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Research Article

Surfactant Proteins A and D in the Seminal Plasma of Stallions

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Abstract: We have previously shown that surfactant protein (SP)-A and SP-D are present in the stallion's genital system. The aim of the present study was to evaluate the expression and presence of SP-A and SP-D in the seminal plasma and accessory sex glands of stallions. We characterized further the SP-A and SP-D in the seminal plasma of stallions by western blotting and immunohistochemically in accessory sex glands. On one-dimensional SDS-PAGE electrophoresis and western blots, bands for SP-A (34 kDa) and SP-D (43 kDa) in seminal plasma and accessory sex gland tissue extracts were detected. The similarity between biochemical properties (relative molecular masses) of SP-A and SP-D in accessory sex glands and seminal plasma imply that SP-A and SP-D proteins are also present in the seminal plasma and accessory sex glands of stallions.

Key Words: Surfactant protein A (SP-A), surfactant protein D (SP-D), western blotting, stallion, immunohistochemistry, seminal plasma, accessory sex glands

Aygır Seminal Plazmasında Surfaktant Protein A ve D

Özet: Daha önceki çalışmalarımızda SP-A ve SP-D'nin aygır genital sisteminde bulunduğunu gösterdik. Mevcut çalışma surfaktant protein A ve D'nin aygır seminal plazma ve eklenti bezlerinde varlığının araştırılması amacıyla tasarlandı ve aygır erkek eklenti bezlerinde immunohistokimyasal olarak, Seminal plazmada ise western blot yöntemi ile SP-A ve SP-D'nin varlığı karakterize edildi. Tek boyutlu SDS-PAGE elektroforezi ve western blot testlerinde surfaktant protein A (34 kDa) ve D (43 kDa)'nin moleküler ağırlıklarda olduğu gösterildi. Seminal plazma ve erkek eklenti bezlerinde SP-A ve SP-D'lerinde moleküler ağırlıklar yönünden elde edilen biyokimyasal benzerlik, aygır erkek eklenti bezlerinde ve seminal plazmada SP-A ve SP-D bulunduğunu göstermektedir.

Anahtar Sözcükler: Surfaktant protein A (SP-A), surfaktant protein D (SP-D), western blotting, aygır, immunohistokimya, seminal plazma, erkek eklenti bezleri

Introduction

Pulmonary surfactant is a barrier material of the lungs and has a dual role: firstly, as a true surfactant, lowering the surface tension; and secondly, participating in the innate immune defense of the lung and possibly other mucosal surfaces. Four surfactant proteins have been identified and characterized in the lung, including surfactant protein-A (SP-A) (1) surfactant protein-B (SP-B) (2), surfactant protein-C (SP-C) (3), and surfactant protein D (SP-D) (4). SP-A and SP-D proteins are members of a family of collagenous carbohydrate binding proteins (collagenous C-type lectins), now generally called collectins. The lung is the major site of synthesis of SP-A and SP-D, produced by alveolar type II cells and Clara cells. Particular locations of SP-A and SP-D in genital system tissues and organs have also been reported, including the testes, prostate, and placenta (5,6). Moreover, the presence of SP-A in the human prostate (5) and human seminal plasma (7) has been suggested. Oberley et al. (8) suggested an important role for SP-D in antimicrobial host defense and inflammatory regulation in the prostate. It was reported that SP-D mRNA and protein are present in glandular epithelial cells of the human prostate gland and SP-D can inhibit Chlamydial infection of prostate epithelial cells (9)

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and SP-D is suggested as an important molecule in antimicrobial host defense and inflammatory regulation mechanism in the prostate. Kankavi et al. (10) showed that SP-A and SP-D are also present in the stallion's genital tract organ and tissues. Moreover, recent studies suggest that SP-A and SP-D inhibit the growth of some strains of gram-negative bacteria, by increasing the permeability of the microbial cell membrane (11). Investigations on SP-A and SP-D antibacterial studies suggested that SP-A and SP-D directly inhibit the proliferation of *P. aeruginosa* (12), *Chlamydia trachomatis* (8), and *Aspergillus fumigatus* (13) in the lung. The present study was designed to evaluate the cellular localization of SP-A and SP-D in stallion seminal plasma and accessory sex glands.

