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# Aerosolized perfluorocarbon reduces adhesion molecule gene expression and neutrophil sequestration in acute respiratory distress

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**Abstract**

In acute respiratory distress syndrome, neutrophil migration into the lung plays a key role in the development of lung injury. To study the effect of different modes of ventilation with perfluorocarbon (FC77®), intrapulmonary neutrophil accumulation and mRNA expression of E-selectin, P-selectin and intercellular adhesion molecule-1 (ICAM-1), mediating leukocyte sequestration, were measured in surfactant depleted piglets. After bronchoalveolar lavage, 20 animals either received aerosolized perfluorocarbon (Aerosol-PFC), partial liquid ventilation (PLV) with perfluorocarbon at functional residual capacity filling volume (FRC-PLV) or at low volume (LV-PLV) or intermittent mandatory ventilation (control). After 2 h of perfluorocarbon application, intermittent mandatory ventilation was continued for 6 h. In the Aerosol-PFC group, all measured adhesion molecules showed a significantly reduced gene expression compared to controls. FRC-PFC treatment was effective in significantly diminishing P-selectin and ICAM-1 mRNA expression. Relative lung tissue neutrophil counts were significantly reduced in the Aerosol-PFC and the FRC-PLV group. Treatment with aerosolized perfluorocarbon is at least as effective as partial liquid ventilation at FRC volume in reducing pulmonary adhesion molecule expression and neutrophil accumulation in acute respiratory distress syndrome.


*Keywords:* Partial liquid ventilation; Acute respiratory distress syndrome; Aerosol; Selectin; ICAM-1 (intercellular adhesion molecule-1)

## 1. Introduction

In the pathogenesis of neonatal respiratory distress syndrome and the progression to bronchopulmonary dysplasia, the early inflammatory response including expression of adhesion molecules and cytokines in the lung with subsequent activation of neutrophils play a pivotal role (Speer, 2001). A considerable part of tissue damage and finally development of lung fibrosis has been attributed to activated neutrophils accumulating in the alveolar and interstitial space of the lung producing, among others, proinflammatory cytokines and fibrogenetic proteins (Downey et al., 1999; Lee and Downey, 2001; Speer, 2001; Strieter and Kunkel, 1994). Recruitment of leukocytes is achieved through a multistep cascade involving selectin-mediated rolling on the vascular endothelium, firm adhesion and transmigration into sites of inflammation mediated by intercellular adhesion molecule-1 (ICAM-1) (Etzioni, 1996; Strieter and Kunkel, 1994; Vestweber and Blanks, 1999).

E-selectin, P-selectin and ICAM-1 are expressed on activated vascular endothelial cells after stimulation with tumor necrosis factor, proinflammatory cytokines or elevated pressure in lung venular capillaries (Etzioni, 1996; Kuebler et al., 1999; Strieter and Kunkel, 1994). ICAM-1, but not P- and E-selectin, is further produced in bronchial epithelial cells, type II pneumocytes and alveolar macrophages (Feuerhake et al., 1998; Hubbard and Giardina, 2000; Piedboeuf et al., 1996). In premature infants with respiratory distress syndrome, as early as 24–36 h after birth, elevated plasma levels of soluble E-selectin and ICAM-1 can be measured and have been proposed to serve as prognostic factor for the severity of bronchopulmonary dysplasia (Little et al., 1995; Ramsay et al., 1998). Soluble

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ICAM-1 concentrations in tracheal aspirates could be shown to correlate with development of bronchopulmonary dysplasia (Kojima et al., 1993) therefore suggesting that these very early inflammatory processes significantly contribute to the pathogenesis of bronchopulmonary dysplasia (Speer, 2001).

The considerable impact of adhesion molecules has also been demonstrated in different animal models with acute lung injury. Mice deficient for ICAM-1 or E-selectin and the use of blocking antibodies against P-selectin and ICAM-1 lead to reduced neutrophil sequestration and lung tissue damage (Bless et al., 1998; Doerschuk et al., 1996; Hayashi et al., 1999; Mulligan et al., 1995; Vestweber and Blanks, 1999).

