

A Study of Surfactant Proteins A and D in Asymptomatic Cigarette Smokers versus Nonsmokers

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ABSTRACT:

Background/Aim: The surfactant proteins, SP-D and SP-A, are collectins that play an important role in the innate immunity of the lungs. They bind to carbohydrate moieties on the surface of microorganisms, promoting their uptake and killing. This study aimed at shedding light on the effect of cigarette smoking on the level of pulmonary surfactant proteins.

Patients & Methods: The levels of SP-A, SP-D and phospholipids in BAL fluids were measured in 16 healthy smokers and 12 non-smokers.

Results: The level of SP-A in BAL fluids of smokers (1.79 ± 0.27 ug/ml) was significantly decreased compared with those in non-smokers (3.22 ± 0.46 ug/ml). BAL SP-D level of smokers was also found to be significantly lower (0.52 ± 0.09 ug/ml) than non-smokers (1.59 ± 0.32 ug/ml). The level of total phospholipid in BAL fluids of smokers (34.26 ± 3.38 nmol/ml) was also lower than that of non-smokers (44.17 ± 5.12 nmol/ml) but with no significant difference. The ratios of SP-A to phospholipid and SP-D to phospholipid were decreased in smokers (53.50 ± 5.15 ng/nmol and 18.39 ± 3.35 ng/nmol, respectively) than in non-smokers (68.83 ± 8.17 ng/nmol and 23.83 ± 3.85 ng/nmol, respectively). The levels of SP-A and SP-D in BAL showed a significant correlation both in smokers and non-smokers. The ratio of SP-A to SP-D in smokers (4.69 ± 0.91) was significantly higher than in non-smokers (2.29 ± 0.14).

Conclusion: These findings of reduced surfactant proteins (SPs) in smokers may have important reflection on the host defense systems and the integrity of the airways and the development of chronic obstructive lung disease in smokers.

Key Words: Surfactant - Cigarette smokers - Nonsmokers.

INTRODUCTION

Surfactant is a complex mixture of lipids and proteins that are synthesized in the alveolar space by alveolar type II cells [1] and in the bronchiolar Clara cells of the airways [2]. Additionally, SP-A is produced in the large airways by submucosal glands [3]. Four surfactant-specific proteins (SPs) have been characterized [4,5] and these proteins promote

lung stability by regulating the surface tension-lowering properties [6], regulate surfactant homeostasis in the alveoli [7] and modulate host defense functions in the lung [8]. Hydrophilic surfactant proteins, surfactant protein A (SP-A) and surfactant protein D (SP-D), are the members of C-type lectin superfamily along with mannose-binding proteins [4,5].

Pulmonary surfactant prevents airspace collapse by reducing surface contractile forces at low alveolar volumes at the air/liquid interface in alveoli [9] and it has been shown to prevent small airway collapse^[10-12]. Surfactant also possesses anti-inflammatory properties, ^[13] facilitates mucus clearance^[14], plays a role in prevention of pulmonary infection^[13] and can scavenge extracellularly generated oxyradicals and enhance intracellular antioxidant enzyme content ^[15].

Surfactant, like lung matrix, represents a likely target for the chronic, neutrophil-dominated inflammation associated with cystic fibrosis. Neutrophil-derived lipases^[16], oxidants^[17], and proteases^[18] can biochemically alter surfactant in vitro. Activated neutrophils can directly orchestrate lipid peroxidation in dilinoleoylphosphatidylcholine vesicles^[19-20] and this peroxidation injury is enhanced by pyrophosphate-chelated iron but attenuated by superoxide dismutase or catalase.

The hydrophilic surfactant proteins SP-A and SP-D, playing an important role in the innate immunity of the lungs, bind to carbohydrate moieties on the surface of microorganisms, promoting their uptake and killing ^[21]. The physiological relevance of these functions has been clearly demonstrated in mice. SP-A and SP-D deficient animals had a reduced clearance of various viruses or bacteria and increased numbers of neutrophils respectively ^[22-24].

Some authors reported that cigarette smoke might alter the surface activity of pulmonary surfactant ^[25-27]. Endobronchial washings from long-term cigarette smokers showed a significant rise in minimum surface tension compared with nonsmokers^[27].

This study was planned to elucidate the effect of cigarette smoking on the level of surfactant proteins (SPs) by determining the contents of SP-A and SP-D and total phospholipids in BAL of healthy smokers compared with nonsmokers.

