Surfactant protein D concentrations in serum and bronchoalveolar lavage fluid from young healthy horses on pasture and in a barn environment

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Received January 16, 2020.
Accepted May 12, 2020.

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OBJECTIVE
To evaluate surfactant protein D (SP-D) concentrations in serum and bronchoalveolar lavage fluid (BALF) from young healthy horses on pasture or housed in a typical barn.

ANIMALS
20 young healthy horses.

PROCEDURES
Horses were randomly assigned to 1 of 2 groups (pasture, n = 10; barn, 10), and serum and BALF samples were collected for SP-D determination at baseline (all horses on pasture) and 2 weeks and 4 weeks after the barn group of horses was relocated from the pasture to the barn. Other evaluations included physical and tracheoscopic examinations. Findings were compared within and between groups.

RESULTS
Physical and tracheoscopic examinations, CBC, and serum biochemical analysis did not reveal evidence of respiratory disease, and no significant differences were present within and between groups. Serum SP-D concentrations did not significantly differ within and between groups, but BALF SP-D concentrations were significantly lower for the barn group at 2 weeks but not at 4 weeks, compared with baseline. The BALF SP-D concentration-to-BALF total protein concentration ratio was < 1.5 and did not significantly differ within and between groups.

CONCLUSIONS AND CLINICAL RELEVANCE
A mild decrease was evident in the concentration of SP-D in the BALF collected from young healthy horses after 2 weeks of exposure to a barn environment. The clinical importance of this finding remains to be determined. (*Am J Vet Res* 2021;82:152–157)

Surfactant protein D is a pattern recognition molecule synthesized by type II alveolar cells in the lung (predominantly) and by club cells in the airways[1,2] but is also found in other organ systems.[2] Surfactant protein D is part of the collectin family (defined by 4 common structural domains) and adopts multimeric structures (eg, cruciform and fuzziball).[2] It plays a critical role in the innate immune defense system of the lung through aggregation of microorganisms, opsonization of pathogens, enhancement of phagocytosis, and inhibition of microbial growth.[2,3] Also, SP-D modulates inflammation, the acquired immune response, and allergic reactions.[2,4] The role of SP-D has been investigated in numerous respiratory diseases, including those that affect the airways (eg, asthma, chronic obstructive pulmonary disease, and cystic fibrosis), the alveoli (eg, pneumonia, emphysema, and pneumoconiosis), and the interstitium (eg, sarcoidosis and idiopathic pulmonary fibrosis).[2] Surfactant protein D has been measured in serum, BALF, and lung tissue and is a potential biomarker for lung integrity and disease progression in respiratory diseases and respiratory complications associated with other diseases.[2,5,6] Increased serum SP-D concentrations are thought to be the result of increased alveolocapillary permeability, modified SP-D synthesis or clearance, and changes in SP-D molecular structure (eg, oxidation and cross-linking).[2] In addition, SP-D concentrations may be influenced by individual (eg, obesity) or environmental (eg, dust) factors.[7–10] Therefore, the BALF SP-D concentration-to-serum SP-D concentration ratio is also affected by the aforementioned respiratory diseases and factors.[11]

ABBREVIATIONS
BALF Bronchoalveolar lavage fluid
SP-D Surfactant protein D

In equine medicine, SP-D was first isolated from the BALF of healthy horses and characterized by Hobo et al.[12] Equine SP-D has high homology with human

SP-D and cross-reacts with antibodies to human SP-D. Subsequent to its first characterization, SP-D concentrations were determined in various respiratory diseases.[13–18] Surfactant protein D concentrations are decreased in the BALF of horses following prolonged road transport (vs before transport).[15] Serum SP-D concentrations parallel the clinical condition of horses with experimentally induced pneumonia,[14] and increased serum SP-D concentrations were described[16] for horses with mild asthma (formerly known as inflammatory airway disease) versus healthy horses. Serum SP-D is considered a biomarker in horses with mild asthma, particularly when combined with haptoglobulin and secretoglobulin.[17,18] The cause of increased serum SP-D concentrations in horses with mild asthma remains unknown. Overall, information on the concentration of SP-D in the BALF of healthy and diseased horses is sparse. The effects of individual (eg, age and breed) and environmental (eg, housing and training) factors on serum and BALF SP-D concentrations of horses have not yet been determined. Therefore, the purpose of the study reported here was to evaluate SP-D concentrations in the serum and BALF of young healthy horses on pasture and in a typical barn environment.

