Consequences of One-lung Flooding – A Histological and Immunological Investigation

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Abstract

Background: Videothoracoscopic lung sonography after partial fluid instillation could be a new method for endoscopic detection of lung lesions. Histopathological consequences of unilateral diagnostic or therapeutic lung flooding under bronchoalveolar lavage has yet to be defined. The aim of the study was to investigate histological and immunohistological alterations induced by one-lung flooding (OLF).

Methods: 13 female pigs were subjected to OLF (15 ml isotonic electrolyte solution per kg for 60 minutes), and lung tissue was collected 30 minutes, 2 hours, 24 hours, 48 hours, 6 days, 8 days, and 10 weeks after flooding. Histological examinations and immunohistochemical labeling for surfactant protein A (SP-A) were performed. Cellular proliferation was measured by Ki67 immunohistochemical labeling. Apoptosis was detected through enzymatic in-situ labeling of apoptosis-induced DNA strand breaks by means of the TUNEL (TdT-mediated dUTP nick end labeling) method.

Results: Histological analyses revealed the presence of inflammatory cell infiltrates in the interstitium at 24 hours after OLF. However, no destruction of the alveolar wall and no pulmonary oedema were observed. In addition, OLF was not associated with any decrease in surfactant protein A immunoreactivity. Two hours after OLF, the number of apoptotic cells was increased (OLF: 7% vs. Control: 0.6%, $p < 0.05$), but cellular proliferation was unchanged. Conversely, at 48 h after OLF, the number of apoptotic cells had returned to control levels, but cellular proliferation had increased (OLF: 5% vs. Control: 1.1%, $p < 0.05$). Cellular proliferation returned to baseline levels eight days after OLF.

Conclusions: These data demonstrate that OLF is not associated with destruction of the alveolar texture, atelectasis-provoking surfactant loss, or any irreversible damage to the pulmonary parenchyma. Lung flooding for the purpose of videothoracoscopic lung sonography is safe and justifiable. But repeated lung flooding under bronchoalveolar lavage involving the same lung area within 1 week is not to be recommended.

Key words: lung flooding; surfactant; histopathology; cell kinetics

Introduction

Instilling saline into one or more lung segments is a procedure under bronchoalveolar lavage (BAL). Furthermore lung flooding enables lung sonography and allows visualization of lung lesions under videothoracoscopy. (Lesser et al. 1998; Lesser et al. 1999). Videothoracoscopic lung sonography can be helpful for endoscopic resection or ultrasound-guided interstitial thermo therapy for treatment of non-resectable primary and secondary lung tumours. One-lung flooding (OLF) has been investigated in animals, and studies have shown that OLF is not associated with a deterioration in hemodynamics or gas exchange (Klinzing et al. 1999). In the current, we set out to investigate the histological consequences induced by OLF as well as the effects of OLF on apoptosis and cellular proliferation.

Materials and Methods

Experiments were carried out on thirteen 3-5 month old female pigs (Deutsches Landschwein) with a mean weight of 37.2 kg (range, 30-47 kg). The present study conformed to the Guidelines for the Care and Use of Laboratory Animals (National Veterinary Protection Law, No. 30, 1998; Thuringia, Germany) and was approved by the Veterinary Department of the Thuringian State Authority for Food Protection and Fair Trading.

Anaesthesia and Artificial Respiration

Anaesthesia was initiated by an intramuscular injection of 10 mg kg⁻¹ ketamine with 150 IU hyaluronidase. Additionally, 6.25 mg droperidol and 10 mg diazepam were applied after cannulation of an ear vein, and the animals were orotracheally intubated during spontaneous breathing with a left-bend Robertshaw double-lumen tube with an extra-long bronchial leg (Model 39 Ch, specially made by Mallinckrodt, Athlone, Ireland). Correct positioning of the tube was verified by flexible bronchoscopy (Olympus BF 3C30 fibre bronchoscope). After relaxation with 1.4 mg kg⁻¹ rocuronium bromide and enhancement of the anaesthesia by 5

