The hydrophilic proteins of lung surfactant as a prognostic marker in experimental pneumonia

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Key words: biomarker, phospholipids, pneumonia, proteins, surfactant

Abstract:
BACKGROUND: SP-A and SP-D are hydrophilic proteins which regulate the inflammatory response of the lung. Pasteurella multocida is one of the most common bacteria isolated from calves suffering from shipping fever pneumonia, one of the major problems in dairy herds. OBJECTIVES: Evaluation of surfactant content may provide a valuable diagnostic tool for detection of calf pneumonia due to Pasteurella multocida and also state of treatment. METHODS: Ten Holstein-Frisian bull calves aged 4 months with body weight of 120 ± 5 kg were selected for study in two groups. The Pasteurella multocida (PMC66 Razi) was used in the present study for inducing pneumonia. The Bronchoalveolar lavage (BAL) process was done in selected calves. BAL fluid was collected and centrifuged and finally the sediment (crude surfactant) was reserved at -20°C. The cytological evaluation and surfactant content was assayed by ELISA, TPL kit assay and HPLC. RESULTS: The serum levels of SP-A and SP-D in pneumonic group were significantly elevated. Although the increased Bronchoalveolar lavage fluid (BALF) level of SP-A in pneumonic cases was found as compared with the control animals, the statistical analysis did not show any significant differences between two groups. The level of SP-D in BALF of pneumonic group significantly elevated. The amount of Dipalmitoylphosphatidylcholine (DPPC) in pneumonic group decreased significantly in comparison with control group. CONCLUSIONS: Pasteurella inducing pulmonary can change the major component of lung surfactant, evaluation of these markers can be helpful as an appropriate tool in diagnostic state of pneumonia and healing.

Introduction

Surfactant is composed of six phospholipids and four apoproteins. The components of surfactant include phospholipids (80% to 86%), neutral lipids, and proteins (Hartl and Griese, 2006; Jobe and Ikegami, 2001; Lewis and Veldhuizen, 2004).

SP-A cooperate with the immune cells of the alveoli in defense system which binds to a wide range of organisms that accelerate phagocytosis of pathogens by macrophages...
SP-D is also a hydrophilic protein with structural closeness to SP-A which is synthesized by type II alveolar cells and Clara cells and bind to bacteria, viruses and fungi. Although it does not play a role in surface activity it has an important role in lung defense (Hartl and Griese, 2006; Kunitake et al., 2001; Phelps 2001).

*Pasteurella multocida* is a gram-negative bacteria belonging to the Pasteurellaceae family (Kuhnert and Christensen 2008). *P. multocida* causes a range of diseases in mammals and birds which can also cause a zoonotic infection in humans (Radostits et al., 2007).

Based on previous researches, bacteria induce changes in pulmonary surfactant directly on secreted surfactant or indirectly through pulmonary type II epithelial cells (Madan et al., 2002; Shu et al., 2013). The gram-negative bacteria or endotoxin can damage type II epithelial cells which cause abnormal quantities of surfactant production, abnormal compositions of fatty acids or phosphatidylcholine and abnormal concentrations of phospholipids (Brogden, 1991).

Anatomical changes during pneumonia can cause the concentrations of surfactant to decrease and distort the surfactant composition (Brogden, 1991).

Although extensive research has been carried out on changes in surfactant during infection, there has been no reliable evidence with regard to experimental pneumonia and alteration of SP-A, D and Dipalmitoylphosphatidylcholine (DPPC) amount (Wang and Notter 1998).

The aim of this study was evaluation of the effect of experimental pneumonia on the levels of SP-A, D as a potent defense factor, TPL and DPPC as the main component of lung surfactant in the peripheral blood and BALF in pneumonic calves with *Pasteurella multocida*.

### Materials and Methods

**Study population:** Ten Holstein-Frisian bull calves aged 4 months with body weight 120 ± 5 kg were selected for study and divided in two groups (control group and experimental group). The calves were purchased from a big dairy farm in good health, located near Tehran. All The calves were fed colostrum (10% BW) within six hours of birth. They were housed in individual stainless steel pens (2m × 1.5 m × 2m) with chaff coated floor and were fed twice daily. The calves’ vital signs (temperature, heart and respiratory rate) were checked before experiment and at adaptation period.

