; Immuno-Biological Laboratories, Gunma, Japan) primary antibody. The sections were individually washed three times for 15 min each with 0.075% Brij detergent in PBS (pH 7.6), followed by three washes with PBS for 15 min.

The sections were incubated with horseradish peroxidase-conjugated goat Anti-Mouse IgG (1:100)-diluted secondary antibody (N-Histofine® Simple Stain MAX-PO, Nichirei Biosciences Inc., Tokyo, Japan) in a moist chamber for 30 min at room temperature. After three washes with 0.075% Brij detergent in PBS for 15 min each, antigens were visualized by incubating the sections in a diaminoazobenzene (DAB) H_2O_2 substrate in Tris HCL (pH 7.6) for 10 min. The appearance of a brown stain determined by light microscopy indicated the target signal regardless of nuclear staining intensity. The slides were washed with distilled water for 5 min.

The sections were counterstained with Harris hematoxylin for 30 s to 10 min, washed with alkaline tap water, then incubated with a bluing reagent for 5–30 min. The sections were dehydrated three times with absolute alcohol for 1 min each, clarified with xylene three times (1, 5, and 5 each), and mounted with dibutylphthalate polystyrene xylene (DPX) and coverslipped. The immunoreactive cells were detected by light microscopy, and the immunohistochemical staining results were interpreted. Alveolar Type II pneumocytes that produced surfactant in the cytoplasm of lung cells were identified by brown staining.

Immunostained lung sections were visualized using a 40× objective of a CX 31 RTSF Olympus Biological microscope connected to a DP22/DP27 HQ-1600 × 1200 microscope digital camera (All from Olympus Life Science, Tokyo, Japan) and photographed at the Common Research Laboratory, University of Medicine-2, Yangon. An average of 21 non-overlapping fields per slide were systematically counted using a HumaScope PAD 500Image Analyzer microscope (Human Diagnostics Worldwide, Wiesbaden, Germany). At least 1000 Type II cells were counted per slide.

Analysis of immunohistochemical staining

The positivity surfactant index was calculated as [17]:

$$Alveolar\ Type\ II\ cells\ secreting\ surfactant\,[\%] = \frac{Number\ of\ positive\ Type\ II\ cells \times 100}{Total\ number\ of\ cells} \quad .$$

We randomly counted positive Type II cells in 21 fields per slide (n = 73) at 22–33 weeks of gestation to determine the total and average numbers of positive Type II cells secreting surfactant. The positivity index is shown as a ratio [%].

Statistical analysis

Data were statistically analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). The results are presented as ratios [%] and the frequency distributions of categorical data are presented as means and standard deviations of continuous data. The statistical methods performed included the chi-square test, one-way ANOVA with Bonferroni post-hoc pairwise comparisons and linear regression[5] to analyze the data whenever appropriate.

RESULTS

Study population

We included 92 human fetuses with known gestational age (22–44 weeks). Table 1 shows the frequency distribution of gestational age and sex in the study population. The gestational ages of 22–24 (25%), 25–27 (16.3%), 28–30 (19.6%), 31–33 (18.5%), 34–36 (6.5%), 37–39 (4.4%), and 40 and above (9.7%) and number of fetuses were 23, 15, 18, 17, 6, 4, and 9, respectively.

Histological studies

We identified fetal lungs with tubular structures and a large lumen lined with simple cuboidal, to pseudostratified columnar ciliated epithelium as bronchial trees from 22–25 weeks of gestation (Fig. 1A, black arrows). Small mesenchymal accumulations resembling cartilage plates were found near the bronchial tubes (Fig. 1A, star and small blood vessels were also evident (Fig. 1A, green arrows). These findings suggested that a gestational age of 22–25 weeks indicates the canalicular stage of development, and that all bronchial airway and vascular development starts before this gestational age.

Bronchioles lined by cuboidal epithelium were detected from 26–27 weeks (Fig. 1B, black arrows). Bronchioles and primitive alveoli (Fig. 1B, black arrowheads) first appeared during this time, after which their numbers increased as gestational age advanced. Several bronchioles were detected from 28 to 33 weeks (Fig. 1C, black arrows), and abundant differentiated primitive alveoli were lined with alveolar wall cells, indicating the saccular stage of development (Fig. 1C, black arrowheads). Small blood vessels were also identified (Fig. 1C, green arrow).