Therapeutic options to improve outcome of neonates with severe respiratory distress syndrome include high frequency oscillation ventilation (Gerstmann et al., 1996) and therapy with inhaled nitric oxide (Demirakca et al., 1996). Partial liquid ventilation with perfluorochemicals as another strategy has proven to be effective in improving gas exchange and pulmonary function in adult patients with acute respiratory distress syndrome as well as in neonates with critical lung disease (Gauger et al., 1996; Greenspan et al., 1997).

This study was conducted to evaluate the modulation of adhesion molecule activation and neutrophil accumulation comparing different ventilatory regimens using aerosolized perfluorocarbon, low-volume partial liquid ventilation or liquid ventilation at FRC volume in surfactant depleted piglets. Therefore, mRNA expression of P-selectin, E-selectin and ICAM-1 and histological neutrophil accumulation was measured in lung tissue.

## 2. Materials and methods

### 2.1. Subjects

Twenty piglets with a body weight of $4.01 \pm 0.35$ kg were included in the study. Data of 18 piglets were available for evaluation. Two piglets were excluded due to early death by pneumothorax in one piglet in the LV-PLV group and due to poor mRNA quality in one piglet of the control group. The animal experiments were approved by the Animal Care Committee of the University and the Government of Mittelfranken, Germany and performed according to the guidelines of the NIH.

### 2.2. Protocol

Animal preparation and perfluorocarbon treatment were performed as recently published (Kandler et al., 2001). Briefly, continuous anesthesia was performed with midazolam, ketamine, and fentanyl. The animals were paralyzed with vecuranium. Lung injury was induced by repeated saline lung lavage. It was considered to be stable, when $PaO_2$ constantly remained below 80 mm Hg for 60 min. The animals were randomized to four different therapy groups (Aerosol-PFC, FRC-PLV, LV-PLV and control). In all animals, respiratory support was held constant at identical respiratory settings. Piglets in the Aerosol-PFC group received 10 ml/kg/h aerosolized perfluorocarbon (PFC, FC77®, 3M, Neuss, Germany). Piglets in the low volume-partial liquid ventilation group (LV-PLV) group received perfluorocarbon at a dose of 10 ml/kg/h, administered endotracheally through the side port of the tube connector. In the group of partial liquid ventilation at functional residual capacity filling volume (FRC-PLV group), 30 ml/kg FC77® were filled into the lung over a period of 30 min. For compensation of evaporative loss FC77® (20 ml/kg/h) was continuously replaced. Therapy with FC77® was stopped after 2 h. After an additional observation period of 6 h, anterior and posterior tissue specimens were taken from the right lung. Four tissue specimens were taken from the inferior lobe (central and basal), two from the superior and two from the middle lobe. Samples were frozen in liquid nitrogen and kept at $-80$ °C until analysis.

### 2.3. Assessment of leukocyte infiltration

Samples for histological examinations were taken from identical sites as described above. The left lung was perfused with 2.5% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer. The slides were stained