The study was carried out on 16 healthy cigarette smokers and a control group of 12 healthy nonsmokers. All subjects were asymptomatic and had no evidence of respiratory disease either by physical examination, chest radiograph, or pulmonary function tests. A written consent was obtained from all subjects.

Bronchoalveolar lavage (BAL) was performed using a fiberoptic bronchoscope (Olympus BF type FIU) under local anesthesia with lidocaine. The bronchoscope was positioned in a subsegmental lobe orifice of the right middle lobe and BAL was performed three times with 50 mL aliquots of 0.9% normal saline solution. The fluids recovered were pooled and immediately passed through several layers of loose cotton gauze to remove mucus and then centrifuged at 250g for 10 min at 4°C to sediment the cells. The supernatants were collected and cryopreserved at -30°C until use. These supernatants were used for determination of SP-A, SP-D, and phospholipid.

Human SP-A and SP-D were measured by sandwich ELISA. For SP-A determination, the samples were extracted in 4 volumes of 10 mM EDTA in TBS, homogenized in a bath sonifier (Bandelin, Berlin, Germany) for 1 min and Triton X-100 was added to a final concentration of 4%. The monoclonal mouse anti-human SP-A antibody PC-6 was used for coating and a polyclonal rabbit antihuman SP-A antibody (Chemicon, Temecula, CA, USA) for detection of bound SP-A. As standard, recombinant human SP-A was used. All samples were analyzed in duplicate and serially diluted on the plate in 4-8 steps to determine if they fall into the linear range of the assays. Plates with 96 wells, were coated over night with a monoclonal mouse anti-rat SP-D (VIF 11, BMA, Augst, Switzerland) cross reacting with human SP-D, at 2 pg/ml in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). After blocking with 10 mg/ml bovine serum albumin in TBS with calcium (10 mM Tris-HCl, 140 mM NaCl, 5 mM CaCl₂, 0.1% Triton X-100, pH 7.4), samples or standard were incubated for 1 h. Bound SP-D was detected with the monoclonal mouse anti-rat SP-D antibody (II E 11- biotin, BMA, Augst, Switzerland) in blocking buffer, followed by

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avidine-peroxidase (Dako, Glostrup, Denmark). Color was generated with ABTS (Boehringer-Mannheim, Mannheim, Germany) and H_2O_2 and read at 405 nm. As standard, recombinant human SP-D was used.

Phospholipids were extracted by the method of Bligh and Dyer^[28] and lipid phosphorus was determined by the method of Bartlett^[29].

Statistical Analysis: Data are presented as means \pm standard error of the mean (SEM). Tables (1 and 2) also show calculated standard deviation (SD) and percentiles (25%, 75% and 95%). These data were compared by the two-sided non-parametric Mann-Whitney U test to compare paired sets of data and the level of critical significance was assigned at p less than 0.05.

RESULTS

Table (1) and Fig. (1) show the concentrations of SP-A, SP-D and total phospholipid in BAL fluids in smokers and nonsmokers. The level of SP-A in BAL fluids of smokers (1.79 ± 0.27 μ g/ml) was significantly decreased ($p < 0.05$)

compared with that in nonsmokers (3.22 ± 0.46 μ g/ml), (Tables 1,3). The SP-D concentration in BAL fluids of smokers was also found to be significantly ($p < 0.05$) lower (0.52 ± 0.09 μ g/ml; $p < 0.05$), than that of nonsmokers (1.59 ± 0.32 μ g/ml). The level of total phospholipid in BAL fluids of smokers (34.26 ± 3.38 nmol/ml) was also lower than that of nonsmokers (44.17 ± 5.12 nmol/ml) but with no significant difference (Tables 1,3).

The ratios of SP-A to phospholipid and SP-D to phospholipid (Table 2 & Fig. 2) were decreased in smokers (53.50 ± 5.15 ng/nmol and 18.39 ± 3.35 ng/nmol, respectively) compared with those in nonsmokers (68.83 ± 8.17 ng/nmol and 23.83 ± 3.85 ng/nmol, respectively) but with no significant difference. The ratios of SP-A to phospholipid were close to three times the ratios of SP-D to phospholipid in both smokers and nonsmokers. The levels of SP-A and those of SP-D in BAL showed a significant correlation in both smokers and nonsmokers ($r = 0.98$) and ($r = 0.95$) respectively, $p < 0.01$, (Figs. 3 & 4). The ratio of SP-A to SP-D in smokers (4.69 ± 0.91) was significantly higher than that in nonsmokers (2.29 ± 0.14), $p < 0.05$, (Tables 2,3).