Clusters of alveolar sacs were detected (Fig. 1D, black arrowheads), with openings from the alveolar ducts (Fig. 1D, blue arrows) from 34 to 40 weeks. All lung components were identified, and the bronchioles had differentiated into terminal and respiratory bronchioles (Fig. 1D, black arrows), with saccular alveolar outpouching and small knobs of smooth muscles (Fig. 1D, star). Respiratory bronchioles and alveolar ducts differed in the smooth muscle knobs on the lining wall The alveolar walls were extremely thinned between 40 and 44 weeks of gestation (Fig. 1E, black arrowheads) with openings from the alveolar ducts (Fig. 1E, blue arrow), indicating the alveolar stage of development that resembled an adult lung.

Immunohistochemical studies

We detected and quantified the immunoreactive alveolar lining Type II pneumocytes using Anti-Human SP-A PE 10 in the lungs of 92 autopsied human fetuses. Of note, 29 fetuses (31.53%) aged 22–25 weeks showed no positive staining reaction, whereas the lungs of 19 fetuses (20.65%) aged 34–44 weeks showed extensive expression of the surfactant. Only 44 cases (47.82%) between 26 and 33 weeks of gestation were counted as positive for Type II cells. Among these, the mean numbers of male and female fetal lungs were 17 (112.76 ± 85.31) and 27 (134.19 ± 76.92). Positive Type II pneumocyte parameters by sex did not differ significantly ($p > 0.05$). Type II cells started to produce surfactant at 26 weeks of gestational age, and the number of positive cells was 1.6 ± 1.14. At 27 weeks, this number increased to 4 ± 0.71, then significantly increased to 67 ± 14.85 at 28 weeks ($p < 0.001$). These numbers progressively increased at 29, 30, 31, 32, and 33 weeks to 155.8 ± 14.45 ($p < 0.001$), 162.4 ± 13.83, 191.0 ± 3.56 ($p < 0.01$), 188.78 ± 7.35, and 230.5 ± 13.99 ($p < 0.001$), respectively (Figs. 2, 3). Brown-stained aggregates were detected all over the intra-alveolar wall and luminal space from 34 to 44 gestational weeks. We identified a correlation between PE10-positive alveolar Type II pneumocytes and gestational ages between 26 and 33 weeks ($R^2 = 0.9212$, $p < 0.001$).

Positive surfactant-producing Type II pneumocytes could not be detected between 22 and 25 weeks; therefore, no positivity index could be calculated. The surfactant was released from 34 to 44 weeks, and spread throughout the alveoli. Table 2 shows details of the positivity index of Type II pneumocytes based on gestational weeks 26 to 33 and sex. The positivity index significantly differed as gestational age advanced. The mean SP-A PE10 positivity indexes at 22 to 25, 26, 27, 28, 29, 30, 31, 32, and 33 weeks were 0.70 ± 0.50, 2.0 ± 0.35, 26.23 ± 5.34, 64.91 ± 2.64, 66.25 ± 3.58, 82 ± 5.65, 87.04 ± 3.84, and 96.72 ± 0.99, respectively. One-way ANOVA with Bonferroni post-hoc pairwise comparisons revealed significant differences between 27 to 29, 30 and 31, and 32 and 33 weeks ($p < 0.001$ for all; Fig. 4). Among 44 fetal lungs at 26 to 33 weeks, 17 and 27 were from males and females respectively, and their mean positivity indices of alveolar Type II pneumocytes secreting surfactant were 28.25 ± 36.66 and 35.41 ± 39.05, respectively. These parameters did not significantly differ between the sexes.

The positivity index of alveolar Type II pneumocytes secreting surfactant and a gestational age between 26 and 33 weeks significantly correlated ($R^2 = 0.9212$, $p < 0.001$). We calculated the positivity index of Type II pneumocytes in fetal lungs expressing SP-A PE10, and estimated the gestational age of fetuses using the correlation and regression formula of: $x = y + 392.61/15.094$

as 26–33 weeks (x stands for gestational age in weeks and y stands for positivity index of Type II cells by %, Fig. 5) [5].