$\mu g\,kg^{-1}$ fentanyl, artificial ventilation was started with a 1.0-1.5 minimum alveolar concentration (MAC) of isoflurane in an oxygen/nitrous oxide mix (FIO_2 = 0.3). Anaesthesia and relaxation were maintained by continuous application of the inhalation aesthetic and regular follow-up injections of 0.5 mg kg^{-1} rocuronium bromide and 5 $\mu g\,kg^{-1}$ fentanyl. Respiration under volume control was provided by an intensive respirator (Servo 900, Siemens, Germany; tidal volume 10 ml kg^{-1}, respiratory rate 16 - 20 min^{-1}, expiratory partial CO_2 pressure 35 - 45 mmHg, PEEP = 6 cm H_2O). During the experiment, 4 - 6 ml/kg/h of a whole electrolyte solution and 2 - 4 ml/kg/h of hydroxyethyl starch were infused as a basic infusion. The body core temperature was maintained to 36 - 37 °C by pre-heating the infusion solutions and covering the animals with heat-insulating foil. An arterial cannula (18 G; Vygon, Ecouen, France) was placed into the right femoral artery. The electrocardiogram, arterial blood pressure, capillary oxygen saturation and expiratory CO_2 concentration were measured and recorded continuously (Datex AS/3™, Helsinki, Finland).

FLOODING AND RE-VENTILATION

Twenty minutes after artificial respiration with an inspiratory oxygen concentration of 100% (PEEP = 6 cm H_2O, V_t = 10 ml/kg), a 7 mm thoracoport was applied in the left pleural cavity, and the endobronchial tube leg was disconnected from the respirator. After spontaneous atelectasis, the left lung was slowly filled through the left tube leg with 15 ml/kg of an isotonic electrolyte solution (E 153®) preheated to body temperature. Filling was performed passively, using gravity to force the flow of liquid from the infusion bottle, which was suspended 50 cm above the heart. The animal was temporarily brought to an anti-Trendelenburg position (30°) during this time. After completion of the flooding, the tube end of the flooded lung was hermetically sealed, and the horizontal dorsal position of the animal was restored. The liquid was left in the lung for 60 minutes and then passively drained by placing animals in the Trendelenburg position. This was followed by simultaneous ventilation of both lungs for 30 minutes. To phase out anaesthesia, animals were switched from intermittent positive pressure ventilation to synchronized intermittent mandatory ventilation. When spontaneous respiration reached a sufficient level, the animals were extubated. The pleural drainage was removed, and the thorax incision was sutured hermetically.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

At 30 minutes, 2 hours, 24 hours and 2 days after OLF, tissue samples were systematically collected from the central (perihilar) and peripheral regions (2 centimetres subpleural) of each pulmonary lobe after fixation of the whole lung that was inflated with a constant airway pressure. Seven animals were sampled at each time point. Additional samples were collected at 6 days, 8 days, and 10 weeks after OLF. Two animals were sampled at each of these time points. The lung

was fixed in buffered formalin or flash-frozen in liquid nitrogen and stored at -80 °C. Lung tissue from healthy, untreated animals that died while anaesthesia was induced served as negative controls, along with tissue from the right lung of each experimental animal. For histological analyses, 4 μm-thick paraffin sections prepared from formalin-fixed tissue were stained with haematoxylin-eosin (HE).

