Preterm birth affects both surfactant synthesis and lung liquid resorption actors in fetal sheep

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ABSTRACT

Introduction: After birth, the lungs must resorb the fluid they contain. This process involves multiple actors such as surfactant, aquaporins and ENaC channels. Preterm newborns often exhibit respiratory distress syndrome due to surfactant deficiency, and transitory tachypnea caused by a delay in lung liquid resorption. Our hypothesis is that surfactant, ENaC and aquaporins are involved in respiratory transition to extrauterine life and altered by preterm birth. We compared these candidates in preterm and term fetal sheeps.

Materials and methods: We performed cesarean sections in 8 time-dated pregnant ewes (4 at 100 days and 4 at 140 days of gestation, corresponding to 24 and 36 weeks of gestation in humans), and obtained 13 fetal sheeps in each group. We studied surfactant synthesis (SP-A, SP-B, SP-C), lung liquid resorption (ENaC, aquaporins) and corticosteroid regulation (glucocorticoid receptor, mineralocorticoid receptor and 11-betaHSD2) at mRNA and protein levels.

Results: The mRNA expression level of *SFTPA*, *SFTPB* and *SFTPC* was higher in the term group. These results were confirmed at the protein level for SP-B on Western Blot analysis and for SP-A, SP-B and SP-C on immunohistochemical analysis. Regarding aquaporins, ENaC and receptors, mRNA expression levels for *AQP1*, *AQP3*, *AQP5*, *ENaCα*, *ENaCβ*, *ENaCγ* and *11βHSD2* mRNA were also higher in the term group.

Discussion: Expression of surfactant proteins, aquaporins and ENaC increases between 100 and 140 days of gestation in an ovine model. Further exploring these pathways and their hormonal regulation could highlight some new explanations in the pathophysiology of neonatal respiratory diseases.

1. Introduction

One of the greatest challenges of transition from intra-uterine to extra-uterine life is the ability for fetal lung to change from a fluid-filled organ to an air-filled organ that is able to provide adequate hematosis to the newborn. Preterm birth, which occurs before 37 weeks of gestational age (GA) in humans, alters this transition, and preterm newborns often exhibit respiratory diseases, with an incidence that is inversely correlated to their GA (Ancel et al., 2015). Fetal fetal sheeps have always been the most chosen animal model regarding birth adaptation, mostly due to their comparable size to the human newborn, and the lung development similarities in both species (Samson et al., 2018). Nevertheless, extensive studies on transition to extra-uterine life cellular and molecular physiology are lacking. To fulfill their hematosis duty quickly after birth, the lungs must both resorb the fluid that they contain and maintain an efficient functional residual capacity to allow breathing. These first steps involve different actors such as surfactant, aquaporins and ENaC channels. The implementation of these different actors is controlled by various hormones and is altered by prematurity (Morton and Brodsky, 2016).

Pulmonary surfactant is a lipid-protein complex and highly surface-active molecule made of many different molecules such as lipids (85% of

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Table 1
Description of the oligonucleotide primers used for gene quantification by real team RT-qPCR.

	Gene	Primer F (5' - 3')	T_mF (°C)	Primer R (5' - 3')	T_mR (°C)	Amplicon size (bp)
House-keeping genes	*RPL19*	CCCCAATGAGACCAATGAAATC	53	CAGCCCATCTTTGATCAGCTT	52	72
	PPIA	TGACTTCACACGCCATAATGGT	48	CATCATCAAATTTCTCGCCATAGA	47	62
Genes of interest	*SFTPA*	GCAGCCACTCTAACTTTGCC	59	CCCAGTGTGGTGCTATGGAG	60	132
	SFTPB	TGTGCGACTCCGTTCAGTCC	62	CAGAGGGGTGAGCGGGTTT	62	147
	SFTPC	CTCAGCATTCTCTGGGGACCT	61	GGATCTCCACTGTCCCTTTGG	60	127
	ENaCa	TTCCAGTGACACAGGCGTTT	60	TCTTCACTACACTGGGCTGC	60	140
	ENaCβ	CTGGACGTCATTGAGTCTGACA	60	CCATATCCATACCAGCACCGT	52	200
	ENaCγ	ACCCACCTCAGCTGCTTTTT	60	AGCCAGCACATGGTTCTTGA	60	188
	AQP1	CATCGAGATCATCGGGACTCT	59	AATTGTGCGTGATCACCGAGG	61	200
	AQP3	TCAGGGCAAAAGACCGAGTG	60	TGAGAAACGCCTGTACCTGG	60	154
	AQP4	GGTTGGACCAATCATAGGAGC	58	ATCAAGTCGTCGGTCTCCAC	59	177
	AQP5	ACTGACCAGCATCAGACAGGG	62	GCTCTTGCTTCCAGTTCTTCAC	60	164
	NR3C1	TGAGTTAAGCAGGCTTCAGGT	59	AAAACCGCTGCCAGTTCTGA	60	194
	NR3C2	TGTAACCGGGTTTGGGATGG	60	TGGGACAGGCATTCAACAGT	60	141
	11βHSD2	CATCGAGCACTTGAATGGGC	60	ATGACCTGGGTAATAGCGGC	60	130