DISCUSSION

Here, we identified bronchial trees at a gestational age of 22–25 weeks as tubular structures with large lumens lined by simple cuboidal, to a pseudo-stratified ciliated columnar epithelium, assuming that all bronchial airways and vascular development started before this canalicular stage. Bronchioles were lined by cuboidal epithelium and primitive alveoli at 28 to 33 weeks of gestation during the saccular stage of the fetal lung. The cluster of alveolar sacs opened from the alveolar duct from 34 to 40 weeks of gestation. The alveolar walls were extremely thinned at 40–44 weeks of gestation and resembled the findings of adult lungs, suggesting the alveolar stage of development. The histogenesis sequences of developing fetal lungs determined herein were consistent with previous reports that divided lung development into canalicular (16 to 25 weeks), saccular, and alveolar stages [9, 10, 20, 32].

Several structures, as well as a few that are serous and resemble adult glands, have been identified in the wall of the large bronchus at 23 weeks of gestation [26]. At this stage, the bronchi are fully differentiated, having a ciliated pseudostratified columnar epithelium with cartilage plates, lymphoid tissue, mucous and serous glands, smooth muscle, and connective tissue in the wall. The distal bronchial tubes are lined with low cuboidal cells surrounded by a few spindle-shaped cells. Although we identified bronchial tubes with a lining of pseudo-stratified columnar to cuboidal epithelium, structures resembling adult glands were undetectable at this gestational age. This dissimilarity in the results might be due to differences in the types of tissues studied and laboratory methods. The bronchioles lined by the cuboidal epithelium and primitive alveoli were obvious at 28 to 33 weeks, and they increased with advancing gestational age. These findings comply with others who have found bronchioles, alveolar ducts with further subdivisions, and an abundance of primitive alveoli at 28 to 33 weeks [21, 33].

The granular reaction product, SP-A, expressed in the cytoplasm of alveolar wall cells of fetal lungs, indicated that these cells are alveolar Type II. The numbers of positively stained cells varied with gestational age. The present study showed that no alveolar Type II pneumocytes in fetal lung tissue stained positive for SP-A PE10 at the gestational age of 22 to 25 weeks. These findings were consistent with other reports indicating that fetal lungs at 22 weeks of gestation do

not stain positive for alveolar Type II cells [4, 23]. Our results are also consistent with those of others who identified an earlier onset of surfactant production using biochemical and electron microscopy [29–32]. These authors concluded that pulmonary surfactants are not synthesized before 22 to 25 weeks of gestation. The present study detected surfactant production at 26, but not found at 22 weeks.

The number of SP-A PE10-positive Type II pneumocytes significantly increased from 27 to 29 weeks of gestational age and then progressively increased as gestational age reached full term. These results are consistent with reports of a progressive increase in the number of Type II pneumocytes secreting surfactant as gestational age advanced from 26 weeks of gestation [22, 34]. The rate of surfactant secretion from Type II pneumocytes significantly changed from 27 to 28 weeks in the present study. This finding is consistent with previous findings of surfactant synthesis in fetuses starting at 28 weeks of gestation and reaching functional levels at 34 weeks [15].

At 34 to 44 weeks of gestation, a large amount of brown granular aggregates were stained all over the intra-alveolar space. Only a few Type II cells with a clear morphological appearance were positive. This is inconsistent with previous findings in which a few scattered cells were positive only at 31 weeks of gestation [4]. However, many cells were positively stained and reaction products were abundant in the alveolar lumen of a newborn lung at 39 weeks of gestation; this was confirmed by the presence of many positively stained reaction products at this time point. The difference might be attributable to methodological and pathological factors. For example, an evaluation of SPA and pro-SPB immunoreactivity in the lungs of 15 fetuses at 14 to 22 weeks of gestation, and 25 neonates from 24 to 41 weeks found the highest reactivity in term neonates. However, one neonate with grade 3 reactivity was detected at 22 weeks and one was negative for both proteins at 31 weeks. These findings revealed obvious inter-individual variability in SPA and pro-SPB production and suggested that other epigenetic factors acting during gestation might influence surfactant production and, consequently, the survival potential of neonates at birth [7].