Table (1): SP-A, SP-D (μ g/ml) and phospholipid (nmol/ml) in BAL of smokers and nonsmokers.

	SP-A		SP-D		Phospholipid		
	Smokers	Nonsmokers	Smokers	Nonsmokers	Smokers	Nonsmokers	
Mean	1.79	3.22	0.52	1.59	34.26	44.17	
SEM	0.27	0.46	0.09	0.32	3.38	5.12	
SD	1.08	1.58	0.36	1.09	13.55	17.74	
Mm.	0.60	0.75	0.15	0.25	11.50	3.00	
Max.	4.00	6.00	1.30	4.00	52.00	69.00	
Percentiles	25	0.81	1.90	0.26	0.74	22.00	41.25
	75	2.59	4.19	0.78	2.50	45.500	54.13
	95	4.00	6.00	1.30	4.00	52.00	69.00

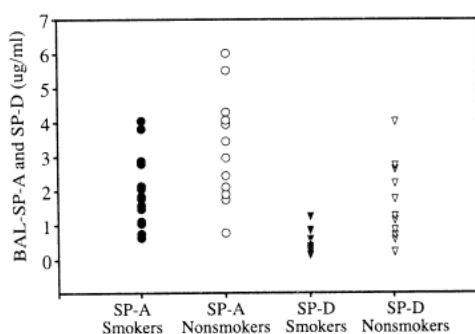
Table (2): Ratios of SP-A/phospholipid, SP-D/phospholipid (ug/nmol) and SP-A/SP-D ratio in BAL of smokers and non-smokers.

	SP-A/ Phospholipid ratio Smokers	SP-A/ Phospholipid ratio NonSmokers	SP-D/ Phospholipid ratio Smokers	SP-D/ Phospholipid ratio NonSmokers	SP-A/SP-D ratio Smokers	SP-A/SP-D ratio Nonsmokers
Mean	53.50	68.83	18.39	23.83	4.69	2.29
SEM	5.15	8.17	3.35	3.85	0.91	0.14
SD	20.60	28.31	13.41	13.34	3.64	0.48
Min.	15.00	35.00	3.80	6.50	1.12	1.50
Max.	85.00	112.00	49.00	48.50	12.00	3.00
Percentiles						
25	40.75	40.00	8.87	13.13	1.92	1.8
75	71.50	95.25	22.37	28.25	7.09	2.70
95	85.00	112.00	49.00	48.50	12.00	3.00

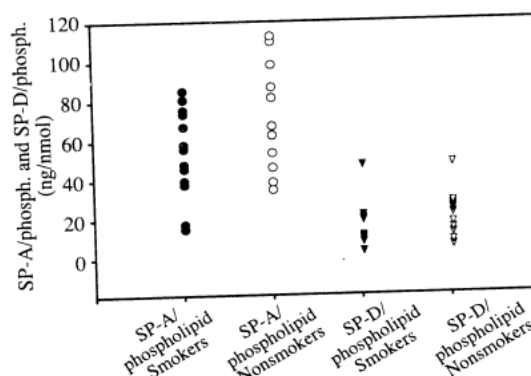
Table (3): Statistical comparison of surfactant proteins (A & D) and phospholipid in BAL of smokers and nonsmokers.

	SP-A	SP-D	Phospholipid	SP-A/ Phospholipid ratio	SP-D/ Phospholipid ratio	SP-A/SP- D Ratio
Mann-whitney (U)	40.500	30.00	59.50	73.00	64.50	47.50
Significance (P value)	0.01*	0.002*	0.09	0.285	0.143	0.024*

* Significant ($P < 0.05$).



Fig(1): SP-A AND sp-d in BAL of smokers and non-smokers.



Fig(2): SP-A/phospholipids and SP-D/phospholipids ratio in BAL of smokers and nonsmokers

