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# Stereological and biophysical characteristics of the ovine surfactant system and its changes caused by ovine pulmonary adenocarcinoma

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ABSTRACT

Surfactant covers the inner surface of lung alveoli and lowers the surface tension to prevent alveoli from collapsing. A lack of surfactant or its dysfunction causes dyspnea. The Jaagsiekte Sheep Retrovirus (JSRV) causes ovine pulmonary adenocarcinoma (OPA), whose typical clinical appearance is fluid running from nostrils. This fluid might contain surfactant as alveolar type II pneumocytes (AEII) are target cells for JSRV. Therefore, the progressive dyspnea during OPA might be caused partially by surfactant alterations.

Bronchoalveolar and intracellular surfactant as well as the biophysical function of surfactant were analyzed in OPA sheep and controls. Transmission electron microscopy and stereological methods were used to characterize ultrastructure and distribution of surfactant subtypes in AEII and bronchoalveolar lavage fluid (BALF). Pulsating Bubble Surfactometry enabled studying the surface activity of the surfactant, while lung volumes were detected by computed tomography.

The methods used are suitable to determine intraalveolar and intracellular surfactant subtypes in OPA sheep and controls. OPA sheep showed more lamellar body-like forms, multivesicular vesicles and tubular myelin in BALF compared to controls. These higher amounts of active surfactant subtypes might be a consequence of a higher surfactant production and release. Surfactant subtypes in AEII of OPA sheep showed smaller and more immature lamellar bodies compared to controls. The surfactant surface activity of OPA sheep does not show obvious defects. In conclusion, the general quality of surfactant in OPA appears to be equivalent to surfactant produced in controls, however, dyspnea of OPA might be triggered by quantity of fluid production.

## 1. Introduction

The Jaagsiekte Sheep Retrovirus (JSRV) causes ovine pulmonary adenocarcinoma (OPA) (Palmarini et al., 1999) and induces oncogenic transformation of alveolar type II pneumocytes (AEII), Clara cells and undifferentiated cells, whereas the predominantly affected cell types are AEII (Platt et al., 2002). Sheep are infected by aerosolic transfer from sick individuals (Dungal, 1946). Mostly young sheep are affected because of having higher amounts of proliferating AEII. Adult sheep with lower proliferation rates are less susceptible unless mild injuries of the respiratory epithelium have caused a rise of proliferation (Murgia et al., 2011). Intratracheal virus application in seven-day-old lambs leads to severe clinical signs within weeks (Salvatori et al., 2004). After infection, proliferation of AEII and their tumor growth is induced, in parts followed by metastasis into lymph nodes. Copious amounts of lung fluid are produced that include $10^7$–$10^{10}$ copies of JSRV RNA per ml (Cousens et al., 2009). Clinical signs are raised breathing rates and a loss of weight until death caused by circulatory failure (Dungal, 1938). To confirm the infection with JSRV intravitally, a hemi-nested PCR of bronchoalveolar lavage fluid (BALF) is the most sensitive method to detect JSRV proviral DNA (Voigt, 2004; Voigt et al., 2007).

Surfactant is necessary to lower the surface tension in airways

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(Clements, 1997) and to keep alveoli open, clean and dry (Ochs, 2006). This is needed to prevent endexpiratory collapses. After being released from AEII via exocytosis into the alveolar lumina, surfactant produces a film that covers the airways of the whole lung (Clements, 1997; Welsch and Deller, 2010). AEII cover 10% of the alveolar surface (Welsch and Deller, 2010) within the lung and contain intracellular surfactant components such as lamellar bodies (lb), multivesicular bodies (mvb) and composite bodies (cb) (Banks, 1986; Chevalier and Collet, 1972; Welsch and Deller, 2010).

Several specimen and methods are available to characterize the intraalveolar lung surfactant. Bronchoalveolar lavage fluid (BALF), which has been used in sheep predominatly to detect the infectious agent, can be used as well to measure surface tension by a Pulsating Bubble Surfactometer (PBS) (Enhorning, 1977; Mottaghian, 1999) as well as to determine the intraalveolar active and inactive surfactant subtypes (Clements, 1997).

Transmission electron microscopy of BALF pellets and lung tissue is also well described for other species (Busley et al., 2016).

A stereological approach is needed to characterize three dimensional structures using two dimensional methods (Hsia et al., 2010). In order to make sure that every part of an organ has the same chance to be sampled, using uniform random sampling is necessary (Knudsen and Ochs, 2011).

To calculate the intravital volume of lung parenchyma in sheep, computed tomography (CT) is a useful tool combined with the Cavalieri principle.

Because of similarities to human cancer and the broad spectrum of studying early stages of cancer, OPA is a unique animal model to understand mechanisms of carcinogenesis (Palmarini and Fan, 2001; Youssef et al., 2015). The production of raised amounts of foamy fluid which typically runs from the nostrils of JSRV infected sheep when animals are lifted by their hindlegs (Sharp and De las Heras, 2000), leads to the hypothesis that this might cause breathing difficulties and consists at least partly of surfactant. This could be of minor quality, since it is unable to sufficiently lower the surface tension. If so, this should be reflected in lowered amounts of active surfactant subtypes in BALF along with raised surfactant production in AEII of JSRV infected sheep.