The present findings suggested that the positivity index of alveolar Type II pneumocytes secreting surfactant increases as gestational age advances in human fetal tissues. Gestational age was estimated by calculating the SP-A PE10 positivity index of Type II pneumocytes in fetal lungs. The gestational age of the fetus could be estimated using a formula ($x = y +$

392.61/15.094) between 26 and 33 weeks [5]. Although this formula was useful for estimating gestational age, it was not applicable to all gestational ages. The formula could not be applied between 22 and 25 weeks of gestation, when SP-A PE10 positive Type II pneumocytes were undetectable. From 33 to 44 weeks of gestation, SP-A PE10 positive aggregates were almost spread across the alveolar surfaces and SP-A PE10 positive Type II pneumocytes were undetectable among cellular features. We assumed that more surfactant was produced and spread over the alveolar surface of the fetal lung.

We found a significant correlation between fetal gestational age and alveolar Type II pneumocytes that secrete surfactant. Alveolar Type II pneumocytes can be immunohistochemically identified in fetal lung tissues based on SP-A PE10 expression, which can help to assess fetal gestational age. This information is valuable for anatomical research, forensic medical investigations, and obstetric and neonatal management.

CONCLUSIONS

The present study investigated SP-A expression during the development of human fetal lungs in Myanmar (Burma). We found that Type II alveolar epithelial cells express SP-A, which significantly increases between 26 and 33 weeks, and that surfactant secretion can begin at 26 weeks of gestation. Furthermore, the number of Type II cells considerably increased from 28 weeks. The surfactant spread throughout the alveoli at 34 weeks. Because the positivity index of these cells correlated with gestational age, we established a formula to estimate the gestational age. These findings should contribute to understanding the development and maturity of fetal lungs and provide valuable insights for the diagnosis and management of respiratory disorders in newborns in Myanmar (Burma). However, further detailed investigation using electron microscopy is needed to define intracellular structures and determine functional constituents at various gestational ages.

Article information and declarations

Data availability statement

Data will be made available upon reasonable request.

Ethics statement

The Board of Ethics and Research Committee at the University of Medicine 1, Yangon, Myanmar, approved the post-mortem collection and investigation of human fetal and neonatal lungs. Gestational age was determined based on the last menstrual period (LMP) of the mother. Guardians of the fetuses and autopsied neonates provided written informed consent and histories after receiving a detailed description of the study.

Authors' contributions

Saung A Kari: literature search, project development, ethical application, data collection and data analysis, figure preparation, manuscript — writing and editing, final approval of the version to be published.

Zaw Myo Hein: literature search, project development, ethical application, data collection and analysis, figure preparation, manuscript — writing and editing, final approval of the version to be published.

Gehan El-Akabawy: literature search, data analysis, figure preparation, manuscript - writing and editing, final approval of the version to be published. **Saung Ya Mone Naing:** literature search, figure preparation and final approval of the version to be published.

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Conflict of interest

The authors declare they have no competing interests.

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Table 1. Frequency distribution of gestational age (weeks) by sex.

Gestational age (weeks)	**Sex**		**Total**
	Male	**Female**	
22–24	10 (10.86%)	13 (14.1%)	23 (25.0%)
25–27	7 (7.6%)	8 (8.7%)	15 (16.3%)

28–30	7 (7.6%)	11 (11.9%)	18 (19.6%)
31–33	5 (5.4%)	12 (13.0%)	17 (18.5%)
34–36	3 (3.26)	3 (3.24)	6 (6.52)
37–39	1 (1.08)	3 (3.24)	4 (4.34)
40+	3 (3.26)	6 (6.49)	9 (9.78)
Total	**39 (42.39%)**	**53 (57.4%)**	**92 (100.0%)**

Table 2. Total cell counts and positive Type II cells at different gestational ages (weeks).