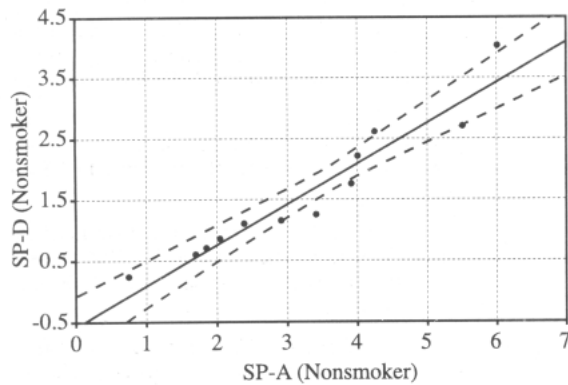


Fig. (3): Correlation between SP-A and SP-D in BAL of smokers

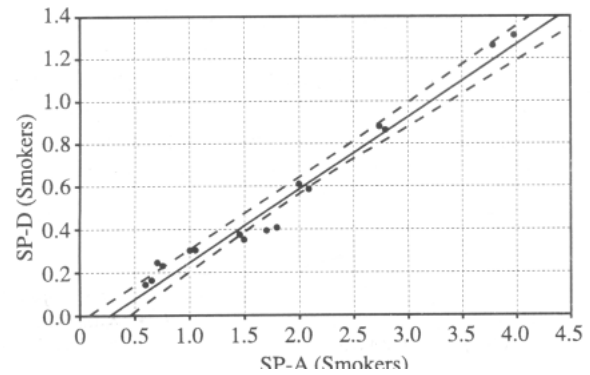


Fig. (4): Correlation between SP-A and SP-D in BAL of nonsmokers

DISCUSSION

Pulmonary surfactant proteins, SP-A and SP-D, are a class of collagenous lectin proteins (collectins) present in pulmonary secretions. The concentrations of SP-A and SP-D in BAL fluids from patients with idiopathic pulmonary fibrosis (IPF), acute respiratory distress syndrome (ARDS) and interstitial pneumonia with collagen vascular diseases (IPCD) are rather lower than those in healthy controls. On the contrary, their serum concentrations are significantly increased in patients with pulmonary alveolar proteinosis (PAP), IPF, ARDS and IPCD. There is a prominent increase of these proteins in BAL fluids and sputum of patients with PAP^[30].

Hartshorn et al.^[31] reported that surfactant proteins, SP-A and SP-D, were shown to enhance neutrophil uptake of bacteria like *Escherichia coli*, *Streptococcus pneumoniae* and *Staphylococcus aureus* through a mechanism that involved both bacterial aggregation and direct actions on neutrophils. The mechanisms of opsonizing activity of SP-D and SP-A differed in important respects from those of opsonizing antibodies. Both SP-A and SP-D are believed to play a role in the innate immunity of the lung not only against bacteria, viruses but also in-

cluding, fungi, yeasts, lipopolysaccharides and allergens^[32].

In this study, BAL levels of SP-A and SP-D obtained from smokers showed a significant decrease in comparison to those in nonsmokers, while there was no significant difference of total BAL phospholipid between smokers and nonsmokers.

Shijubo et al.^[33] observed that mean SP-A levels in BAL fluids of healthy smokers were significantly lower than those of healthy nonsmokers.

The earliest pathologic abnormalities to develop within the lungs of smokers consist of accumulations of alveolar macrophages within the alveoli^[34]. The surfactant in cigarette smokers is being sequestered from the alveolar epithelial surface into alveolar macrophages, reducing the alveolar pool size of surfactant^[35].

It seems that there are additional mechanisms that reduce surfactant in smokers other than phagocytosis by alveolar macrophages. Linnoila et al.^[36] have reported that lung tumor cells with SP-A immunoreactivity were seen more frequently in patients with a lighter smoking

history. The production of SPs from alveolar type II cells might be reduced in smokers.

It has been reported that SP-A and SP-D play crucial roles in host defense mechanisms and immunomodulation of the peripheral airways, possibly by enhancing microbial recognition, phagocytosis and/or killing by resident phagocytic cells^[37-41]. The significant reductions of BAL fluid SP-A and SP-D in smokers might attenuate the host defense functions of surfactant in the peripheral airways of smokers and may lead to direct toxic injury of the lung and the development of pulmonary disease including chronic obstructive pulmonary disease (COPD).

Conflicting results have been published about the influence of tobacco smoking on alveolar phospholipid level with no clearly confirmed evidence about the contents of phospholipid in BAL fluids of smokers. Finley and Ladman^[42] and Wurtemberger et al.^[43] observed a lower total phospholipid content in human smokers. However, Low et al.^[44] reported no difference in lavage phospholipid concentration between smokers and nonsmokers. On the other hand, Hughes and Haslam^[45] reported that smokers had significantly higher levels of total phospholipid compared with nonsmokers. The result of phospholipid in BAL fluids of this study is consistent with those of Low et al.^[44].