Table 1
Primers and TaqMan probes used in this study

| | |
|---|---|
| *Hypoxanthine-guanine-phosphoribosyl-transferase* | |
| Forward | 5′-TGGAAAGAATGTCTTGATTGTTGAAG-3′ |
| Reverse | 5′-ATCTTTGGATTATGCTGCTTGACC-3′ |
| TaqMan probe | 5′ (FAM)-ACACTGGCAAAACAATGCAAACCTTGCT-(TAMRA)3′ |
| *β-actin* | |
| Forward | 5′-TCATCACCATCGGCAACG-3′ |
| Reverse | 5′-TTCCTGATGTCCACGTCGC-3′ |
| TaqMan probe | 5′ (FAM)-CCTTCCTGGGCATGGAGTCCTGC-(TAMRA)3′ |
| *E-selectin* | |
| Forward | 5′-AGCTCTGACGTGTGGTGCC-3′ |
| Reverse | 5′-ACTCCACCAGCAGCAAGTCC-3′ |
| TaqMan probe | 5′ (FAM)-TGCCTACTTGTGAAGCTCCTGCTGAGTCC-(TAMRA)3′ |
| *P-selectin* | |
| Forward | 5′-CTGTGATGAAGGCTCGTCCC-3′ |
| Reverse | 5′-CGTATTCAAGCCGAAGGTTCC-3′ |
| TaqMan probe | 5′ (FAM)-AAGTGTGCTGCAATGCTTGGAGACGG-(TAMRA)3′ |
| *Intercellular adhesion molecule (ICAM-1)* | |
| Forward | 5′-CTGGCAGACGAGAAGGTGGT-3′ |
| Reverse | 5′-GCTCGCTCAGGGTCAGGTT-3′ |
| TaqMan probe | 5′ (FAM)-TGACCTTCTACAGCTTCCCACCTCCCA-(TAMRA)3′ |

with chloracetate esterase. A blinded expert pathologist examined the sections and attributed them to a four-point score (Quintel et al., 1998): 0: None, 1: mild, 2: moderate and 3: severe neutrophil accumulation.

### 2.4. RNA quantification

Total RNA was extracted from the tissue using guanidine-thiocyanate acid phenol (RNAzol, WAK Chemie®, Bad Homburg, Germany). One microgram of RNA was reverse transcribed in a volume of 20 μl at 39 °C for 60 min (chemicals from Boehringer®, Mannheim, Germany).

The use of TaqMan real time polymerase chain reaction (PCR) for mRNA quantification in porcine lung tissue was recently published (von der Hardt et al., 2002). The method has been described in detail before (Dötsch et al., 1999; Heid et al., 1996). Primers and TaqMan probes are listed in Table 1. Gene expression was related to the housekeeping genes β-actin (A) and hypoxanthine-guanine-phosphoribosyl transferase.