No.	Case No.	Sex	GA	Positive cell	total cells	PI [%]
1	36	F	26	2	221	0.9
2	82	M	26	1	210	0.5
3	89	F	26	3	223	1.3
4	92	M	26	2	259	0.8
5	84	M	27	4	227	1.8
6	60	M	27	4	188	2.1
7	5	F	27	3	198	1.5
8	11	M	27	4	180	2.2
9	19	F	27	5	210	2.4
10	56	F	28	58	256	22.7
11	62	F	28	68	254	26.8
12	31	M	28	45	270	16.7
13	26	M	28	87	265	32.8
14	8	F	28	55	228	24.1
15	77	F	28	72	278	25.9
16	35	F	28	87	265	32.8
17	39	F	28	64	228	28.1
18	86	M	29	143	217	65.9
19	69	F	29	157	256	61.3
20	80	M	29	148	235	63.0
21	55	F	29	180	269	66.9
22	34	F	29	151	224	67.4
23	29	M	30	144	220	65.5
24	9	M	30	152	219	69.4
25	3	M	30	173	248	69.8
26	18	F	30	167	274	60.9
27	30	F	30	176	268	65.7

28	78	F	31	188	231	81.4
29	91	M	31	191	257	74.3
30	4	F	31	189	217	87.1
31	85	F	31	196	230	85.2
32	68	M	32	193	230	83.9
33	43	F	32	189	212	89.2
34	54	F	32	178	202	88.1
35	10	F	32	180	211	85.3
36	12	F	32	194	216	89.8
37	59	F	32	187	224	83.5
38	61	M	32	193	210	91.9
39	15	M	32	184	228	80.7
40	17	F	32	201	221	91.0
41	42	F	33	223	230	97.0
42	57	M	33	249	254	98.0
43	65	F	33	233	243	95.9
44	38	F	33	217	226	96.0

GA — gestational age; PI — positivity index.