**Experimental process:** The *Pasteurella multocida* (PMC66 Razi) was chosen in the present study because of its availability and being rapidly phagocytized. Pneumonia induction in case group (five calves) was carried out by insertion of tracheal tube in the trachea and lavage catheter passed to entrance of crania, in a sedation situation, and a suspension of *Pasteurella multocida* (2×10⁹ cfu/ml, 300 ml/ calf) was administered (Dowling A et al., 2002). After inoculation the bacteria, clinical signs were checked hourly and blood samples were collected until observation of the most severe signs of pneumonia.

Before challenge, after observing most signs of pneumonia and one day after completion of treatment and elimination of clinical signs, bronchoalveolar lavage was
performed for collection of BALF which injected 250 ml normal saline at room temperature, given in five divided doses into lung via BAL catheter and negative pressure is applied to aspirate lavage fluid. Calves were under critical care support after BAL procedure. In control groups the bronchoalveolar lavage was performed by injecting 250 ml normal saline (given in five divided doses) in parallel challenge group and blood samples were collected every hour until observation of pneumonia symptoms in test group.

**Isolation surfactant:** BAL fluid was centrifuged at 250 g for 15 min and sedimentation was referred for preparation smear. The supernatant fluid was collected and centrifuged at 20000g for 60 min (Robert et al., 2002). Finally, the sediment part named crude surfactant was preserved at -20˚C. For cytological evaluation 1-2 ml of each lavage fluid sample was sent to clinical pathology laboratory and was studied using Nihon Kohden 6450 apparatus and, slide smears prepared from the first sedimentation of BAL fluid evaluated cytologically. One sample of each BALF referred to bacteriology laboratory to confirm the bacteria in fluids after challenge.

**Surfactant content extraction:** Whole lung surfactant (LS) was extracted with chloroform-methanol (Modified Bligh and Dyer, 1959).The chloroform layer collected for assaying phospholipids and aqueous layer were collected to evaluate hydrophilic substances.

**Analysis process:** The total protein of aqueous portion of surfactant was measured by micro Bradford method (Bradford, M 1976). SP-A and D contents were measured by enzyme-linked immune sorbent assay (ELISA) kit (EIAab Pulmonary surfactant-associated protein A and D Cas No= E0890b and E1039b).

The total PL content was measured by Phospholipid Assay Kit (Sigma, Cat No: MAK122). Phospholipids content was determined from the inorganic phosphate content of the lipid extracts.

**Treatment:** Treatment began 24h after bacteria administration with Enrofloxacin 10% (Enrovet® Aburaihan Pharmacy Co.) at dose of 5mg/kg SC based on antibiogram test, Flunixin meglumin 5% (Razak Pharma Co.) 2.2 mg/kg BW, IM for 3 days and, AD3E (Razak Pharma Co.) 10 ml per calf as single dose.

**Statistical analysis:** The data were analyzed in two groups with t-test using SPSS version 16.0 and significance level was considered as p≤ 0.05. For comparing the level of surfactant proteins and PL before challenge and after revealing symptoms Paired t-test was used.

**Results**

**Hematological and BALF cytological values:** Significant increase in segmented neutrophil, neutrophil band and WBC occurred in pneumonic calves as compared with control calves, indicating the presence of an inflammatory reaction (p<0.05) (Table 2).

The cytological evaluation of BALF revealed significant differences in neutrophil

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Table 1. Cell count variation in bronchoalveolar lavage fluid in calf after challenge.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=5)%</th>
<th>Case (n=5)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Cells</td>
<td>13.7</td>
<td>11.4</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>15.8</td>
<td>10.9</td>
</tr>
<tr>
<td>Macrophage</td>
<td>64.1</td>
<td>46.8</td>
</tr>
<tr>
<td>Neutrophile</td>
<td>5.6</td>
<td>30.3</td>
</tr>
</tbody>
</table>
and macrophage cell counts in case groups in comparison to control group (p<0.05) (Table 1).