phospholipids, 5% of neutral lipids) and proteins (SP-A, SP-B, SP-C and SP-D), and that covers lungs alveolar surface (Pioselli et al., 2022). It is synthesized by type II pneumocytes and prevents the alveoli for collapsing during the exhalation phases. Type II pneumocytes appear during the saccular phase of lung development, which begins at 24 weeks in humans (Yamamoto et al., 2018) and around the 100th day of gestation in sheep (Lock et al., 2015). Glucocorticoids (GC) stimulate the synthesis of surfactant and the differentiation of type I and type II pneumocytes (Ballard et al.1997; Tan et al., 1999). Therefore, they are the gold-standard preventive treatment in women at risk of preterm birth, to prevent and minimize respiratory distress syndromes in preterm infants (Gyamfi-Bannerman et al., 2016). Although the fetal adrenal produces very little quantities of glucocorticoids before the third trimester of gestation, their secretion increases in order to accelerate fetal maturation and induce labor (Ben-David et al., 2007). Maternal glucocorticoids do not cross the placental barrier as they are inactivated by 11beta-hydroxysteroid dehydrogenase (11β HDS2), an enzyme which transforms active cortisol into inactive cortisone. GC bind specific nuclear receptors, the glucocorticoid receptors (NR3C1) which act on target regions of the genome called Glucocorticoid Responsive Elements (GRE) transcription (Labbe et al., 1990; Bolt et al., 2001).

Lung liquid must be resorbed between the onset of labor and the hours that follow birth (Houeijeh et al., 2017). The physiological mechanisms underlying this phenomenon are poorly understood but must involve sodium and water transfer from the alveolar compartment to the interstitium compartment, presumably via the epithelial sodium channel (ENaC) and aquaporins, respectively.

ENaC channels are sodium channels that allow sodium transport across the plasma membrane by generating a concentration gradient, which causes an osmolarity difference on either side of the membrane, thus allowing aquaporins water absorption (Dagenais et al., 2001). ENaC channels are made up of three subunits: α, β and γ. Their expression is regulated by aldosterone via the mineralocorticoid receptor (MR), a nuclear receptor which also has a strong GC affinity. To prevent GC from competing with aldosterone, cortisol is transformed to inactive cortisone by 11β HSD2 in aldosterone target cells (Garbrecht et al., 2006; Martinerie et al., 2013). In the kidney, MR induces ENaC channels transcription. Several studies suggest that MR therefore plays an important role in the resorption of pulmonary fluid by acting on ENaC channels production in fetal lung (Jesse et al., 2009; Keller-Wood et al., 2009, 2011).

Aquaporins are trans-membrane proteins that form pores which allow the water to cross the membrane according to an osmotic gradient (Liu et al., 2003). Aquaporins initially allow the lungs to fill with pulmonary fluid, which is crucial for the lung development, then they help its resorption around birth which allows the newborn to breathe (Houeijeh et al., 2017; Liu et al., 2003; Li et al., 2009). Four aquaporins are expressed in the lungs: AQP1, AQP3, AQP4, AQP5 (King et al., 2004). Their kidney expression are linked to vasopressin concentration, a peptide hormone secreted by neurohypophysis (Wilson et al., 2013), but no study has ever shown this correlation at the pulmonary level (Chen et al., 2022).

In this study, to test our hypothesis that surfactant, ENaC and aquaporins are all involved in respiratory transition to extrauterine life, altered by prematurity, we compared these actors within preterm and term fetal sheeps.

2. Materials and methods

2.1. Animals and sampling

Tissues were collected from time-dated pregnant ewes' fetuses, from "Ile-de-France" sheep lineage. At 100 (n = 4) and 140 (n = 4) days of gestation (normal term = 145–150 days), caesarean sections were performed with a paralumbar approach after local anesthesia. The fetal sheeps were euthanized immediately after removal from the womb (Dolethal®, pentobarbital, 10ml, IM, Vetoquinol, Lure, France).

After an anatomical dissection, lungs were isolated and sampled. Half of the samples were immediately frozen in dry ice and stored at −80 °C until extraction of RNA, the other half were placed in PFA 4% (paraformaldehyde 4%) and stored at 4 °C for 3 days before their fixation in paraffin.