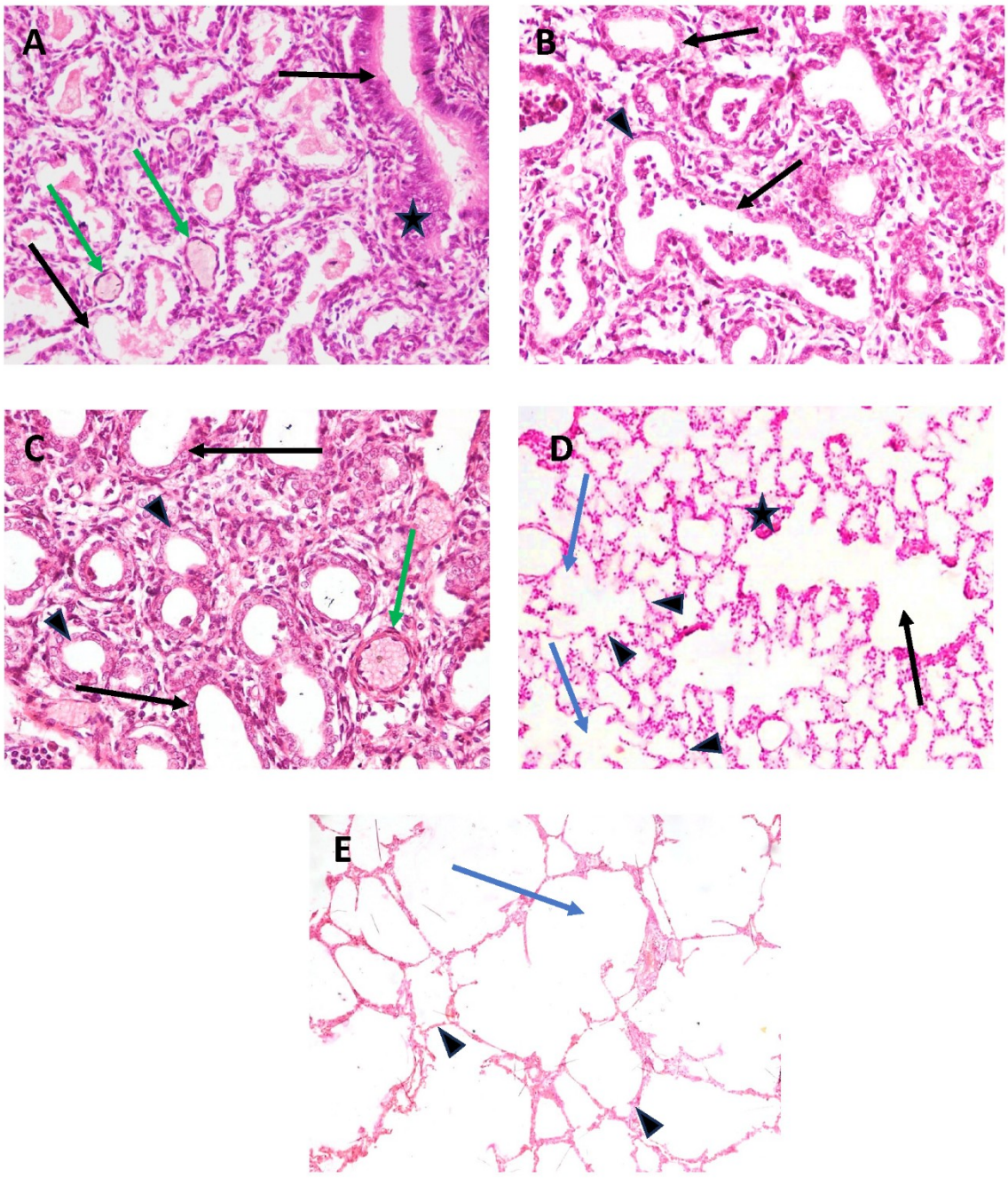

Figure 1. Representative images of hematoxylin and eosin staining; **A.** Bronchial tubular structures are lined by simple cuboidal to pseudo-stratified columnar ciliated epithelium (black arrows), cartilage plates (star), and small blood vessels (green arrows) from 22–25 gestational weeks; **B.** Bronchioles (arrows) and primitive alveoli (arrowhead) are evident from 26–27 weeks; **C.** Bronchioles (black arrows) and abundant primitive alveoli (arrowheads) pouch lined by alveolar wall cells from 28 to 33 weeks; **D.** From 34–40 weeks, clusters of alveolar sacs

(black arrowheads) have openings into alveolar ducts (blue arrows), bronchioles are well differentiated into terminal and respiratory types (black arrows) with saccular alveolar outpouching and small knobs of smooth muscle (star); **E.** From 40–44 weeks, alveolar walls (black arrowheads) are extremely thinned with openings from alveolar ducts (blue arrows) and resemble adult lungs.