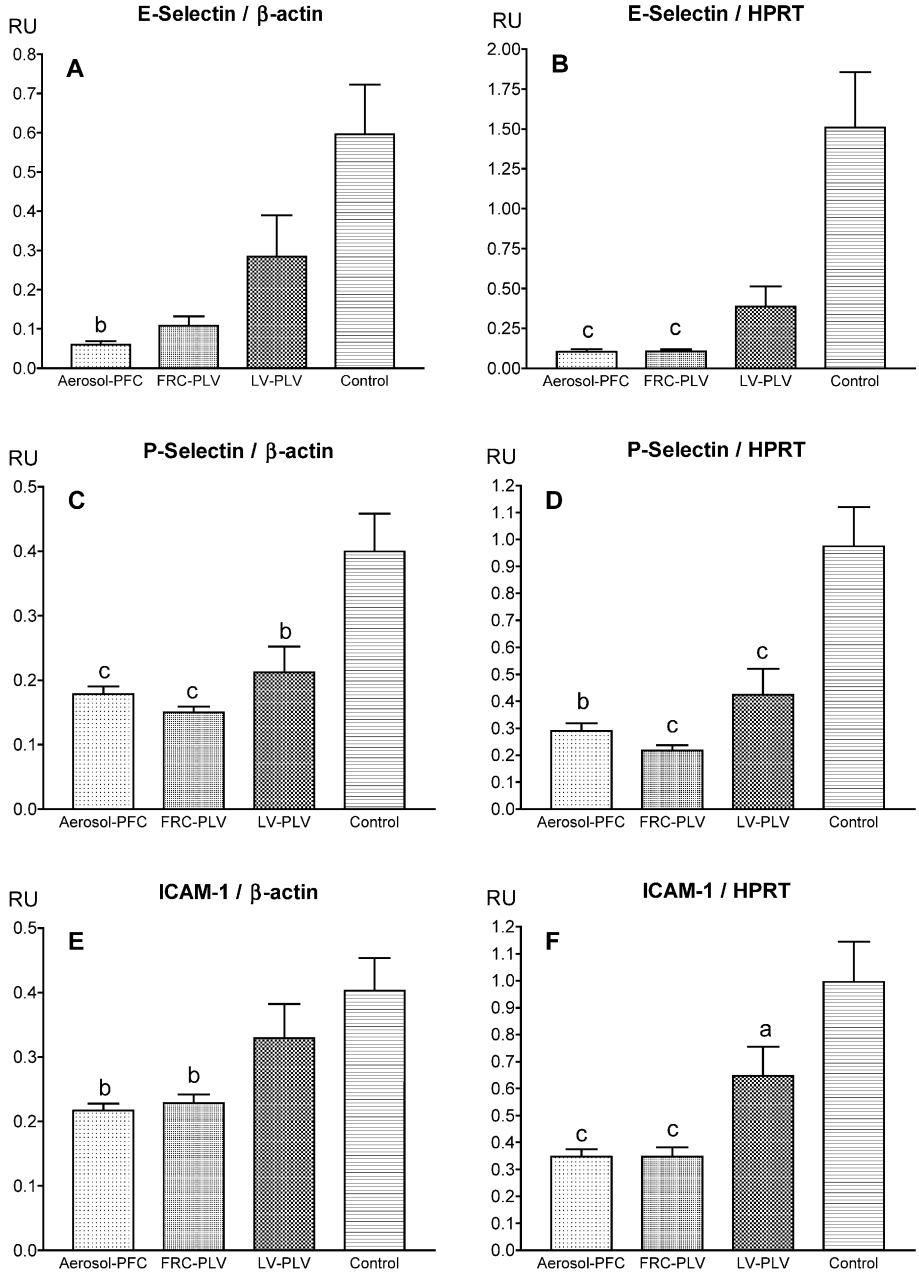


Fig. 1. Pulmonary E-selectin mRNA expression (A: related to β-actin, B: related to hypoxanthine phosphoribosyl transferase (HPRT)), P-selectin mRNA expression (C: related to β-actin, D: related to HPRT) and ICAM-1 mRNA expression (E: related to β-actin, F: related to HPRT) (RU: relative units) in the lung of surfactant depleted piglets after a therapy period of 2 h with either aerosolized perfluorocarbon (10 ml/kg/h) (Aerosol-PFC), partial liquid ventilation with functional residual capacity filling volume (30 ml/kg) (FRC-PLV), low-volume (10 ml/kg/h) partial liquid ventilation (LV-PLV) or intermittent mandatory ventilation (control), and an observation period of 6 h. [a]: $P<0.05$; [b]: $P<0.01$; [c]: $P<0.001$ compared to control.

### 2.5. Statistics

Values are expressed as mean ± S.E.M. Depending on the presence of Gaussian distribution, either ANOVA (analysis of variance) or Kruskal–Wallis test was used for comparison of the groups. In case of significance, Bonferroni and Dunns post-hoc tests were applied, respectively. A difference was only considered as significant, if a $P$-value of less than 0.05 was found for normalization to both housekeeping genes.

## 3. Results

The Aerosol-PFC group was the only group showing significantly lower mRNA expression of E-selectin than the control group. When normalized to β-actin, there was a 10-fold reduction in E-selectin mRNA expression ($P<0.01$, Fig. 1A). The FRC- and LV-PLV groups showed no significant differences when compared to controls. Normalization to hypoxanthine phosphoribosyl transferase confirmed the reduced E-selectin mRNA levels of the Aerosol-PFC group ($P<0.001$) and showed an additional significant difference between the FRC-PLV and the control group ($P<0.001$, Fig. 1B). However, significance for both housekeeping genes was required to count as significant difference between the groups.