2.2. RT-qPCR

Total RNAs were isolated from 100 mg lung tissue after crushing using Trizol solution (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's recommendations. They were then purified using Qiagen columns (RNeasy mini-kit; Qiagen, Courtaboeuf, France) according to the manufacturer's protocol and treated with 1 μL of DNase I (Qiagen). They were quantified by spectrophotometry (NANODROP ND-3300©, Labtech, Wilmington, USA). Their quality and integrity were determined by Agilent® 2100 bio analyzer (Agilent, Massy, France). The RNA integrity number (RIN) of all the RNA samples was over 7 (Mueller et al., 2004). For each sample, cDNA was synthesized from 1 μg of the total RNAs which was retro transcribed using dNTP, oligo dT and retro transcriptase from ThermoFischer Scientific (Waltham, Massachusetts, USA) according to manufacturer's recommendation. For each sample, two retro transcriptions were performed and pooled. Then, cDNA were diluted in DNase free water and stored at −80 °C until further processing. Specific primers for thirteen genes of interest (*AQP1, AQP3, AQP4,* and *AQP5,* which code for the four aquaporins known to be expressed in lungs; *ENaCα, ENaCβ* and *ENaCγ,* which code for the three subunits of ENaC; *SFTPA, SFTPB* and *SFTPC,* which code for the three main proteins composing surfactant; *NR3C1* and *NR3C2* which code for the receptor of glucocorticoids and mineralocorticoids respectively and *11BHSD2,*

which code for 11b-hydroxysteroid dehydrogenase) and two ubiquitary housekeeping genes commonly used in our laboratory (*RPL19, PPIA*), were created using Primerblast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and produced (Eurogentec, Lièges, Belgium) (Table 1). To assess that cDNA amplification generated the expected fragment, every amplicon was sequenced and blasted on NCBI RNA ovine database to confirm its identity.

The RT-qPCR were produced using 50 ng of cDNA, 15 μM of primers and 7.5 μL of SYBR green Mastermix (Applied Biosystems, Waltham, Massachusetts). RNase DNase-free water was finally added to reach a final reaction volume of 15 μl. The PCR reactions were carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems), according to the relative standard curve method (Larionov et al., 2005). All PCR reactions were carried out in duplicate in the following cycling conditions: denaturation at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s and DNA synthesis at 72 °C for 40 s. The reaction products specificity was controlled with their respective melting curves. According to the relative standard curve method, the relative quantification of the amount of mRNA of the selected genes was calculated against a normalization factor generated by the qBasePLUS® software (Biogazelle, Zwijnaarde, Belgium) from the two housekeeping genes *PPIA* and *RPL19*.

2.3. Western-blot

Proteins were extracted from 100 mg of lung sample in a buffer Tris (0.02 M), EDTA (5 mM), NaCl (0.15 M) and, 3[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) as detergent. The grind was centrifuged at 4 °C for 15 min at 13,000 *g*. Protein quantification was carried out using the Bradford method with bovine serum albumin (BSA) as standard (Sigma-Aldrich, Saint-Louis, Missouri). Leammli buffer (4X) and β Mercaptoetanol was added to the proteins before denaturation of the proteins in a double boiler.

For each sample, 20 μg of protein were deposited on a 4–15% TGX gel (Biorad, Hercules, California, USA) for migration. Protein molecular mass markers (Hyper page, Biorad) were run simultaneously as molecular mass standards. The migration was performed in a Tris Glycine SDS 1X buffer with a constant intensity of 20 mA per gel. The proteins were then transferred to a polyvinylidene difluoride membrane (Trans-blot® Turbo Midi Size PVDF Membrane, BioRad).

The membranes were stored for 2 h at room temperature in a solution of Tris base (50 nM), Tris HCl (50 nM), NaCl (2.5 M), Régilait© (5%), TBS Tween (0.05%). The membranes were then incubated overnight at 4 °C in solutions of TBS Tween Regilait (5%) with primary rabbit polyclonal antibodies SP-A, SP-B and SP-C at the respective dilutions of 1/2000 (SP-A Merck Millipore AB3420-I, Merck, Darmstadt, Germany), 1/500 (SP–B Merck Millipore ABS21) and 1/1000 (SP–C Merck Millipore ABC99). The membranes were then incubated with anti-rabbit HRP antibody conjugated to peroxidase (diluted 1:5000; Intercim UP559721, France, Montluçon). Actin B (ACTB) was evaluated as load control, using mouse monoclonal anti-ACTB antibody (diluted 1:2000; Sigma-Aldrich) and anti-mouse IgG antibody conjugated to goat peroxidase (diluted 1:5000; Santa Cruz Biotechnology, Dallas, Texas).

The revelation was performed with a Pierce ECL 23 Western Blotting Substrate kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The chemiluminescence signal was visualized using the ChemiDoc reader (Biorad). The fluorescence was read using the LAS 1000 camera (Fujifilm Life Science, Valhalla, New York, USA) and converted in quantity using the FUJIFILM LAS-4000 Luminescence Image Analyzer software. The data were then normalized against the ACTB values.