Materials and Methods

Preparation of Seminal Plasma Samples: Single ejaculates from each of 5 stallions (Arabian and English breeds), between 5 and 11 years of age, were collected using a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) with an in-line gel filter (Animal Reproduction Systems, Chino, CA, USA). Seminal plasma from each stallion was removed by centrifugation at 1000 × g for 15 min at 4 °C and pooled seminal plasma was again centrifuged at 27,000 × g for 40 min at 4 °C to remove any remaining spermatozoa. Following this, supernatant was examined using phase contrast microscopy (200× magnification) to ensure that no spermatozoa remained in the seminal plasma. To these seminal plasma samples was added protease inhibitor to prevent nonspecific proteolysis.

Collection of Tissue Samples: To define the tissue distribution of SP-A and SP-D, we harvested tissues from stallions 22-26 years old (5 stallions). The horses had no history of clinical signs of genital system disease and were housed on pasture. These studies were carried out with Ethics Committee approval.

Antibodies: SP-A (SP-A, 8) (0.1 mg/0.1 ml) and SP-D (SP-D, 13) (0.1 mg/0.1 ml) anti-horse monoclonal primary antibodies were kindly donated by Dr. Seiji Hobo, (Equine Research Institute, Japan Racing Association, Japan).

Protein Assay: The protein concentration of the stallion accessory sex gland tissue sample and seminal plasma extractions were measured by a protein assay (14).

SDS-Page and Western Blotting: Seminal plasma from each stallion contained 20 µl of protease inhibitor cocktail (Sigma P8340; St Louis, MO, USA). Cells and debris were removed by centrifugation at 4 °C. Samples containing ~100-150 µg of protein were subjected to one-dimensional SDS/PAGE (12% w/v, 1.5 mm thick gel polyacrylamide gel) by the method of Laemmli (15), with a Mini Protean II apparatus (Bio-Rad, California, USA) in the presence of 5% β-mercaptoethanol. After electrophoresis, gels were used in Western blotting of antigens by transferring separated proteins onto the nitrocellulose membrane. Blots were probed with monoclonal antibodies directed against horse SP-A (SP-A, 8) and SP-D (SP-D, 13) diluted 1:500 with 0.05% TTBS and incubated for 2 h at room temperature. After 3 × 10 min washes, blots were incubated for 30 min with anti-mouse IgG antibody (2 mg/ml) diluted 1:1000 with 0.05% TTBS (M-8642, Sigma). After a further 3 × 10 min washes, the blot was incubated for 15 min with peroxidase-conjugated anti-goat IgG antibody (1 mg/ml) 1:2000 with 0.05% TBST (A-4174, Sigma). After a final wash with Tris-buffered saline, the blots were developed with a chemiluminescent detection system (ECL, Amersham Life Science).

Immunohistochemistry: Formalin-fixed, paraffin-embedded tissue sections (4 µm) were deparaffinized and rehydrated serially in 100%, 95%, 75%, and 50% EtOH. The slides were boiled in 0.1 M sodium citrate (pH 6.0) for 10 min and then cooled for 20 min for antigen retrieval. The slides were rinsed in phosphate buffered saline (PBS) and endogenous peroxidase activity quenched by incubating in 0.3% H_2O_2 in methanol for 30 min. The slides were rinsed with PBS and incubated in Ultra V Block solution for 10 min (LabVision Corporation, Fremont, CA, USA). The slides were then incubated with SP-A and SP-D monoclonal primary antibodies (1:500) at 4 °C overnight. For negative controls, some cases slides were incubated overnight with PBS or rabbit IgG molecules in place of the primary antibody. The slides were rinsed in PBS and incubated in a secondary antibody kit, firstly with Biotinylated Goat Anti Polyvalent (LabVision Corporation) for 15 min at room temperature and then the slides were rinsed and incubated in Streptavidin peroxidase agent (LabVision Corporation) for 15 min at room temperature. The slides were rinsed in PBS and incubated in diaminobenzidine (DAB) chromogen (Dako liquid DAB). The slides were counterstained with Mayer's hematoxylin (Sigma, USA) for 30 s to 1 min, and then mounted using paramount mounting medium (containing 15 mM NaN_3, Dako, USA). Non-specific immunoglobulin binding was

blocked by incubating sections in 5% bovine serum albumin (BSA) for 2 h. In further experiments, as a positive control for SP-A and SP-D staining, adult lung tissue sections were stained using the SP-A and SP-D antibodies.

Bronchoalveolar lavage fluid (BALF) was taken during autopsy from the same animals. One hundred milliliters of BN buffer containing 0.01 M sodium borate pH 7.4, 0.15 M NaCl, and 3 mM $CaCl_2$ was used for lavage of each lung. The lavage was centrifuged at 250 × g for 10 min at 4 °C to remove cellular debris, the supernatant was subsequently centrifuged at 27,000 × g for 2 h at 4 °C, and the resulting pellet was resuspended in 100 µl of BN buffer and kept in –20 °C (16).