**Serum SP-A and SP-D content:** The results obtained from the analysis revealed that the level of SP-A in serum in control group (n=5) was 85.52 ± 5.6 ng/ml and in pneumonic group (n=5) was 177.7±6.3 ng/ml which was significant (p=0.05).

The maximum serum SP-D level was 65.33 ± 2.67 ng/ml and 180.9 ± 12.87 ng/ml in the control and pneumonic group, respectively. There was a significant difference between the two conditions (p=0.002).

The level of serum SP-A and SP-D in pneumonic group after treatment was recorded 95.24± 9.5 ng/ml and 80.3±2.5 ng/ml which reveal significant reduction in comparison to before treatment (p<0.05) (Table 3).

**BALF SP-A and SP-D Content:** The level of SP-A in BALF was 90.7±5.14 ng/ml and 61.5±2.54 ng/ml in pneumonic and control group respectively. Although the amount of SP-A in BALF of pneumonic cases increased compared to the control cases, no significant difference was observed between two groups (p>0.05).

The level of SP-D in BALF in pneumonic group was 155.32±18.12 ng/ml and in control group was recorded 89.24±4.6 ng/ml, which indicates extremely significant differences between two groups (p=0.001).

The level of BALF SP-A and SP-D in pneumonic group after treatment was reported 69.12± 6.5 ng/ml and 84.4± 0.7 ng/ml.
ml which indicate significant reduction in comparison to before treatment (p<0.05) (Table 3).

**BALF phospholipids content:** The level of total phospholipids content in pneumatic calves measured 76±8.5 mg/ml and in control group 89.23±14.1 mg/ml, which was not a significant difference between the two conditions, but the amount of DPPC in pneumatic group (DPPC concentration: 3.3 mg/ml) decreased significantly in comparison with control group (DPPC concentration: 10.5 mg/ml) which enhanced after treatment in pneumatic group (DPPC concentration: 7.5 mg/ml and TPL 81.13±04.8 mg/ml) (Table 3).

**Discussion**

The hydrophilic proteins, SP-A and SP-D, are glycoproteins that play a significant role in lung host defense and are now recognized as important components of innate lung immunity (Atochina et al., 1991; Khubchandani and Snyder, 2001; Rooney et al., 1994; Wright, 1997).

SP-A and SP-D as antimicrobial peptides are part of the innate immune response of lung that have been demonstrated to kill gram negative and gram positive bacteria and may also have the ability to enhance immunity by functioning as immunomodulators. In the present report a well-characterized *pasteurella* infection was used to evaluate changes in expression and allocation of the most important components of the surfactant system that occur during pneumonia.

In this model, *pasteurella* noticeably changed the total alveolar pool size of the SP-A and SP-D, whereas levels of hydrophobic surfactant proteins were increased.

In patients with interstitial lung disease, pulmonary alveolar proteinosis and patients with idiopathic pulmonary fibrosis the serum level of SP-D was significantly higher than those of healthy subjects (Takahashi et al., 2000; Tzouvelekis et al., 2005; Wang and Notter, 1998) which have assessed the value of SP-D serum levels as a disease marker for human lung disease. In pneumatic cases, due to *Pneumocystis carinii* marked elevation in alveolar collection level was observed because of increased expression and accumulation of SP-A and SP-D protein (Atochina et al., 1991).

The evaluation of surfactant proteins and DPPC as prognostic markers before induction of the inflammation and after challenge revealed the phases of lung infection, thus the elevated SP-D and SP-A in serum after challenge and reduction after treatment can be considered as a marker in infection process. SP-D could also be up regulated as the infection begins to be cleared and the anti-inflammatory properties of SP-D are needed.

Normally, SP-D is only present in alveoli. However, when the permeability of the alveoli increases as a result of damage due to inflammation, it appears to leak out into the bloodstream. Based on our results, there was a significant correlation between the alterations of SP-A and SP-D in serum and lung infection in calves with pneumonia.

In past decades evaluation of the epithelial lining fluid by bronchoalveolar lavage (BAL) was done as a common way of studying the proteins secreted by the lung epithelium and investigating their alterations in lung disorders (Reynolds and Newball 1974). However, scientists tried to show the presence of these proteins in the bloodstream with different lung disor-
ders using enzyme-linked immunosorbent assays (ELISA) for evaluation of different situations of disease or level of healing.