Immunohistochemical labeling for surfactant protein A (SP-A) antigen was performed on both paraffin and frozen microsections. Immunohistochemical labeling for Ki67 antigen was performed on paraffin microsections. Prior to incubation with primary antibody, 4 μm-thick paraffin sections were deparaffinized and washed in tris buffer. On the other hand, frozen sections, which had been allowed to air dry for over two hours, were fixed in acetone for 10 minutes and blocked for 30 minutes. Primary antibody incubations were performed in humidified chambers at 4 °C overnight. Rabbit anti-human surfactant protein A polyclonal antibody (1 : 50, Chemicon International Inc., USA) or Klon MIB1 (1 : 10 in a solution containing RPMI medium, PBS, and inactivated bovine normal serum; Dianova, Germany) were employed. Following primary incubations, sections were washed in tris buffer and incubated with appropriate secondary antibody. For analysis of SP-A, sections were incubated with mouse-anti-rabbit antibody (1 : 400 in dilution buffer for bridging antibody, Dako, Denmark) at room temperature for 45 minutes. Sections were subsequently incubated with rabbit-anti-mouse antibody (1 : 70, Dako, Denmark) at room temperature for 45 minutes. For the analysis of Ki67 antigen, sections were incubated with rabbit-anti-mouse serum (1 : 70 in dilution buffer for bridge antibodies, Dako, Denmark) at room temperature for 45 minutes. SP-A and Ki67 immunoreactivity was visualized by incubating sections with alkaline phosphatase - antialkaline phosphatase (APAAP) complex (1 : 70 in dilution buffer for APAAP complex, Dako, Denmark) at room temperature for 45 minutes. The rabbit-anti-mouse and APAAP complex incubation steps were repeated twice for 10 minutes each to increase sensitivity. Bound alkaline phosphatase was detected with naphtol-AS-bis-phosphate/new fuchsine. Following detection, sections were washed in tris buffer and water, and nuclei were counterstained with hemalum for one minute. The specimens were then mounted in glycerinated gelatine (Merck, Germany). To confirm the specificity of immunohistochemical labeling, the staining procedure was carried out using non-immune serum in the place of primary antibody.

The percentage of Ki67-labeled nuclei was employed as an index of cellular proliferation. Labeled cell nuclei were manually counted, with the aid of a counting grid plate, at 300X magnification. Nuclei were counted from ten randomly selected lung tissue regions. A total of 1000 cells were counted per lung specimen.

MEASUREMENT OF APOPTOSIS

Apoptosis was detected through enzymatic in-situ labeling of apoptosis-induced DNA strand breaks by

means of the TUNEL (TdT-mediated dUTP nick end labeling) method (Gavrieli et al. 1992; Junghänel et al. 1998). TUNEL was performed using the In-Situ Cell Death Detection Kit, AP (Boehringer, Mannheim, Germany), according to the manufacturer's instructions. Negative controls, which were included in each labeling experiment, consisted of reaction solutions that did not contain any terminal deoxynucleotidyl transferase. TUNEL-labeled nuclei were visualized on a binocular light microscope (Zeiss, Germany) and manually counted with the aid of a counting grid plate at 300x magnification. The percentage of TUNEL-labeled cells was determined by counting ten randomly selected lung tissue regions. A total of 1000 cells were counted per lung specimen.

STATISTICS

All values are expressed as mean ± standard deviation (SD). Comparisons of means from the left and right lungs, within the same treatment group, were performed using the Wilcoxon signed-rank test. Comparisons of means among the three groups (control, 2 h and 48 h post OLF) were performed using the Kruskal-Wallis test. In case of significance, a paired comparison was performed using the Mann-Whitney U test. In all cases, values of $p < 0.05$ were accepted as significant.

RESULTS

OLF results in a reversible infiltration of inflammatory cells but does not disrupt the alveolar wall.

Thirty minutes after OLF, the alveolar surface was intact, without any ruptured alveoli, desquamated pneumocytes, or interstitial oedemas (Fig. 1). Up to two hours later, slight capillary hyperemia was apparent. A few alveoli with residual liquid and small intra-alveolar haemorrhages were also conspicuous. The basic alveolar architecture of the pulmonary parenchyma was preserved 24 hours after OLF, although collapse of some alveoli was apparent, along with a broadening of the alveolar septa. The latter was due to slightly increased infiltration of the lung interstitium with inflammatory cells. 48 hours after OLF inflammatory cell infiltrates was decreased and the alvealor septa were smaller. Six days after OLF, inflammatory cell infiltrates and the broadening of alveolar septa were no longer detectable. Except for the collapse of individual alveoli, the alveolar wall was regular. No histological abnormalities could be detected at 8 days and 10 weeks after OLF.