Materials and Methods

Horses

Twenty young healthy horses from a research herd at the Department of Veterinary Science, University of Kentucky, were selected for this study. These horses were born at the research farm in September 2014 (n = 3) and between May and September 2016 (17) and included 13 colts and 7 fillies. Horses had been housed on pasture since weaning, were routinely vaccinated against tetanus and rabies, and received an anthelmintic for prevention of intestinal parasitism. All horses were considered healthy on the basis of physical examination, CBC, and serum biochemical analysis findings. Serum and BALF samples were collected from June 2017 to July 2017. The study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol No. 2017-2617).

Protocol

Horses were randomly assigned to 1 of 2 groups. Horses on pasture (pasture group; n = 10) consisted of 6 colts and 4 fillies of various breeds and ages (two 3-year-olds and eight 1-year-olds), had free access to grass, and received a grain mix once a day. Horses housed in a typical barn (barn group; n = 10) consisted of 7 colts and 3 fillies of various breeds and ages (one 3-year-old and nine 1-year-olds), were bedded on shavings, and were fed hay ad libitum and a grain mix once a day. All horses were sampled at baseline (when all horses were on pasture) and then 2 and 4 weeks after 10 of the horses were relocated from the pasture to the barn (barn group).

Patient examination and sample collection

Each horse was briefly examined prior to each sample collection. Horses were sedated with detomidine[a] (0.01 mg/kg, IV) and butorphanol[b] (0.01 mg/kg, IV). Blood was collected from the jugular vein by use of evacuated blood collection tubes with and without EDTA. Venous blood was allowed to clot for 60 minutes, and serum was then harvested after centrifugation at 2,000 × *g* for 15 minutes. Serum was stored at –80°C until further analysis.

An endoscope was passed through the nasal passages and into the trachea (tracheoscopy) to check for the presence of mucus.[19] Then, a cuffed BALF collection tube[c] was passed through the nasal passages and into the trachea. Next, approximately 60 mL of a 0.4% lidocaine solution was infused through the collection tube to reduce coughing; the collection tube was advanced until resistance was met (the tube was wedged in an airway) and its cuff was inflated with 6 mL of air. Two aliquots of 120 mL of sterile saline (0.9% NaCl) solution were infused with 60-mL syringes through the collection tube and manually aspirated with 60-mL syringes immediately after infusion. Recovered BALF from each horse was mixed, pooled, and placed in a sterile specimen cup on ice. Recovered BALF volume and quality were recorded, and samples were processed within 30 minutes after collection. Nucleated cell counts were determined by use of an automated cell counter, and a WBC differential cell count was determined through immersion microscopic examination of 400 cells on a cytospin slide stained with a modified Wright stain. Cell-free BALF was obtained by means of centrifugation of BALF at 400 × *g* for 10 minutes and aspiration of the resulting cell-free supernatant. Cell-free BALF was stored at –80°C until further analysis.

Protein and SP-D measurement

Protein content of the cell-free BALF supernatant was measured with the bicinchoninic acid method that included bovine serum albumin as the standard.[d] Serum and BALF SP-D concentrations were determined with a commercially available ELISA kit designed for the detection of SP-D in people.[e] Samples of BALF were diluted 184 times with saline solution, whereas serum samples were diluted 11 times according to the manufacturer's instructions. All samples were run in duplicate, and only results with a coefficient of variation of < 5% were included.

Statistical analysis

Normal probability plots showed that data were skewed. Subsequently, data were summarized as median and range. Effects of time (baseline and 2 and 4 weeks) and group (pasture and barn) were assessed by use of linear generalized estimating equations. The statistical model specified time, group, and the interaction between time and group as fixed effects. Repeated measures within horse were modeled by spec-

Table 1—Median (range) for percentage of BALF recovered (%), total nucleated cell count (No./μL), and WBC differential cell count (%) at baseline, 2 weeks, and 4 weeks for young healthy horses on pasture (n = 10) and housed in a barn (10). Horses housed in a barn were relocated from the pasture after baseline samples were collected.