P-selectin gene expression was significantly reduced in all of the perfluorocarbon-treated groups compared to controls. When related to β-actin, P-selectin gene expression was reduced by 55% in the Aerosol-PFC group ($P<0.001$), by 62% in the FRC-PLV group ($P<0.001$) and by 47% in the LV-PLV group ($P<0.01$, Fig. 1C). The data were confirmed when P-selectin was related to hypoxanthine phosphoribosyl transferase (Fig. 1D). Regarding ICAM-1/β-actin, only the Aerosol-PFC (46% reduction, $P<0.01$) and the FRC-PLV group (43% reduction, $P<0.01$) showed significantly lower gene expression than controls (Fig. 1E). Similar results were obtained after relation to hypoxanthine phosphoribosyl transferase (Fig. 1F). There was no difference in mRNA levels of P-selectin, E-selectin or ICAM-1 between the perfluorocarbon-treated groups.

Relative neutrophil infiltration revealed to be significantly lower in the Aerosol-PFC and the FRC-PLV group than in the control group. Compared to the LV-PLV group, there were significant differences to the Aerosol-PFC and the FRC-PLV group (Fig. 2).

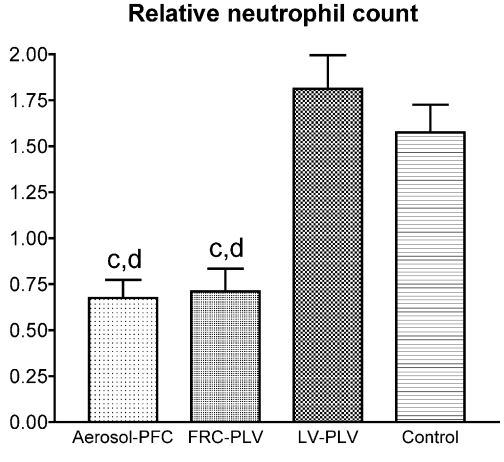


Fig. 2. Relative neutrophil infiltration in lung tissue of surfactant depleted piglets after a therapy period of 2 h with either aerosolized perfluorocarbon (10 ml/kg/h) (Aerosol-PFC), partial liquid ventilation with functional residual capacity filling volume (30 ml/kg) (FRC-PLV), low-volume (10 ml/kg/h) partial liquid ventilation (LV-PLV) or intermittent mandatory ventilation (control), and an observation period of 6 h. Relative neutrophil count was assigned to a four-point score by a blinded expert pathologist. [c]: $P<0.001$ compared to control. [d]: $P<0.001$ compared to LV-PLV.

## 4. Discussion

On endothelial cells, P-selectin, E-selectin and ICAM-1 are constitutively expressed at very low levels. P-selectin can be detected on the surface of endothelial cells within 5–10 min after stimulation (Bless et al., 1998; Etzioni, 1996; Strieter and Kunkel, 1994). Maximal surface expression of E-selectin after activation has been measured after 4–6 h and of ICAM-1 after 24 h (Etzioni, 1996; Pober et al., 1986). Proinflammatory cytokines, tumor necrosis factor and elevated pressure in postcapillary pulmonary venoles are inductors of mRNA synthesis and protein formation of P-selectin, E-selectin and ICAM-1 preceding neutrophil activation and sequestration into the inflamed tissue (Berton et al., 1996; Bevilacqua et al., 1987; Kuebler et al., 1999). To detect differences in gene expression already 8 h after initiation of lung injury, we used TaqMan real time PCR for mRNA quantification as a highly sensitive method (Dötsch et al., 1999; Heid et al., 1996).