SURFACTANT IS NOT DEPLETED BY OLF

As shown in Fig. 2, SP-A was successfully detected in porcine lung tissue by means of a polyclonal antibody against the human protein. Moreover, SP-A could be detected in both the cryostat and formalin-fixed sections. In the untreated lung, SP-A immunoreactivity appeared as a thin, discontinuous band along the alveolar surface. Thirty minutes after OLF, the SP-A immunoreactivity was still present on the alveolar surface, with no apparent change in staining intensity.

INCREASED PROGRAMMED CELL DEATH IS AN EARLY EVENT ASSOCIATED WITH OLF

As expected, the percentage of TUNEL-labeled nuclei in lung tissue from untreated animals was relatively low (0.6%; Fig. 3). In contrast, a substantial increase in TUNEL-labeled nuclei was noted 2 hours after OLF (7.0%, $p < 0.05$ vs. untreated control). At this time, there also was a distinct, but statistically insignificant, difference between the left, flooded lung and the right, unflooded lung. The increase in TUNEL-labeled nuclei seen at 2 hours was transient, as the percentage of labeled nuclei returned to baseline levels (0.8%) at 48 hours.

INCREASED CELL PROLIFERATION IS A DELAYED EVENT ASSOCIATED WITH OLF

The proliferation index in lung tissue collected from untreated animals was 1.1%, and the index in lung tissue (right and left) collected 2 h after OLF was comparable (0.7%; Fig. 4). However, at 48 h after OLF, the number of proliferating cells in left lung tissue had increased to 5% ($p < 0.05$ vs. control, 1.1%). At that time, there was also a trend towards increased proliferation in the left lung compared to the right lung. Eight days after OLF, the proliferation index in the left lung had returned to baseline, control levels.

DISCUSSION

Lung flooding is a technique performed under diagnostic or therapeutic bronchoalveolar lavage. Furthermore lung flooding enables lung sonography. In this way videothoracoscopically undetectable lung lesions can be visualized (Lesser et al. 1998; Lesser et al. 1999). In comparisen with laparoscopic radiofrequency or laser ablation of colorectal liver metastases videothoracoscopic application is limited (Siperstein et al. 2007). Lung sonography after instilling saline into one or more lung segments could be a useful method to control the placement of the probe and coagulation procedure of lung tumours by videothoracscopy.

Little information is available on the pathophysiological significance of lung flooding. OLF is not associated with a deterioration in hemodynamics or gas exchange. On the contrary, oxygenation was improved, and the pulmonary right-to-left shunt was reduced in size, compared to classical one-lung ventilation and atelectasis of the dependent lung (Klinzing et al. 1999). Up to 24 hours after re-ventilation of the flooded lung, the pulmonary right-to-left shunt volume is slightly increased under spontaneous respiration (Klinzing et al. 2000; Klinzing et al. 2001). Subsequent studies have shown that, one hour after OLF, extravascular water in the lung increases by 5% (Klinzing et al. 2000). Measurement of the phospholipoid content in the recovered liquid has revealed that, at maximum, approximately one-half of the calculated surfactant pool is lost (Klinzing et al. 2000). Here, we have further investigated the morphological changes associated with these conditions.