In this study, both levels of BAL fluid SP-A and SP-D were decreased in smokers. This result was not consistent with the findings reported by Hamm et al. ⁽⁴⁶⁾ who found that the levels of BAL fluid SP-A were slightly higher in smokers than that in nonsmokers (4.5 µg/mL and 3.4 µg/mL, respectively), although not significant.

In the present study, a significant correlation was found between the contents of SP-A and those of SP-D in BAL fluids both in smokers and nonsmokers in addition to a significant difference of SP-A/SP-D ratio between the two groups. This seems acceptable as both SP-A and SP-D are synthesized in alveolar type II cells and nonciliated epithelial cells and secreted into the alveolar spaces.

Cigarette smoking is considered as a major risk factor for the pathogenesis of COPD. The role of surfactant proteins being decreased by smoking is not yet clearly clarified in the evolution of obstructive lung disease. Lusuardi et al. ⁽⁴⁷⁾, in a series of 20 smokers non-asthmatic COPD patients compared with 5 nonsmokers healthy controls, found a marked decrease (about 6-7 times) of total phospholipids in BAL in smokers COPD. An alteration of mucociliary clearance and an impairment of antimicrobial defense might be important factors related to surfactant status in COPD. However, no definitive data are available.

In conclusion, this study revealed that the BAL surfactant proteins are decreased in smokers. This reduction of these hydrophilic surfactant proteins (SPs) in smokers may have important reflections on the host defense systems of the peripheral airways and might be a crucial cause of chronic obstructive lung disease. It would be interesting to determine whether patients with chronic obstructive lung disease have reduced levels of SP-A and SP-D in BAL fluids. Further studies are needed to confirm the possible roles of these hydrophilic SPs in the development of chronic obstructive lung disease, particularly, in smokers and to the potentials of surfactant replacement in arresting disease progress.

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دراسة بروتينات السيرفاكتانت أ و د في المدخنين بالمقارنة إلى غير المدخنين

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مقدمة : يتكون مركب السيرفاكتانت من مزيج من البروتين والدهون الذي يتم تصنيعه في الخويصلات الهوائية بالرئتين و أيضا في الشعب الهوائية. تم التعرف على أربعة أنواع من هذه البروتينات التي لها وظيفة هامة في الحفاظ على ثبات الرئة بالتحكم في التوتر السطحي للرئة فتمنع غلق الممرات الهوائية بالإضافة إلى وظيفة منع الالتهابات و التحكم في المخاط الذي يمنع إصابات الجهاز التنفسي و أيضا بالاتحاد مع الكائنات الدقيقة عن طريق الاتحاد مع الكربوهيدرات الموجودة على سطح الأجسام الدقيقة فيساعد على التخلص منها ، ومن هنا تتضح أهمية السيرفاكتانت في زيادة مناعة الجهاز التنفسي .

الهدف من البحث : تهدف هذه الدراسة إلى إيضاح تأثير التدخين على بروتينات السيرفاكتانت أ و د عن طريق مقارنة نسب تركيزها في الأشخاص المدخنين الذين لايعانون من شكاوى بالجهاز التنفسي و الأشخاص غير المدخنين.

الأشخاص وطرق البحث: أجريت هذه الدراسة على مجموعة مكونة من ١٦ فردا من المدخنين بالمقارنة إلى مجموعة مكونة من ١٢ فردا من الأصحاء غير المدخنين. تم أخذ عينات من المخاط بالشعب والخويصلات الهوائية عن طريق منظار الألياف الضوئية تحت محذر موضعي و بعد موافقة كتابية لجميع الأشخاص المشاركين في البحث ، تم عمل غسيل للشعب و الخويصلات الهوائية بحلول الملح ٠,٩ % ثلاث مرات و تجميع عينات الغسيل و معالجتها للتخلص من الخلايا و المخاط و تم حفظ السائل النقي في درجة حرارة ٣٠ درجة تحت الصفر لحين إجراء قياس السيرفاكتانت أ و د و فوسفاتيدات الدهون .

النتائج : أوضحت النتائج انخفاض ملحوظا في نسبة تركيز بروتينات السيرفاكتانت أ و د في المدخنين بالمقارنة إلى غير المدخنين.

الاستنتاج : يمكن استنتاج التأثير السلبي للتدخين على انخفاض نسبة بروتينات السيرفاكتانت أ و د في سائل غسيل الشعب و الخويصلات الهوائية مما يترتب عليه التأثير المباشر على نقصان مناعة الجهاز التنفسي و الإصابة بالتهابات الشعب الهوائية المستمر الذي يؤدي بدوره إلى الإصابة بالتهابات الرئوية المزمنة .