	Pasture			Barn		
Variable	**Baseline**	**2 weeks**	**4 weeks**	**Baseline**	**2 weeks**	**4 weeks**
Recovery	54 (40–73)	77 (58–100)	63 (51–78)	55 (23–67)	71 (53–87)	65 (32–100)
Total nucleated cells	375 (180–990)	455 (140–930)	520 (150–940)	310 (70–780)	430 (120–1,050)	265 (30–1,250)
Macrophages	66 (51–89)	68 (55–81)	58 (48–75)	65 (53–88)	63 (53–73)	59 (46–73)
Neutrophils	1 (0–2)	2 (1–4)	2 (0–3)	1 (0–2)	1 (0–3)	2 (1–4)
Lymphocytes	33 (8–48)	31 (17–44)	40 (24–51)	35 (10–47)	36 (25–45)	41 (26–50)
Eosinophils	0 (0–0)	0 (0–1)	0 (0–0)	0 (0–0)	0 (0–1)	0 (0–0)

Table 2—Median (range) for SP-D concentration in BALF (ng/mL) and serum (ng/mL), BALF SP-D concentration-to-serum SP-D concentration ratio, and BALF SP-D concentration-to-BALF total protein concentration at baseline, 2 weeks, and 4 weeks for the horses of Table 1.

	Pasture			Barn		
Variable	**Baseline**	**2 weeks**	**4 weeks**	**Baseline**	**2 weeks**	**4 weeks**
SP-D BALF	3,086 (1,362–8,192)	3,347 (1,654–5,870)	3,750 (1,671–5,212)	4,208* (1,822–8,699)	2,188* (1,069–3,968)	3,407 (1,711–4,511)
SP-D serum	25 (20–50)	26 (12–55)	17 (12–30)	24 (16–43)	24 (11–90)	18 (12–72)
BALF SP-D-to-serum SP-D ratio	135 (44–327)	146 (30–499)	187 (64–366)	131 (70–297)	80 (25–285)	175 (45–299)
BALF SP-D-to-BALF total protein ratio	1.3 (0.7–2.4)	1.1 (0.9–1.8)	1.1 (0.9–1.9)	1.3 (0.9–1.7)	1.3 (0.9–1.8)	1.4 (1.1–1.9)

*Significant difference in BALF SP-D concentration between baseline and 2 weeks.

ifying the compound symmetry correlation matrix. The interaction between time and group was further analyzed to compare the groups at each time point and to compare time points within each group. For the latter, *P* values were adjusted for multiple comparisons with the Tukey method. All analyses were performed with commercial statistical software.[f] Values of $P < 0.05$ were considered significant.

Results

For each horse and sample time, physical examination and CBC findings were within reference limits and no tracheal mucus was noted with tracheoscopy. Cytologic findings for BALF were summarized **(Table 1)**. Recovery of BALF was < 50% in 5 horses (pasture group at baseline, n = 3; barn group at baseline, 1; and barn group at 4 weeks, 1). Total nucleated cell counts were greater than that reported[20] for healthy horses (< 530 cells/μL) for 6 horses in the pasture group and 5 horses in the barn group. None of the horses developed a cough or tracheal mucus or had BALF with neutrophilia. Physical examination, CBC, tracheoscopy, and BALF cytology results did not significantly differ within and between groups.

Serum SP-D concentrations did not significantly differ within and between groups **(Table 2; Figure 1)**. Two horses in the barn group had serum SP-D concentrations that were higher at 2 and 4 weeks, compared with baseline. Surfactant protein D concentration in BALF was significantly ($P = 0.02$) lower for the barn group at 2 weeks, compared with baseline, but this difference was not noted at 4 weeks **(Figure 2)**. The BALF SP-D concentration-to-BALF total protein

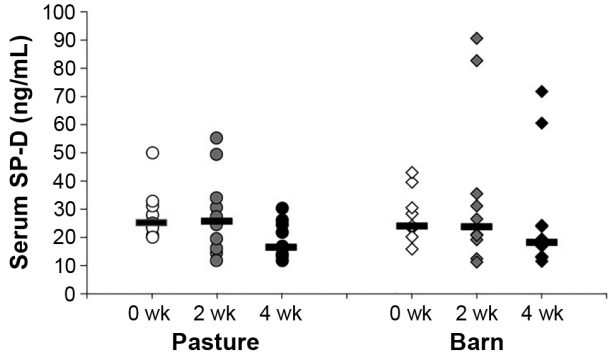

Figure 1—Serum SP-D concentrations for young healthy horses on pasture (n = 10) or housed in a barn (10) at baseline (0 weeks), 2 weeks, and 4 weeks. Horses housed in a barn were relocated from the pasture after baseline samples were collected. Horizontal bars indicate the median.