2.4. Immunohistochemistry

From each lung sample, sections of 7 μm were carried out and fixed on slides. Hematoxylin and eosin stains were performed using a Multistainer automaton (Leica ST 5020, Leica Biosystems France, Nanterre, France) to highlight the structure of the tissue. Reading and digitization of the slides was made with the ×20 magnification using Aperio® AT2 microscope slide digitizer (Leica Biosystems).

The immunohistochemical markings were carried out using a Bonde III automaton (Leica Biosystems). The slides were then dewaxed and incubated with a citrate solution to allow access to antigenic sites. Non-specific sites were blocked using bovine serum albumin solution (BSA 5%). Incubating the slides in hydrogen peroxide solution for 20 min blocked the activity of endogenous peroxidases. The primary antibody was then added to 1/100 for SP-A, 1/500 for SP-B and 1/500 for SP-C and incubated for 1 h at 37 °C. The revelation was carried out using a secondary antibody, kit provided by Leica for immunohistochemistry (Bond Polymer Refine Detection), which reacts with DAB (3,3′-diaminobenzidine). At the end of the markings, the slides were dehydrated using a Multistainer controller (Leica ST 5020). The reading and the digitization of the slides were performed with ×40 magnification using Aperio® AT2 microscope slide digitizer (Leica Biosystems).

Table 2
Description of the two experimental groups.

Description	Preterm	Term	p.value
Pregnancy stage (in days)	100	140	
Number of ewe	4	4	
Number of fetal sheep	13	13	
Male	8	7	ns
Female	5	6	ns
Weight in g (mean [min-max])	953 [675–1200]	3455 [2370–4875]	<0.001
Offspring per litter			
Ewes A and B	Male 970g	Female 4070g	
	Male 1080g	Female 3375g	
	Female 970g	Male 4140g	
	Female 1050g	Male 3805g	
Ewes C and D	Male 675g	Male 2540g	
	Male 985g	Female 2370g	
	Male 750g	Female 2635g	
	Female 865g	Female 2620g	
Ewes E and F	Male 925g	Female 3750g	
	Male 875g	Male 2825g	
	Male 1035g	Male 3400g	
Ewes G and H	Female 1200g	Male 4875g	
	Female 1005g	Male 4510g	

ns. non-significant.

2.5. Statistical analysis

All RNA and proteins quantities of preterm fetal sheeps (100 days) were compared to term fetal sheeps (140 days). In case of normal distribution of the data (Shapiro-Wilk test), the data were analyzed with a *t*-test (Student). If the distribution was not normal, a non-parametric test, the Mann-Whitney test, was performed. The sex ratio between the two experimental groups was analyzed with a Fisher test.

The analysis was carried out with the statistical software GraphPad Prism 115 8.1. P-values <0.05 were considered statistically significant.

3. Results

3.1. Animal comparison

In this study, a caesarian section was performed on 8 time-dated pregnant ewes at 100 days of pregnancy (preterm, n = 4) and 140 days of pregnancy (term, n = 4). In the preterm group, we collected 13 fetal sheeps (8 males and 5 females) with a mean weight of 953g [675g–1200g]. In the term group, we collected 13 fetal sheeps (7 males and 6 females) with a mean weight of 3455g [2370g–4875g].

No difference was reported concerning the number of offspring per litter. The description of the two experimental groups is provided in Table 2.

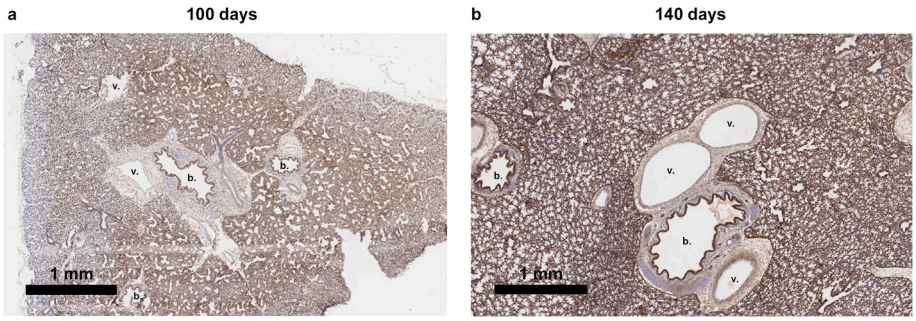

Fig. 1. Lung histological analysis in fetal sheep lungs at 100 days, preterm (a) and 140 days, term (b) after hematoxylin & eosin staining.
b. bronchial tubes, v. blood vessels.