Results

Western blotting and immunohistochemistry were used to identify SP-A and SP-D in stallion seminal plasma, prostate, and seminal vesicle. Western blot analysis also showed the molecular weights of SP-A and SP-D in stallion seminal plasma samples. In extracts from the stallion BALF and seminal plasma samples 34 and 43 kDa monomeric bands were detected (Figure 1A and 1B). These bands correspond to the SP-A and SP-D proteins and they have been previously described within ranges described for SP-A and SP-D. The Western blotting results shown in Figures 1A and 1B were chosen randomly from 1 stallion that represents 5 stallions.

SP-A and SP-D appeared to be present in stallion prostate, seminal vesicle, ductus deferens, and seminal plasma, as was evidenced by specific horse monoclonal antibody labeling. Moreover, the protein content of seminal plasma of stallions was approximately 10 mg/ml (8.6-11.2 mg/ml). Controls were done in the presence of horse BALF or in the absence of primary antibodies during the blocking step and/or using egg yolk as a negative control tissue. Epithelial cells lining the stallion prostate glands show positivity for SP-A and SP-D (Figure 2A SP-A and Figure 2B SP-D). Furthermore, secretory materials were strongly reactive with monoclonal horse SP-A and SP-D antibodies, indicating that the glandular epithelium of stallion prostate secretes SP-A and SP-D proteins (Figure 2A for SP-A, Figure 1B SP-D). However, SP-A and SP-D proteins were not uniformly present in all tubuloalveolar glands of the prostate. The secretory end pieces of seminal vesicles lined with simple columnar epithelium were SP-A and SP-D positive (Figure 2C SP-A and Figure 2D SP-D). SP-A and SP-D were prominent in the stallion seminal vesicle epithelium. The lumen of the seminal vesicles and secretory material were strongly reactive with monoclonal SP-A and SP-D antibodies (Figure 2C SP-A and Figure 2D SP-D). SP-A and SP-D immunohistochemistry showed staining of the cell surface in the pseudostratified columnar epithelium of vesicular glands as shown in Figure 2C SP-A and Figure 2D SP-D. Ductus deferens is also lined by a stereociliated pseudostratified columnar epithelium. Stereocilia are actually nonmotile, long microvilli that serve to increase the absorptive and/or secretory surface of this epithelium. Lamina propria layer of ductus deferens (Figure 2E for SP-A and Figure 2F for SP-D) was also stained with basal cells by SP-A and SP-D monoclonal antibodies. Remnant seminal plasma material within the secretory glands was stained with SP-A and SP-D antibodies (Figure 2E SP-A and Figure

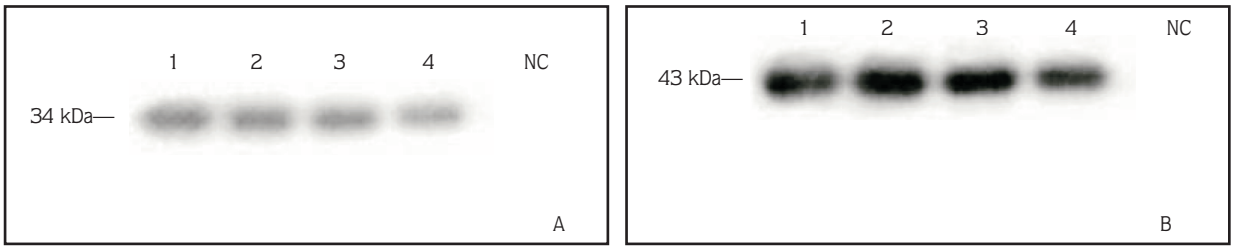

Figure 1. Western blot analysis for surfactant proteins A (Figure 1A) and D (Figure 1B) after one dimensional electrophoresis under reducing conditions in which stallion accessory sex gland tissue extract and seminal fluid proteins were compared with surfactant from stallion bronco-alveolar lavage fluid as a positive control. Samples from the stallion seminal plasma were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection was done with anti-equine SP-A and SP-D antibodies, an exposure time of 1 min, and enhanced chemiluminescence. The immunoblot in Figure 1A was produced by incubating the membrane with a monoclonal antibody against equine SP-A. The immunoblot in Figure 1B was produced by incubating the membrane with a monoclonal antibody against equine SP-D. In total 100-150 mg of proteins were loaded in each line for SP-A and SP-D detection, respectively. Legend: 1: lung, 2: seminal plasma, 3: prostate, 4: seminal vesicle and NC: negative control.