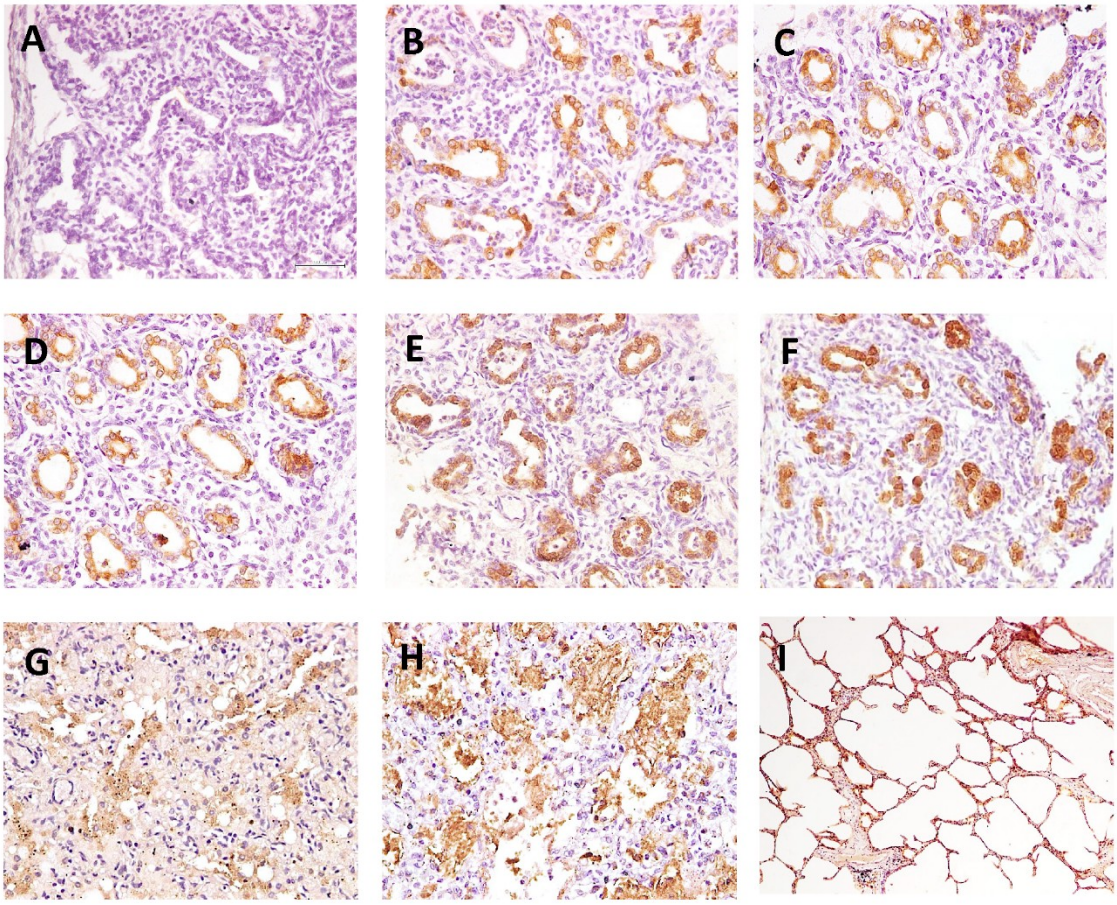

Figure 2. Anti-human surfactant protein (SP-A, surfactant protein-A) staining of fetal lungs at different gestational ages. Representative images of fetal lungs at weeks 27 (**A**), 28 (**B**), 29 (**C**), 31 (**D**), 32 (**E**), 33 (**F**), 34 (**G**), 38 (**H**), and 44 (**I**), show a gradual increase in numbers of positive cells as gestational age advances.

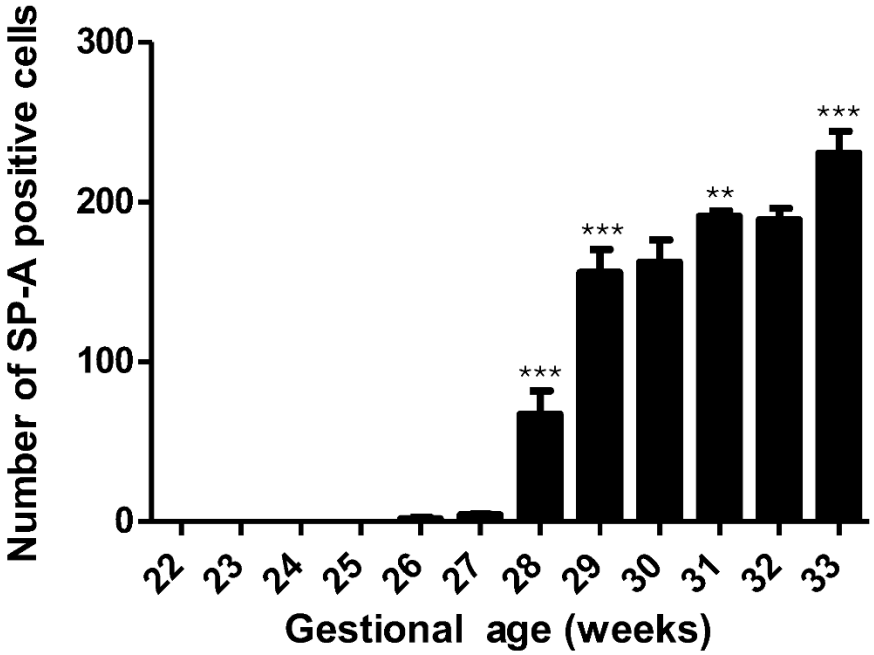

Figure 3. Numbers of surfactant protein-A positive cells at gestational ages between 22 and 33 weeks. Numbers of cells significantly differ between 27 to 29 ($^{***}p < 0.001$), 30 and 31 ($^{**}p < 0.01$), and 32 and 33 weeks ($^{***}p < 0.001$); one-way ANOVA with Bonferroni post-hoc pairwise comparisons.