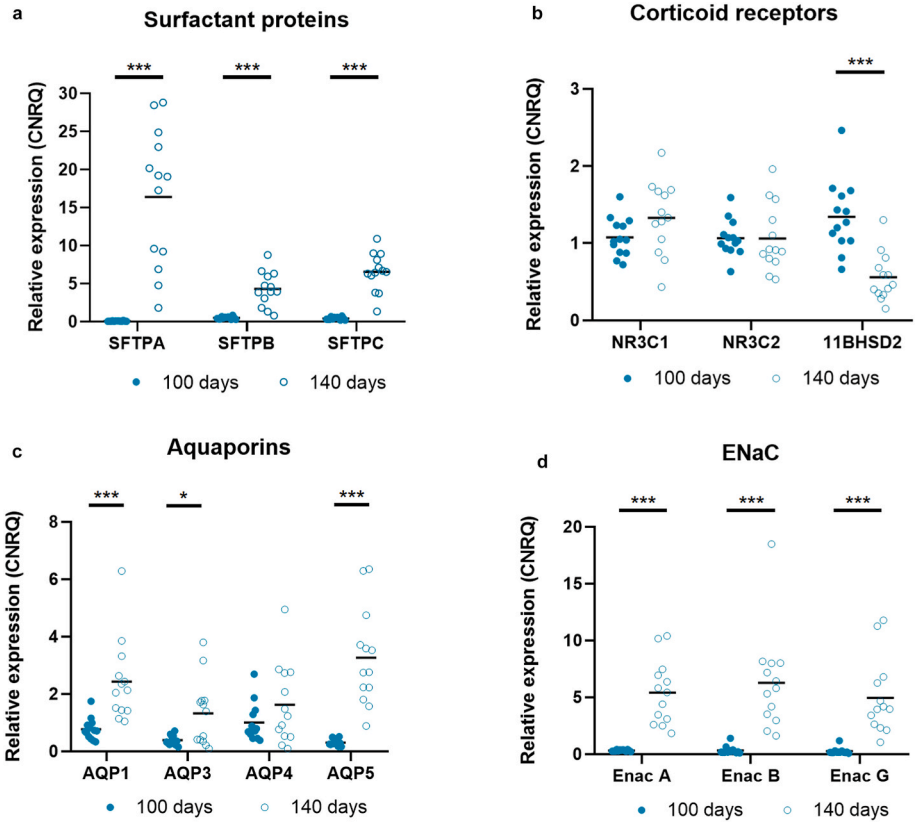

Fig. 2. Calibrated Normalized Relative Expression (CNRQ) evaluated by real-time quantitative PCR at 100 days (preterm, solid circles) and 140 days (term, blank circles) in fetal sheep lungs of: a. *SFTPA, SFTPB* and *SFTPC*; b. glucocorticoid receptor (*NR3C1*), mineralocorticoid receptor (*NR3C2*) and 11b-hydroxysteroid dehydrogenase (*11β HDS2*); c. aquaporins; and d. ENaC isoforms
* $p < 0.05$, ***$p < 0.001$.

3.2. Lung aeration and surfactant synthesis

The histological study of the lungs reveals major differences in lung structure between preterm (100 days) and term (140 days) fetal sheeps. At 100 days, the lung tissue showed blood vessels, bronchi and bronchioles but the alveoli were sparse and the lung tissue remained compact (Fig. 1a). At 140 days, the lung tissue was mature and consisted mostly of large alveoli (Fig. 1b).

The mRNA expression level of *SFTPA*, *SFTPB* and *SFTPC* (Fig. 2a) was significantly ($p < 0.001$) higher in the term (140 days) than in the preterm group (100 days) with large inter-individual variations that were not correlated with fetal sheep weight, sex or number of fetal sheep per litter. At the protein level, Western Blot analysis (Fig. 3) showed a significantly ($p < 0.001$) higher amount of SP-B in the term than in the preterm group. SP-C tended ($p = 0.07$) to be more abundant in full-term fetal sheeps as well. No blotting was observed for SP-A. Immunohistochemical analysis showed the presence of the proteins SP-A (Fig. 4a and b), SP-B (Fig. 4c-d-e) and SP-C (Fig. 4f-g-h) around the alveoli and in the bronchial tubes with significantly higher levels of proteins in full-term compared to the preterm fetal sheeps (respectively $p < 0.001$, $p < 0.05$ and $p < 0.05$). No group effect between fetal sheeps of different ewes was observed concerning results in RT-PCR, western-blotting or immunohistochemistry.

3.3. Resorption of pulmonary fluid actors

Regarding aquaporins, mRNA expression levels for *AQP1*, *AQP3* and *AQP5* mRNA were respectively 3.1-fold ($p < 0.001$), 3.4-fold ($p < 0.05$) and 10.8-fold ($p < 0.001$) higher in the term fetal sheeps compared with the preterm fetal sheeps, and no significant difference was found for