The present study is the first demonstrating the effective use of different ventilation strategies with perfluorocarbon in reducing the early pulmonary gene expression of adhesion molecules. In a piglet model of acute respiratory distress syndrome due to surfactant depletion, mRNA expression of E-selectin, P-selectin and ICAM-1 has been most effectively suppressed by the novel approach using perfluorocarbon as aerosol. The Aerosol-PFC group was the only group in which a significant reduction of E-selectin gene expression occurred. Some minor effects in reducing P-selectin gene expression could be seen after treatment with partial liquid ventilation at low volume. These data are in line with the previous studies of our group showing a profound reduction of interleukin-1β, interleukin-6, and interleukin-8 gene expression in porcine lung tissue after treatment with aerosolized perfluorocarbon and perfluorocarbon at FRC volume (von der Hardt et al., 2002). Neutrophil accumulation in the lung was probably attenuated by treatment with aerosolized perfluorocarbon and perfluorocarbon at FRC volume as a consequence of increased cytokine and adhesion molecule expression upre-

gulated on gene level. There is consensus about the fact that neutrophils play a central role in the pathogenesis of acute lung injury enhancing the inflammatory response and leading to tissue damage (Downey et al., 1999; Lee and Downey, 2001; Rinaldo, 1986).

During partial or tidal liquid ventilation, perfluorochemicals have been shown to be distributed into the blood stream and into several tissues (Reickert et al., 2001; Stavis et al., 2002). Adhesion molecule expression might therefore be directly inhibited on the pulmonary vascular endothelium by perfluorocarbon. However, the same efficacy in reducing ICAM-1 and P-selectin gene expression and with respect to E-selectin, a more profound effect was achieved in animals treated with aerosolized perfluorocarbon requiring two- to three-fold lower perfluorocarbon doses. For this reason, we suppose that in our model, improved lung compliance leading to reduced shear forces rather than direct anti-inflammatory mechanisms have led to the observed effects of reduced adhesion molecule mRNA expression. Previous animal studies of our group and others could demonstrate that treatment with perfluorocarbon at FRC volume in contrast to low volume-PLV leads to temporary improvement of gas exchange and lung mechanics therefore reducing shear stress and hypoxia (Colton et al., 1998; Hirschl et al., 1995; Kandler et al., 2001; Overbeck et al., 1996; Quintel et al., 1998). This effect was persistent in animals that had received aerosolized perfluorocarbon (Kandler et al., 2001). High peak inspiratory pressures and large tidal volumes have been shown to damage the alveolar–capillary barrier of the pulmonary microcirculation and to increase microvascular permeability promoting inflammation (Egan, 1982; Parker et al., 1990). Additionally, shear stress on endothelial cells is a well-known factor to induce adhesion molecule expression (Chien et al., 1998). The reduced early inflammatory response in our model might therefore be a consequence of reduced barotrauma and improved compliance.

All these perfluorocarbon effects might be of clinical relevance in adult patients with acute respiratory distress syndrome and neonates with severe lung disease. Premature infants with respiratory distress syndrome who later developed bronchopulmonary dysplasia, revealed elevated plasma levels and tracheal aspirate concentrations of E-selectin and ICAM-1 already 1–7 days after birth (Kojima et al., 1993; Little et al., 1995; Ramsay et al., 1998). In adult patients with acute respiratory distress syndrome, soluble ICAM-1 in pulmonary edema fluid was elevated compared to patients with hydrostatic lung edema (Conner et al., 1999). Early initiation of partial liquid ventilation might therefore not only provide improved gas exchange and reduced mechanical trauma but might also serve to suppress the early pulmonary inflammatory response. Further studies have to be performed to show whether beneficial long-term effects of partial liquid ventilation in terms of reducing lung fibrosis can be achieved. Other encouraging approaches include treatment with antibodies against selectins and ICAM-1 that have been proven to successfully reduce neutrophil accumulation and tissue damage in animal models of lung injury (Bless et al., 1998; Doerschuk et al., 1996; Hayashi et al., 1999).

In summary, we could show that after induction of lung injury due to surfactant depletion in neonatal piglets, respiratory support with aerosolized perfluorocarbon and partial liquid ventilation led to a significant reduction in the pulmonary gene expression of E-selectin, P-selectin and ICAM-1 and neutrophil accumulation. The most pronounced effect was achieved when using perfluorocarbon as aerosol.

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