A single complete filling of the lung with isotonic balanced electrolyte solution for one hour does not
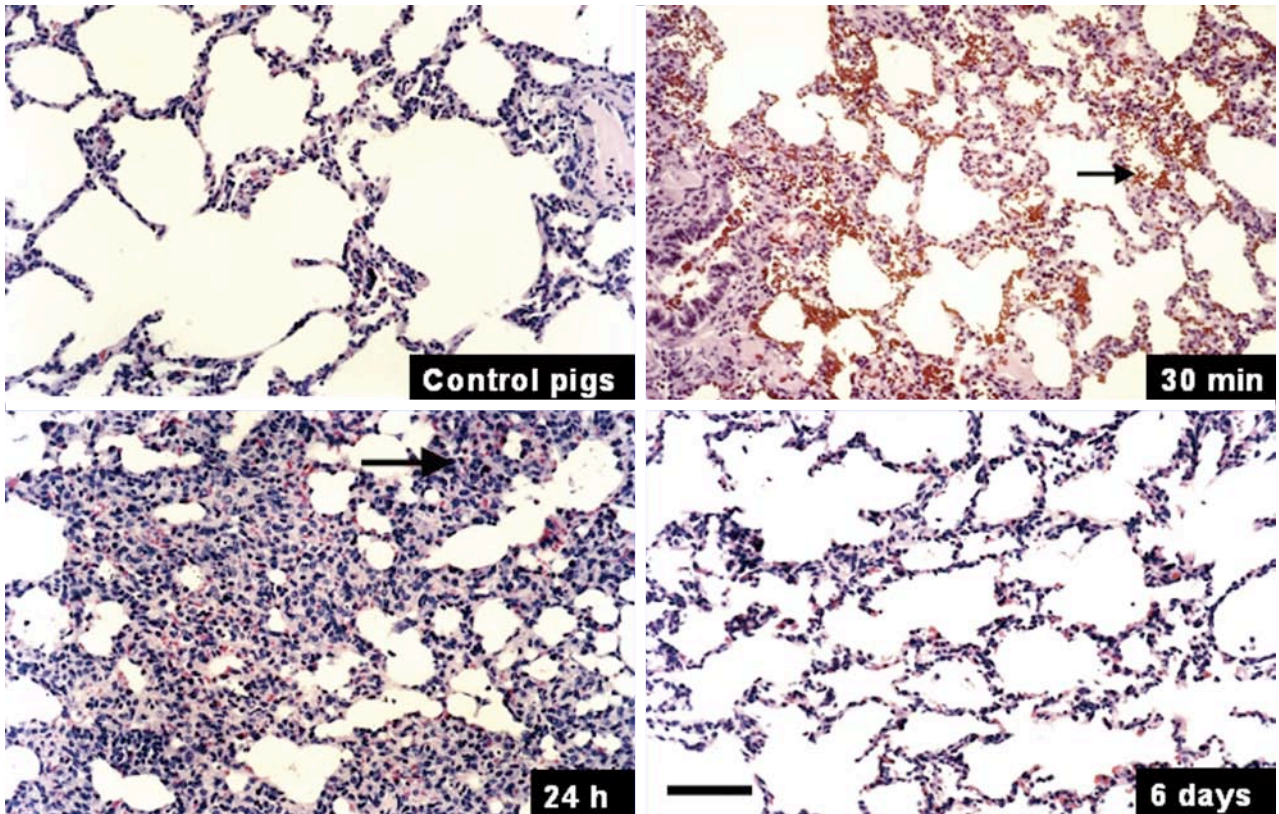
Fig. 1. Haematoxylin and eosin stained sections of the left inferior pulmonary lobe. Representative images are shown for an untreated control animal and for animals subjected to one-lung flooding (OLF). 