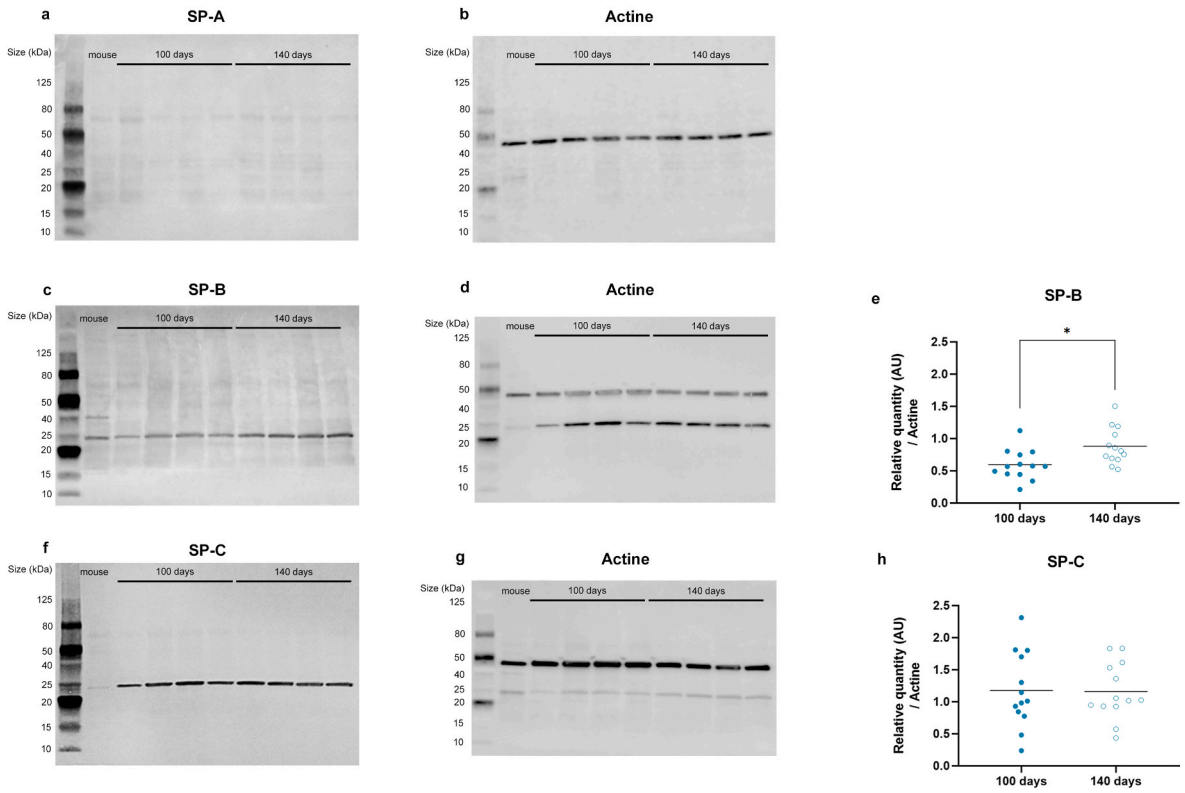

Fig. 3. Western blot evaluation of the relative quantity (AU, Arbitrary Unit) of SP-A (a–b), SP-B (c-d-e) and SP-C (f-g-h), normalized with actine; n.d. non determined, *p < 0.05.

AQP4 (Fig. 2c). Similarly, for ENaC, mRNA expression levels for *ENaCα, ENaCβ* and *ENaC* mRNA were respectively 17.5-fold, 19.1-fold and 17.8-fold higher ($p < 0.001$) in the term fetal sheeps compared with the preterm fetal sheeps (Fig. 2d). No group effect between fetal sheeps of different ewes was observed.

3.4. Steroids receptors

No significant difference was found in the steroid receptors (*NR3C1* and *NR3C2*) mRNA levels between the two stages of development (100 days and 140 days) but the mRNA level of *11β HDS2* was 2.4-fold higher ($p < 0.001$) in the preterm fetal sheeps (100 days) compared with the term fetal sheeps (140 days) (Fig. 2b). No group effect between fetal sheeps of different ewes was observed.

4. Discussion

The results of this study confirm that lung developmental stages and surfactant synthesis in fetal sheep at 100 and 140 days are close to what is observed at 24 and 36 weeks of gestation in humans, respectively, and allow new insights regarding pulmonary liquid resorption at the end of gestation.

Histological analysis shows canalicular then saccular stage at 100 and 140 days of gestation respectively, with progressive alveolarization, which matches with human gestational ages of 24–25 weeks and 35–36 weeks, as previously described (Joshi and Kotecha, 2007). In parallel, we showed that surfactant production increases between 100 and 140 days of gestation in the preterm fetal sheep, at transcription, at translation and at translocation levels. As this was expected, it confirms the accuracy of the fetal sheeps as a model used to study the adaptation to extra uterine life. Previous studies already demonstrated an increase in SP-A, SP-B, SP-C at expression level between preterm and term fetal sheep (Sozo et al., 2006) and in tracheal aspirates between newborn and 1 week preterm babies (Ballard et al.2003). Although we did not find differences in GR expression in the fetal sheep lungs, glucocorticoids have been clearly associated to lung maturation and surfactant production (Bolt et al., 2001) therefore we can hypothesize that there is a difference in cortisol concentrations depending on gestational age, adrenal progressively increasing its cortisol secretion throughout the pregnancy (Lalli, 2010). Unfortunately, we did not explore hormonal assays to test this hypothesis in our study.