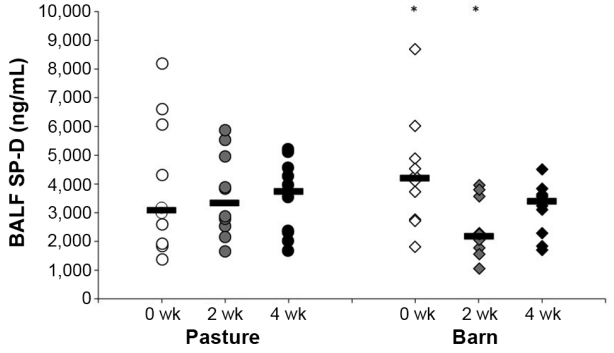

Figure 2—Bronchoalveolar lavage fluid SP-D concentrations for the horses of Figure 1. *Significant ($P = 0.02$) difference between baseline and 2 weeks. ***See*** Figure 1 for remainder of key.

concentration ratio was < 1.5 and did not significantly differ within and between groups. Concentrations of SP-D in the BALF were 157 ± 97 (mean ± SD) times that of serum.

Discussion

Surfactant protein D plays a role in pulmonary immunomodulation and is considered a potential serum biomarker of horses with mild asthma.[16,18] The purpose of the present study was to determine serum and BALF concentrations of SP-D in young healthy horses on pasture or housed in a typical barn. Results revealed that concentrations of SP-D in BALF were significantly lower in horses housed in a barn for 2 weeks, compared with baseline (on pasture), but this difference was not present at 4 weeks. Additionally, concentrations did not significantly differ between horses in the pasture and barn groups and within the pasture group. Serum SP-D concentrations also did not significantly differ between and within groups. The clinical importance of these findings remains to be determined.

In horses with mild to moderate asthma, SP-D concentrations were previously analyzed in serum but not in BALF.[16–18] The relationship between SP-D in BALF and SP-D in serum is complex and influenced by a number of factors, including local synthesis, airway inflammation, airway infection, allergen load, and alveolocapillary membrane permeability.[2,21] Evaluating SP-D concentrations in both BALF and serum is an important step toward understanding the role of SP-D in the pathogenesis of respiratory diseases of horses.

The effect of exposure to organic dust on the concentrations of SP-D was assessed in 2 in vitro studies.[9,10] One research group reported[9] that exposure to organic dust does not affect SP-D concentrations in human lung epithelial cells, whereas another research group reported[10] that organic dust inhibits SP-D production in human alveolar cells. Possibly, SP-D concentrations change over time, with this change being dependent on the agent (eg, dust) and its concentration and the duration of exposure. For example, rats exposed to bleomycin and sensitized mice exposed to an allergen had an increase in the SP-D concentration in the BALF 1 to 3 days after exposure, with a gradual return to baseline concentration over 2 to 4 weeks.[22,23] In the present study, samples were not collected the first few days after horses were moved from the pasture to the barn (barn group); therefore, an initial increase in SP-D concentration in the BALF may have been missed. A decrease in SP-D concentration in the BALF at 2 weeks for the barn group may have also been the result of a response to an allergen (eg, aggregation of allergens and their accelerated removal by alveolar macrophages) with subsequent adaptation to the air particulates in the barn by 4 weeks. Significantly lower concentrations of SP-D in the BALF of healthy horses after prolonged transport were previously described,[15] and prolonged transport has been associated with an increased risk for respiratory disease.

Several studies[21,24–26] that involved the assessment of SP-D concentrations in the BALF from people with asthma yielded conflicting results. In 1 study,[24] increased SP-D concentrations were reported in patients with severe refractory asthma, compared with those with mild to moderate asthma. In other studies,[25,26] high SP-D concentrations were detected in mild asthmatic patients versus healthy patients; in one of these studies,[25] concentrations further increased when patients received an intrabronchial allergen challenge. Yet a study[21] of people with severe, refractory asthma revealed that concentrations of SP-D were decreased in BALF and increased in serum. Results may have differed among studies because people had various phenotypes of asthma and other individual factors or treatments. To the authors' knowledge, studies regarding SP-D concentrations in the BALF of horses with severe asthma have not been published.