The amount of DPPC as the main lipid component of surfactant which reduces surface tension can be changed during pneumonia, as seen in our results the amount of DPPC significantly decreased in pneumonic group in compared to control group. The decrease in the amount of DPPC can cause problems in surface tension and performance of lung (Schürch et al., 2002).

Sheehan et al., mentioned that *P. carinii* pneumonia in adult rat model causes an alveolar surfactant phospholipid insufficiency which is in concordance with our results that amount of DPPC after pneumonia decreased in BALF and after treatment increased, and can be due to bacteria induction and surfactant changes during lung injury (Lewis and Veldhuizen 2004, Sheehan et al., 1986).

Takahashi et al., in 2001, detected SP-A and SP-D in sera of patients with radiation pneumonitis and mentioned specificity and sensitivity of diagnostic method was about 85% (Takahashi et al., 2001).

In this study we tried to compare both methods (BALF and serum) for evaluation of lung disorder situation during pneumonia and, as mentioned, the level of sera SP-A and SP-D significantly changed but the level of SP-A in BALF was not significantly different in two groups. Kunitake et al. reported SP-D levels increased in patients with sarcoidosis compared with healthy controls (Kunitake et al., 2001). Similarly, Janssen et al. found increased SP-D levels in the serum of patients with sarcoidosis as compared with healthy controls (Janssen et al., 2007).

In conclusion, the serum assays of surfactant proteins A and D may provide a value diagnostic tool for detection of pneumonia and state of treatment. The interaction of bacteria or endotoxin with secreted surfactant can cause changes in the physical properties of surfactant such as surface tension due to alteration of DPPC amount or injuring type II epithelial cells which consequently produce abnormal quantities of surfactant and anomalous concentrations of phospholipids.

In addition, *Pasteurella* inducing pulmonary can change the major component of lung surfactant, the evaluation of this marker can be helpful as appropriate tool in diagnostic state of pneumonia and healing.

**Acknowledgments**

The authors gratefully acknowledge the financial support provided by the Institute of Biomedical Research of Veterinary Medicine, University of Tehran.

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کاربرد پروتئین‌های هیدروفوب سورفکتانت ریه به عنوان بیومارکر در تشخیص پنومونی تجربی

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چکیده

پروتئین‌های هیدروفوب و جزئی از سورفکتانت‌های سطح‌پوستی SP-D و SP-A تنظیم‌هایی که پاسخ‌های التهابی را در ریه کنند. باکتری پاستورلا موالتیسیدا یکی از عوامل اصلی ایجاد کننده پنومونی یا تب حمل و نقل در گله‌های گوساله‌ها می‌باشد. هدف از این مطالعه بررسی محتوای سورفکتانتی به عنوان بیومارکری از وضعیت بیماری‌های ریوی و شرایط درمانی مورد ارزیابی گزارش شد. با استفاده از بکتری پاستورلا موالتیسیدا (Rutzi PMCS656) در مطالعه حاضر 10 راس گوساله به هر گروه 20 Kg وزن انتخاب شدند. تغییرات آرام‌ترین جهت ایجاد پنومونی تجربی انتخاب شد. لاواژ برونکوآلوئولار تحت شرایط آرام تحت نگهداری در یخچال 200 C فراگیره شدند. بررسی سیتو‌لیوژیک آنها به صورت پایدار می‌شد. وسیع‌ترین پروتئین‌های مورد تحقیق در این مطالعه از گروه‌های مرتبط با ریه (SP-D و SP-A) به شکل‌های مختلف انتخاب شدند. افزایش نسبی میزان پروتئین‌های انتخابی در نتیجه ایجاد پنومونی با وسیع‌ترین پروتئین‌های مورد تحقیق در این مطالعه بیان می‌شود. انتخاب نتیجه‌گیری‌های بالا با استفاده از متأثرگی پنومونی ریه با وسیع‌ترین پروتئین‌های مورد تحقیق مربوط به حالت ایجاد پنومونی ریه می‌باشد.