Other limitations of our study are the lack of exploration of surfactant quality, regarding phospholipids for example, and the immediate euthanasia which did not allow an optimal constitution of functional residual capacity (Joshi and Kotecha, 2007). We observed major inter individual variations of surfactant proteins in “140 days” fetal sheeps, which were not correlated to birth weight or sex. This could explain why respiratory distress syndrome, which is due to surfactant deficiency, is possible but rarely observed in late preterm babies (Ahimbisibwe et al., 2019).

On the other hand, transitory tachypnea of the newborn (TTN), due to delayed lung liquid resorption, is frequent among late preterm neonates and may be associated to other biochemical pathways, such as ENaC and/or aquaporins (Li et al., 2009). Our results showed significant differences between 100 and 140 days of gestation for all subunits of ENaC and several aquaporins, at transcription levels, allowing us to suggest a graduate maturation of the lung liquid resorption during the end of pregnancy. These results confirm previous published data, where both ENaC subunits and AQP1, 3, 4 and 5 were described to be expressed in the fetal fetal sheep’s lung (Liu et al., 2003; Farman et al., 1997). Alpha ENaC may be the most important for adaptation to extra-uterine life, with death of inactivated αENaC mutant mice shortly after birth (Hummler et al., 1996). Quantitative studies of lung fluid are consistent with this hypothesis. In ovine fetus, lung fluid is secreted at 2 ml/kg/h at mid gestation and gradually increases to 5 ml/kg/h near term (Kotecha, 2000). At the end of gestation, there is a need for reversal of this process from secretion to resorption to ensure normal transition to extra-uterine life without TTN (Jain and Eaton, 2006). There is a massive decline in