30 min after OLF (top right): capillary hyperemia and intraalveolar haemorrhages (arrow), 24 hours after OLF (bottom left): broadening of alveolar septa through inflammatory cells (arrow), and 6 days after OLF (bottom right): regular alveolar wall. Scale bar, 200 μm; Images are 150 x magnification.
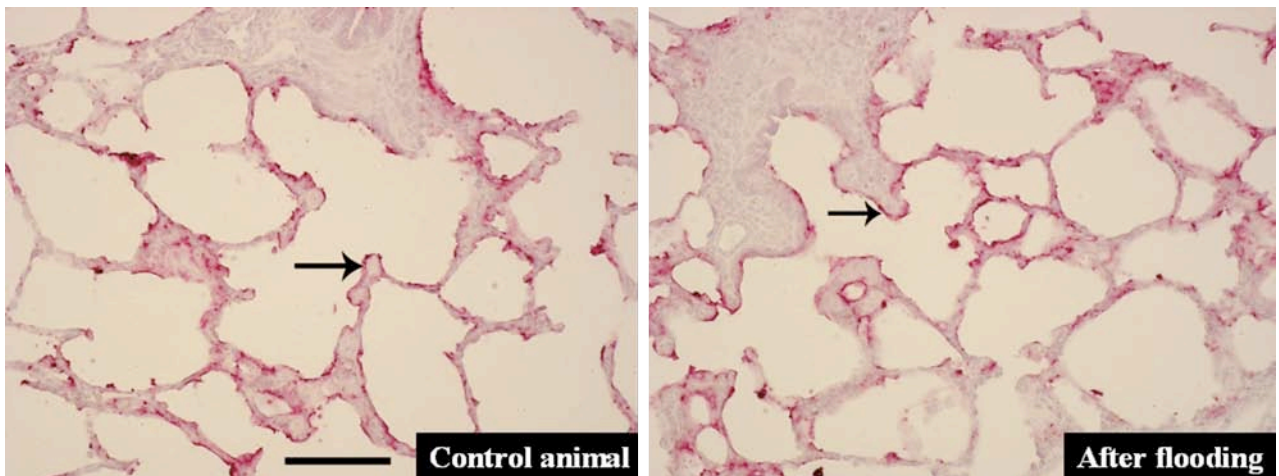