Serum concentrations of SP-D did not significantly differ within and between groups in the present study. However, 2 horses in the barn group had much higher serum SP-D concentrations at 2 and 4 weeks, compared with baseline. Although the relevance of this finding is unclear, monitoring serum SP-D concentrations and for the development of respiratory disease in these 2 horses may be interesting. Serum SP-D concentrations have been assessed in horses with bacterial pneumonia,[14] lower respiratory tract disease,[13,16,17] and mild asthma.[13,14,16–18] In horses with experimentally induced bacterial pneumonia, serum concentration of SP-D paralleled changes in rectal temperature, serum amyloid A concentration, and fibrinogen concentration,[14] and horses with unspecified lower airway disease and coughing had higher serum concentrations of SP-D versus healthy controls.[13] Serum SP-D may be a biomarker of mild to moderate asthma and therefore useful as an aid for the diagnosis of asthma when concurrently assessed with haptoglobulin and secretoglobulin.[17,18] Increased serum SP-D concentrations have also been described for people with various lung diseases, including asthma.[2] In asthmatic people, increased serum SP-D concentrations are associated with decreased lung function parameters and bronchial inflammation.[21]

Causes of increased serum SP-D concentrations may differ with various respiratory diseases depending on their underlying pathogeneses.[11,22,23] Increased serum SP-D concentrations may develop because of increased alveolar expression of SP-D or changes in alveolocapillary membrane permeability or the structure of SP-D.[2] Changes in SP-D structure have been described in the BALF and serum of asthmatic patients.[21] The SP-D structure most likely changed because of hydrolytic enzymes produced by inflammatory cells and bacteria.[24] The ratio of BALF SP-D concentration-to-serum SP-D concentration, which is routinely assessed in studies[11,22] to investigate the role of SP-D in respiratory diseases of people, integrates information from the alveolar space and sys-

temic circulation. The present study did not reveal significant changes in the BALF SP-D concentration-to-serum SP-D concentration ratio over time for the barn group. The relationship between BALF and serum SP-D in horses with respiratory disease remains to be determined. Furthermore, causes for high serum SP-D concentrations in asthmatic horses warrant further investigation.

Limitations of the present study were the inclusion of only young healthy horses and the timing and frequency of sample collection (only 3 sample times: baseline and 2 and 4 weeks after exposure). Other limitations included that the barn group was exposed to a typical barn environment rather than to a challenge environment (ie, exposure to air particles in the present study was likely lower), BALF recovery was < 50% for 5 horses, total nucleated cell counts for the BALF were greater than the reference limit for 11 horses, and the commercially available SP-D kit did not differentiate between the various forms of SP-D (eg, high- vs low-molecular-weight forms). In retrospect, evaluation of SP-D concentrations shortly after exposure to the barn environment may have yielded important information about the time course of SP-D concentrations. Additionally, SP-D concentrations could have been assessed in healthy horses in response to a more challenging environment (vs barn) that can concurrently induce airway inflammation. However, the present study was not designed to evaluate these aspects. Results from approximately the same number of horses in each group were affected by poor recovery of or high total nucleated cell counts in BALF; therefore, results from these horses were still included in the data set. Eleven of the horses had a total nucleated cell count above the reference limit reported[20] for healthy horses. Nucleated cell counts in young horses are generally higher than in old horses.[25,26] Additionally, the volume of instilled saline solution for BALF collection and method of analysis may have affected total nucleated cell counts. A slightly lower volume of saline solution was instilled for BALF collection, compared with the recommended volume (240 mL vs 250 or 300 mL), to adjust for the young age and small size of the horses. Lastly, nucleated cell counts were determined with an automated cell counter rather than manually with a hemacytometer. Future studies should include a more detailed assessment of SP-D structure and degradation products in both serum and BALF of horses with and without respiratory disease.

In conclusion, young healthy horses that were exposed to a barn environment had a mild decrease in the concentration of SP-D in BALF at 2 weeks, but not at 4 weeks, compared with SP-D concentration prior to relocation from pasture to the barn. Serum SP-D concentrations, BALF SP-D concentration-to-serum SP-D concentration ratio, and BALF SP-D concentration-to-BALF total protein concentration ratio were unaffected by exposure to a barn environment. The effects of individual and environmental factors on SP-D concentrations in the BALF and serum of horses warrant further investigation. Also, the effect of airway inflammation and respiratory disease status on SP-D concentrations should be further assessed.

Acknowledgments

Supported by a grant from the Gluck Equine Research Center, University of Kentucky.

The authors declare that there were no conflicts of interest.

Presented in abstract form at the 36th Annual Symposium of the Veterinary Comparative Respiratory Society, Auburn, Ala, September 2018.

The authors thank Joey Morgan, who provided valuable expertise and assistance with laboratory analysis.

Footnotes

a. Dormosedan, Zoetis, Parsippany, NJ.
b. Torbugesic, Zoetis, Parsippany, NJ.
c. Jorgensen Laboratories, Loveland, Colo.
d. Pierce Distribution Services Co, Rockford, Ill.
e. Biovendor, Asheville, NC.
f. SAS, version 9.4, SAS Institute Inc, Cary, NC.

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