Until now no reference values for characterization of intracellular and intraalveolar surfactant of sheep are available to our knowledge. First values for intracellular surfactant of sheep have been published earlier (Lakritz et al., 1992). Further values of healthy sheep are essential to study the effects of surfactant caused by OPA, which is the aim of this study.

## 2. Material and methods

### 2.1. Animals

The JSRV infected sheep were part of a study published earlier (Humann-Ziehank et al., 2011a; Humann-Ziehank et al., 2011b). In brief, nine adult Cameroon crossbreed sheep, naturally infected with JSRV, were bought from a sheep flock in northern Germany. For the study, sheep were kept under standardized conditions over two years to follow up the clinical development of OPA. Finally, the sheep were euthanised and postmortal samples were taken for the study reported here. Six adult Cameroon crossbreed sheep held under the same feeding conditions as the OPA sheep were used as a control group.

The study was approved by the Ethical Commission of the Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany (AZ 33.12-42502-04-08/1579 and AZ 33.9-42502-05-12A254).

### 2.2. Volume of lung parenchyma

CT scans of the lungs were performed during general anesthesia as previously described (Humann-Ziehank et al., 2011a). Afterwards sheep were euthanized using 1–1.5 ml/10 kg bodyweight (bw) pentobarbital natrium (Release®, WDT, Garbsen, Germany) intravenously (i.v.). The CT scans were used to estimate the lung volume by Cavalieri principle. The CTs were evaluated stereologically by using the STEPanizer© (Tschanz et al., 2011) after being transformed from efilm™ into JPEG data formate. Hits on lung parenchyma and nonparenchyma (airways, blood vessels) were counted in a counting window with line pairs and the following settings: Nbr of tiles 36, no subsampling, line width 2, T-bar 0, frame/circle/test system (see Fig. 1) being activated. Following formula was used with slight modifications (Fehrenbach and Ochs, 1998; Hsia et al., 2010):

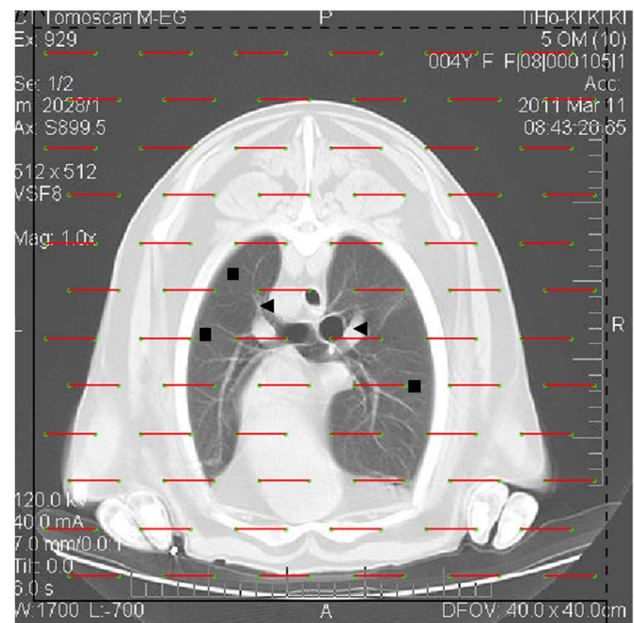


**Fig. 1.** Example for a CT scan of control sheep in the counting window of the STEPanizer©: lung parenchyma (■) and non-parenchyma (arrow head ◄).

$$\text{lung parenchyma volume} = \text{hits on lung parenchyma} \times t \times d^2$$

t = distance between CT slices (7 mm)
d = distance between counting points in respect to magnification (= 0.638)

### 2.3. Sampling

For a postmortal bronchoalveolar lavage (BAL), 100 ml 154 mM saline solution (B. Braun Melsungen AG, Melsungen, Germany) were instilled intratracheally after lungs have been taken out of the thorax. The lungs were gently massaged and the sampling liquid was regained (Dawson et al., 2005). BALF was centrifuged (10 min, 200 × *g*, 4 °C) to separate cells (Voigt et al., 2007). The cell-free supernatant of BALF was stored at − 80 °C for the examination of surfactant.

Sheep lung samples of macroscopically unremarkable as well as of tumorous areas of the OPA positive animals were taken randomly, while tissue blocks of control lungs were sampled according to uniform random sampling method.

### 2.4. Virology

All sheep were tested for proviral JSRV-DNA by hemi-nested PCR (Voigt et al., 2007) of postmortal BALF, lung lymph nodes, lung parenchyma and the buffy coat of centrifuged blood. They were also tested for Maedi-Visna-Virus (MVV) antibodies using serum for an enzyme linked immunosorbat assay (ELISA, Small Ruminant Lentivirus

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