Fig. 2. Immunohistochemical labeling of left inferior pulmonary lobe tissue for surfactant protein A. Representative images are shown for an untreated control animal (left) and for an animal subjected to one-lung flooding (OLF). Tissue was collected 30 min after flooding. No decrease in immunoreactivity (red) along the alveolar surface could be detected in OLF specimens (arrow). Scale bar, 200 μm; Images are 200x magnification.

cause destruction of the alveolar basic architecture. Alveolar ruptures or desquamated epithelia were not observed. After the first hour after OLF, the morphological changes were limited to capillary hyperaemia, intraalveolar haemorrhage, and isolated occurrences of intraalveolar liquid. These morphological changes are not consistent with pulmonary oedema. Rather, they are due to residual liquid that cannot be eliminated; residual volumes may be as large as 40% of the volume instilled (Klinzing et al. 2000). This also explains the increase in extravascular lung water (Klinzing et al. 2000). Active transepithelial transport of sodium from the airspaces to the lung interstitium is the primary mechanism driving the clearance of alveolar fluid
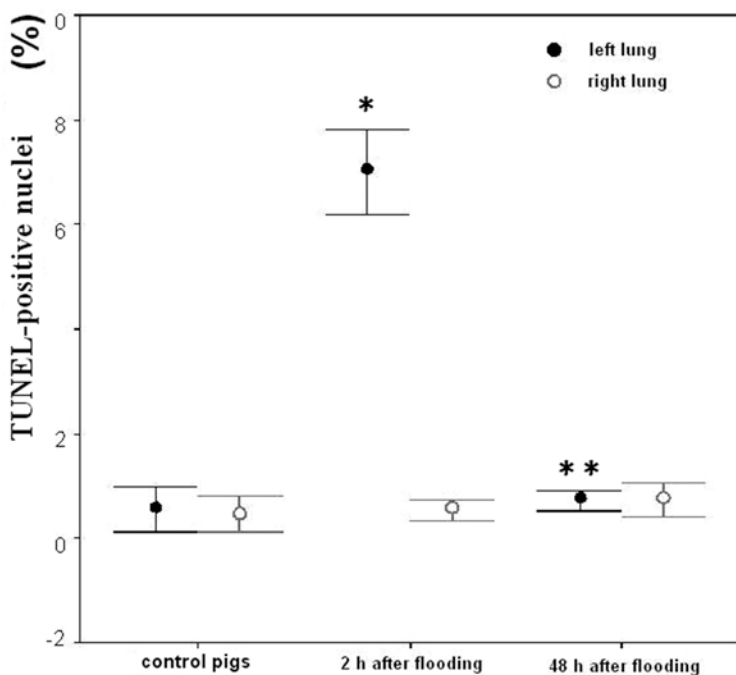
Fig. 3. One-lung flooding (OLF) induces apoptosis. The percentage of TUNEL-positive nuclei was determined in untreated control animals (right and left lung) and in animals subjected to OLF (right and left lung). Tissue was collected 2 and 48 hours after flooding of the left lung. Values are expressed as mean ± SD. *p < 0.05 vs. controls; **p < 0.05 vs. left lung at 2h.
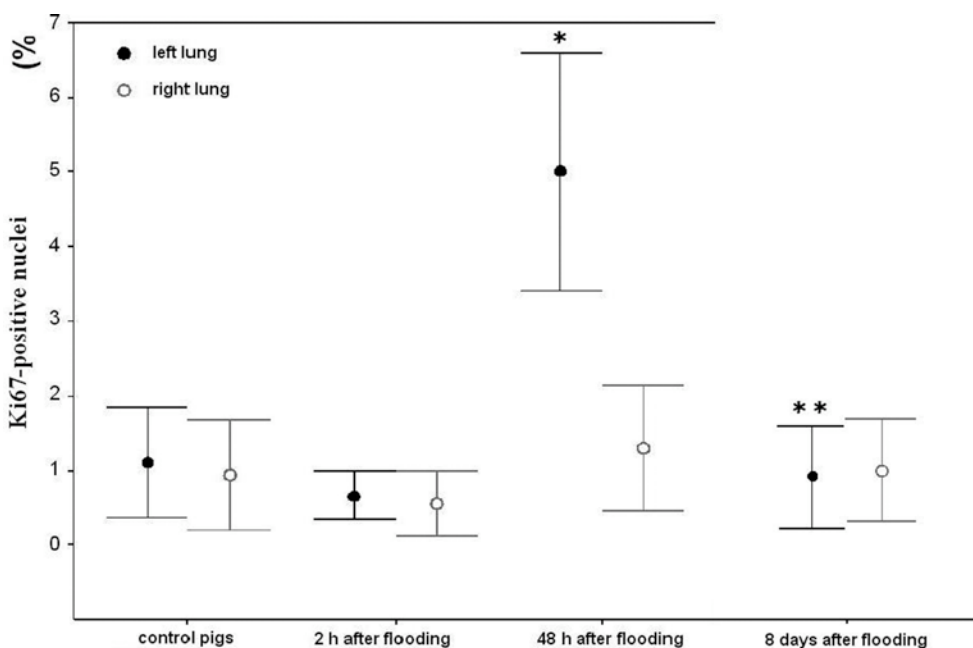

Fig. 4. One-lung flooding (OLF) induces cellular proliferation. The percentage of Ki67-positive nuclei was determined in untreated control animals (right and left lung) and in animals subjected to OLF (right and left lung). Tissue was collected 2 and 48 hours and 8 days after flooding of the left lung. Values are expressed as mean ± SD. *p < 0.05 vs. controls; **p < 0.05 vs. left lung at 48h.