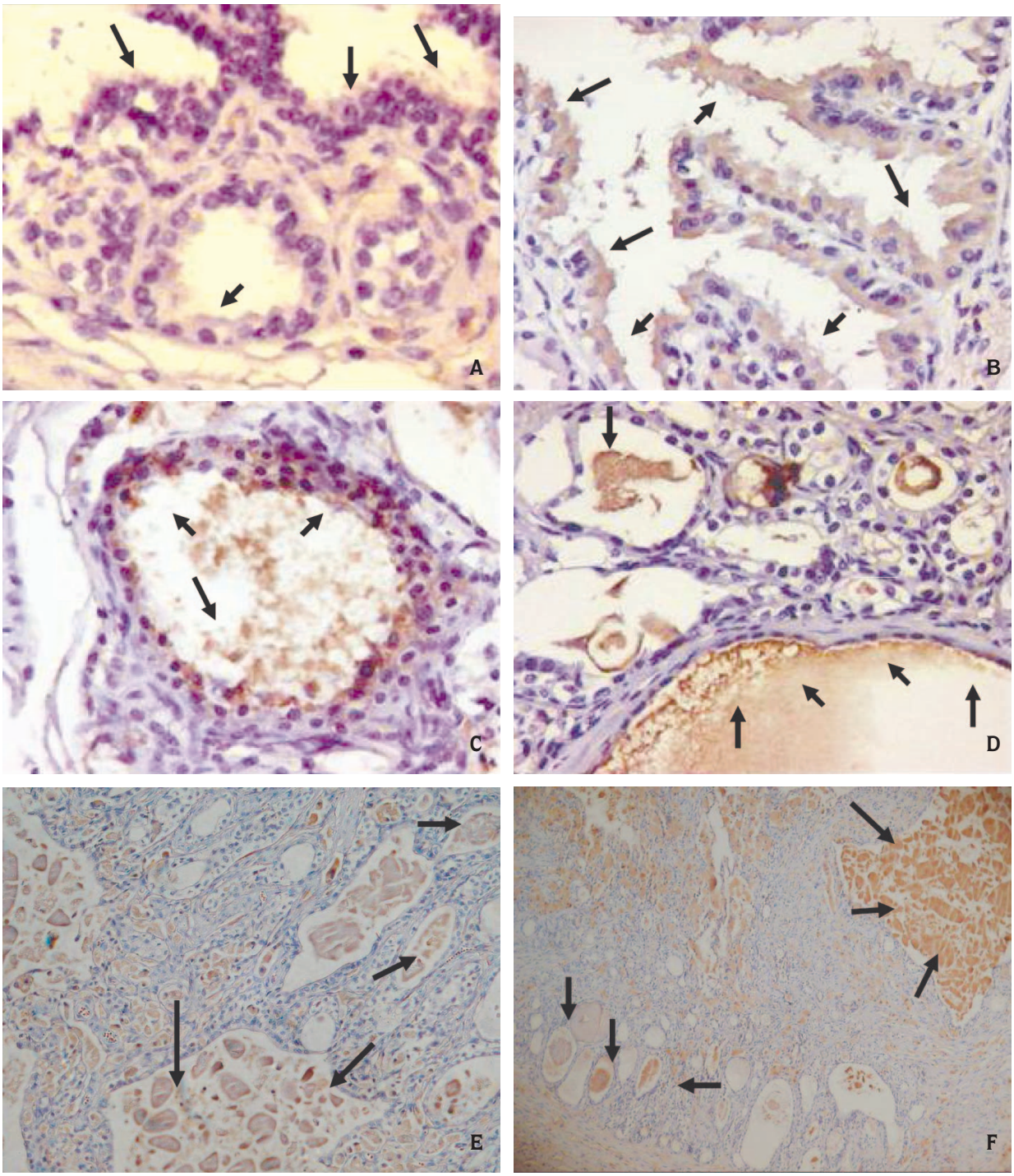

Figure 2. Paraffin sections (4 µm) from the prostate, seminal vesicle, and ductus deferens were incubated with horse SP-A and SP-D monoclonal antibodies against SP-A and SP-D. SP-A and SP-D immunostaining in stallion prostate was observed in the epithelium lining the prostate gland ((Figure 2A) SP-A and (Figure 2B) SP-D, respectively). The secretory end pieces of seminal vesicles are lined with simple columnar epithelium, where epithelium was positively stained with surfactant protein antibodies ((Figure 2C) SP-A and (Figure 2D) SP-D, respectively). Secretory material of seminal vesicles was also stained ((Figure 2C) SP-A and (Figure 2D) SP-D, respectively). The lamina propria layer of ductus deferens and secretory canal was also stained with basal cells by SP-D monoclonal antibody ((Figure 2E) SP-A and (Figure 2F) SP-D. Control sections preincubated with secondary antibodies were uniformly negative (results not shown). Original magnifications: (40×; A, B, C, D) (10× E, F).

2F SP-D). As a positive control for SP-A and SP-D, stallion lung tissue was used (figure not shown). For proof of SP-A and SP-D specificities and demonstration of negative control, the paraffin sections were incubated with normal sheep serum using the same concentration as the monoclonal surfactant protein antibodies in immunohistochemistry. Strong SP-A and SP-D immunoreactivity was observed in alveaolar type II cells and in non-ciliated bronchial cells (Clara cells) in stallion lung tissue (figure not shown).

Discussion

In this paper we describe the location and characterization of surfactant proteins, designated SP-A and SP-D, found in stallion seminal plasma and accessory sex glands (seminal vesicle, prostate, and ductus deferens) as shown by western blotting and immunohistochemistry with monoclonal horse SP-A and SP-D antibodies. Previously it was shown that the equine SP-A cDNA sequence of 747 bp (base pairs) was translated into 248 amino acids in horses after translation (17). In humans, there are 2 highly similar SP-A genes, SP-A1 (18) and SP-A2 (19). The partial amino acid sequence of equine surfactant protein was almost identical to that of humans (20). SP-A and SP-D participate in the innate response to inhaled microorganisms and organic antigens, and contribute to immune and inflammatory regulation within the lung (21,22). In the lung, the specific interactions of lung collectins with microorganisms result in opsonization, growth inhibition, and viral neutralization (23). Therefore, the presence of SP-A and SP-D in the stallion seminal plasma and accessory sex glands supports the hypothesis that surfactant proteins are primarily located in those regions exposed to extraneous stimuli such as