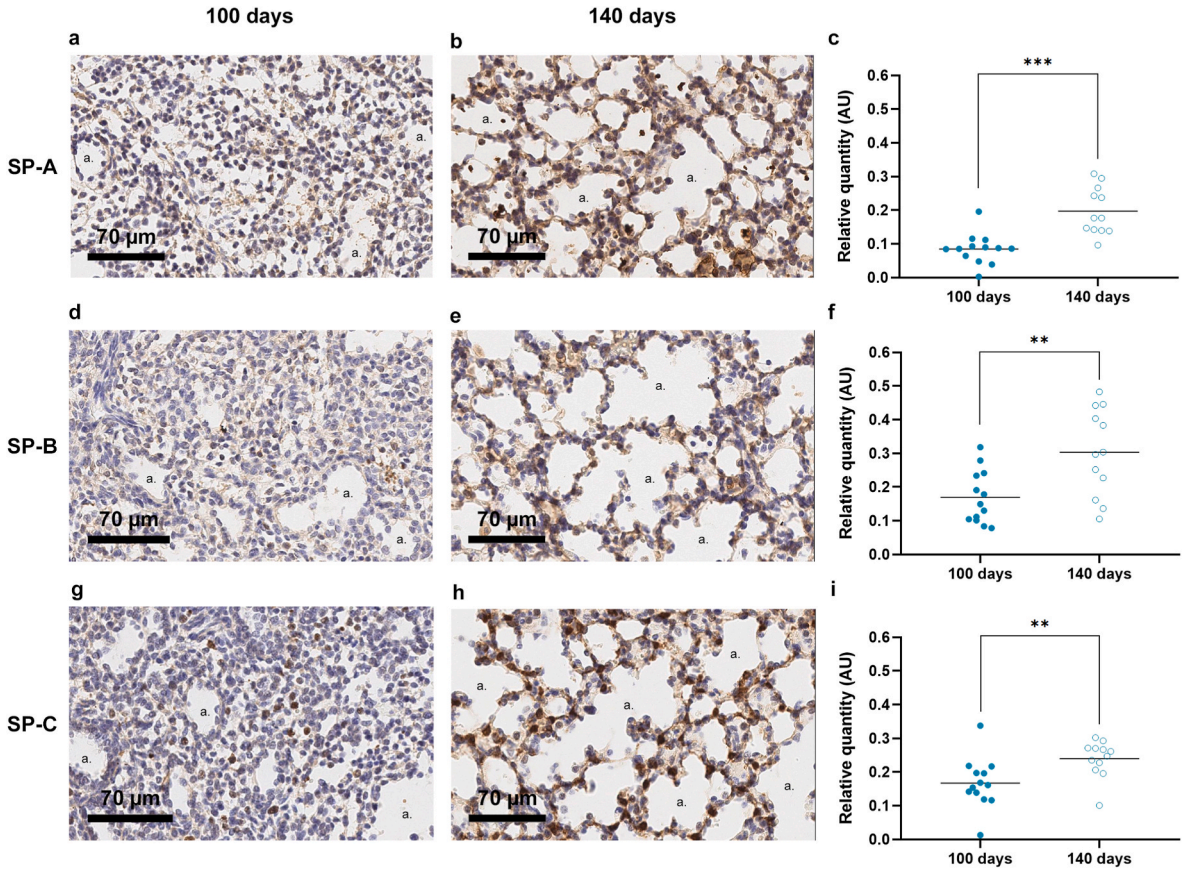

Fig. 4. Immunohistological analysis and immunohistochemistry evaluation of the relative quantity (AU, Arbitrary Unit) of SP-A (a–c), SP-B (d–f) and SP-C (g–i) in fetal sheep lungs at 100 days, preterm (solid circles) and 140 days, term (blank circles)
a. alveolus, $^{**}p < 0.01$, $^{***}p < 0.001$.

lung liquid during vaginal delivery at term in fetal fetal sheep (28 ml/kg before labor vs 7 ml/kg after labor (Berger et al., 1998), which shows that lung liquid clearance begins before labor in the full term fetal sheep (Pfister et al., 2001).

Actions mediated by the adrenal steroids may induce changes in ENaC and aquaporins over the last weeks of gestation in the ovine fetus. Cortisol production increases in the last few days before birth and during active labor (Rose et al., 1978), and expression of ENaC and aquaporins is under regulation of steroids (Dagenais et al., 2001; Jesse et al., 2009; Liu et al., 2003; Venkatesh and Katzberg, 1997). Mineralocorticoids signalization may also have a role in the regulation of lung fluid production and resorption. First, low plasma cortisol concentrations during pregnancy are expected to cause greater occupancy and action via MR than GR (Keller-Wood et al., 2011; Richards et al., 2003), because MR is a high affinity receptor which can respond to low cortisol levels or normal/high aldosterone and because the fetal lung has low expression and activity of the cortisol inactivating enzyme 11betaHSD2 allowing cortisol binding to both GR and MR (Keller-Wood et al., 2009; Wood and Srun, 1995). MR expression is also considerably greater in the fetal lung than in the newborn (Keller-Wood et al., 2005). A study analyzed ovine lung ontogenesis and showed that low endogenous fetal cortisol may increase expression of ENaC at mRNA and protein levels. They also found a discrete increase for GR and MR that we did not find and confirmed our results for 11bHDS2 with a decrease between 100 and 140 days (Keller-Wood et al., 2009).

Last but not least, although surface tension reduction is widely considered as the main activity of the lung surfactant system, it is also established that a low enough surface tension is a key factor to facilitate a rapid liquid clearance from the airways, explaining why the lack of surfactant in immature lungs affects both lungs mechanic and lung liquid resorption. Deeply exploring these candidate pathways could highlight some new explanations in the lung liquid resorption pathophysiology during adaptation to extra-uterine life. It would be interesting to explore the possible effect of hormones involved in respiratory adaptation to extra-uterine life on mRNA and protein levels. Thus, we plan to pursue the use of this experimental ovine model with prenatal conditions modifications, such as maternal administration of cortisol, aldosterone, vasopressin or oxytocin. Vasopressin, which increases during labor, is well known for its role of water reabsorption in the kidneys (Stark et al., 1979) and may also have some effects on the lungs: it stimulates surfactant secretion through activation of AVPR1 (Brown and Wood, 1989; Brown and Chen, 1990), and slows down lung liquid secretion (Perks and Cassin, 1989) and reabsorption (Wallace et al., 1990) through both AVPR1 and AVPR2 (Albuquerque et al., 1998; Fay et al., 1996). Interestingly, the vasopressin structure is similar to the oxytocin structure, which is responsible for labor induction, and these two hormones can cause some cross-reactions (Song and Albers, 2018).

5. Conclusion

The surfactant proteins' and lung liquid resorption actors' expression increases between 100 and 140 days in an ovine model. The impact of the hormonal regulation is possible and would be worth analyzing, for glucocorticoids, mineralocorticoids, vasopressin and oxytocin. For example, it would be interesting to assess the concentration levels of these hormones in mothers and fetuses and study the effects of their administration on candidate mRNA and protein levels.

Statement of ethics

All procedures carried out in the present study were approved by the French Ethical Committee n°16 under protocol APAFIS#22584–2019102411142765.

Conflicts of interest

The authors have no conflict of interest to declare.

Funding source statement

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Authors contributions

EMS and VM conceptualized the study. LO achieved all experimental procedures, with some regular help from EMS, VM, FV, YR, AL, CG and MLY. LO collected data and drafted the initial manuscript. EMS, VM and FV reviewed the manuscript for important intellectual content. All authors revised the manuscript and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Data availability

Data will be made available on request.

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