(Check et al. 1989; Matthey and Wiener-Kronish 1990; Sartori et al. 2001). Hypoxic stress can disrupt this mechanism, leading to pulmonary oedema (Hoschele and Mairbaurl 2003; Roux et al. 2005; Dehler et al. 2006). The rapid removal of the residual alveolar liquid and the lack of interstitial oedema seen here suggests that sodium transport remains intact. Others have reported only slight histological changes after lung flooding with isotonic saline, which are comparable to those noted here. For example, in lung flooded with saline for two hours, a slight interstitial oedema, but otherwise normal alveolar structures was noted (West et al. 1965). Also, no severe tissue damage following 30 minutes of intermittent intratracheal instillation of 3 L saline into spontaneously respiring dogs

was found (Winternitz and Smith 1920). Except for slight alveolar haemorrhage, desquamated alveolar cover cells, and isolated intraalveolar leukocytes and macrophages, they did not observe any destruction of pulmonary tissue.

Visualization of the surfactant complex is possible only through electron microscopy and requires vascular perfusion and laborious fixation techniques (Gil and Weibel 1971; Weibel and Gil 1971). As an alternative, we employed immunohistochemical labeling of SP-A, the main protein constituent of surfactant. The finding that SP-A immunoreactivity was unchanged after reventilation of a flooded lung shows that a single flooding has no appreciable wash-out effect. It is important to note that one cannot conclude that the

function of the surfactant is normal based on the immunohistochemical visualization of SP-A. However, since atelectatic regions were not found by radiography or histology, one can assume that a single lung flooding does not give rise to any lasting disorder of the surface activity of surfactant. This assumption is supported by the rapid normalization of pulmonary gas exchange. Even if a single flooding washes out or inactivates a portion of the surfactant (Klinzing et al. 2000), this loss seems to be compensated for by increased surfactant synthesis by type-II alveocytes. Because surfactant has a half-life of between 15 and 30 hours (Goerke and Clements 1986), disturbances in surfactant metabolism would be morphologically and functionally manifest within a relatively short time. This is illustrated by animals that are incapable of rapid surfactant synthesis (Jobe 1993). In case of drowning in isotonic saline, part of the surfactant is washed out; however, this does not affect the surface activity of the surfactant that is remaining in the liquid-filled lung (aspirated volume 17.2 ml/kg, which is comparable with the volumes used in the present study) (Giammona et al. 1967).

OLF results in a significant increase in programmed cell death. It is possible that cell damage was caused by the ischemia and reperfusion of the tissue. Lung transplantation studies have revealed that warm ischemia induces dysfunction of adenosine triphosphate-dependent enzymes, resulting in intracellular influx of calcium and subsequent activation of xanthinoxidase and phospholipase (Moore et al. 1996). This, in turn, gives rise to toxic oxygen (Mc Cord 1987) or arachidonic acid metabolites (Gee et al. 1985; Ljungmann et al. 1991), which cause parenchymal and endothelial cell damage and liberate inflammatory mediators. The liberation of proteases and toxic oxygen metabolites from inflammatory cells serves as a secondary source of cell damage (Senior and Campbell 1983; Saugstedt et al. 1984; Steinhoff et al. 1997). Thus, the infiltration of inflammatory cells observed at 24 hours after lung flooding may be a source of cell damage. Single-lung ischemia-reperfusion has been shown to result in damage to the contralateral lung (Watanabe et al. 1996); however, the contralateral lung was unaffected under our experimental conditions. Nevertheless, in support of an ischemic origin for OLF-induced cell damage, we have found inflammatory cell infiltrates and a significant increase in programmed cell death in atelectatic lungs, in the absence of liquid filling. 48 hours after OLF inflammatory cell infiltrates were decreased and the alvealor septa were smaller. Therefore the increase of cell proliferation 48 hours after OLF cannot be interpreted by inflammatory cells. Presumable cell proliferation might be resulted from alveolar epithelial cells to compensate for cell damage.

Conclusions

The pathomorphological changes associated with OLF are tolerated by animals. Flooding of lung tissue does not cause destruction of alveolar texture and significant surfactant loss. In the end, there are no detectable morphological indications of irreversible lung tissue damage. Lung flooding for the purpose of videothoracoscopic lung sonography is safe and justifiable. But repeated lung flooding under bronchoalveolar lavage involving the same lung area within 1 week is not to be recommended.

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