pathogens, allergens, and other foreign materials eliciting the attention of immune cells and may be important molecules in a 3-fold innate defense. Khubchandani and Snyder (7) reported the presence of SP-A mRNA in human prostate by northern blot analysis using a human SP-A cDNA probe and ~35 kDa SP-A immunoreactive protein in human seminal plasma. speculated that the SP-A present in the prostate and prostatic secretions may play a role in host defense mechanisms. The data outlined here provide new evidence in stallion reproduction and reproductive immunity that surfactant proteins in these tissues are of possible clinical significance for diagnosis of various reproductive tract disorders. One of the roles of SP-A and SP-D in seminal plasma may be protecting the stallion reproductive tract from these causative pathogens. Additionally, seminal plasma has been shown to modulate immune responses in the uterine environment in pigs and horses (24,25). Seminal plasma components have been reported to elicit inflammatory response in the female genital tract (26). We have been studying SP-A and SP-D and recent studies have shown that seminal plasma proteins participate in the formation and rearrangement of the protein coating of the sperm surface, which changes its composition in different steps of the fertilization process (27). There is increasing appreciation of the key role played by SP-A and SP-D, through specific carbohydrate binding to microorganisms that infiltrate the seminal plasma and its secreting accessory sex glands, the prostate, vesicula seminalis, and ductus deferens, as well as the lung. In this manner, they might serve as a first line defense against the various potential pathogenic organisms that infiltrate the stallion accessory sex glands. It is possible that SP-A and SP-D within seminal plasma function as in the lung.

We propose that, through their local barrier and defense functions, SP-A and SP-D in seminal plasma likely play an important role in immunity and host defense and more detailed work is needed to clarify the roles of SP-A and SP-D in stallion seminal plasma. The presence of SP-A and SP-D in the seminal plasma and accessory sex glands' mucosa and secretory epithelia indicate that they may be the integral component of the stallion genital tract and adaptive immunity of the lower genital tract. In conclusion, future studies should focus on the roles of seminal plasma SP-A and SP-D in fertilization, e.g. capacitation, gamete interactions, and innate host defense function. Furthermore, analysis of the expression of surfactant proteins by seminal plasma in pathologic situations will be extremely interesting and may have potential for clinical application.

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