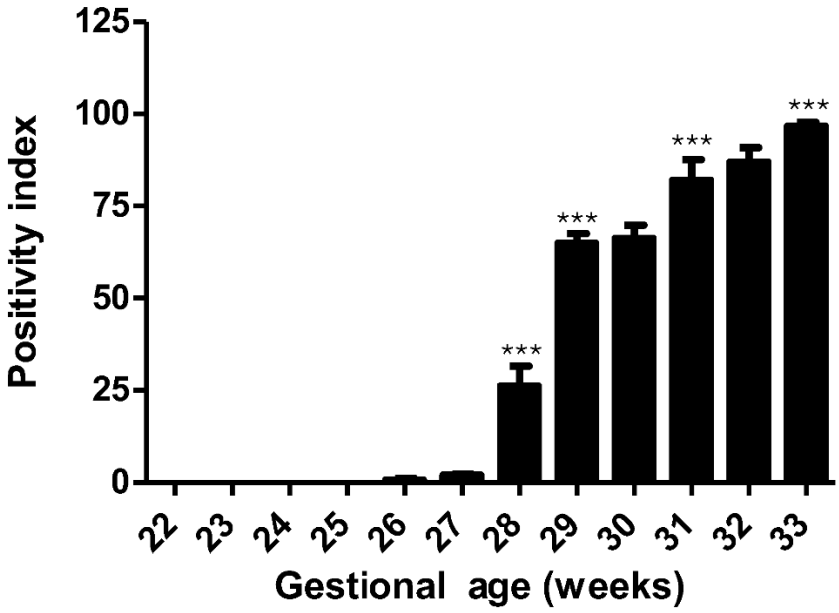

Figure 4. Positivity index of surfactant protein-A positive cells at gestational ages between 22 and 33 weeks. Index significantly differs among 27 to 29, 30 to 31 and 32 and 33 weeks ($^{***}p < 0.001$ for all; one-way ANOVA with Bonferroni post-hoc pairwise comparisons).

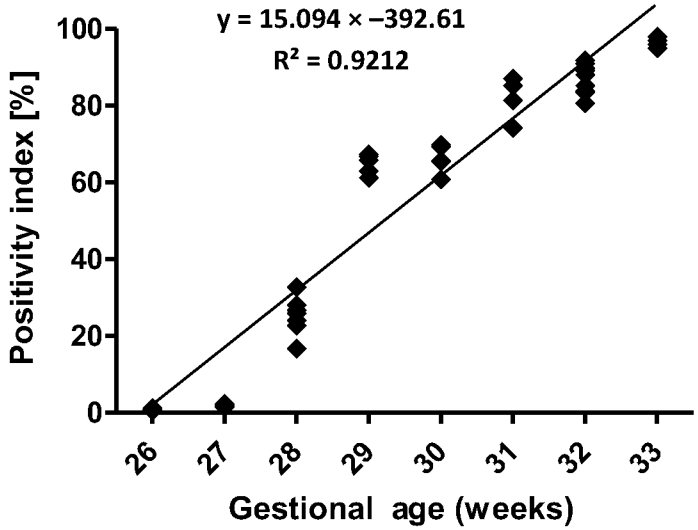

Figure 5. Scattered diagram of positivity index [%] by gestational weeks between 26 to 33 weeks. By linear regression analysis, y = 15.094 x –392.61, x stands for gestational age in weeks,

y stands for positivity index of Type II cells by %, 15.094 is the gradient of the line and –392.61 is the intercept.