

# Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri

A S Alghamdi<sup>1</sup>, D N Foster<sup>2</sup> and M H T Troedsson<sup>3</sup>

Departments of <sup>1</sup>Clinical and Population Sciences and <sup>2</sup>Animal Science, University of Minnesota, St Paul, 495 ASVM Building, 1988 Fitch Avenue, Minnesota 55108, USA and <sup>3</sup>Department of Large Animal Clinical Sciences, University of Florida, Gainesville, Florida 32618, USA

Correspondence should be addressed to A S Alghamdi; Email: algh0007@umn.edu

## Abstract

Seminal plasma (SP) is known to have immunosuppressive properties in several species. Equine SP has been reported to reduce or inhibit chemotaxis, phagocytosis and complement activity *in vitro*. The type and amount of the SP component that suppresses sperm– polymorphonuclear neutrophil (PMN) binding *in vitro* was determined, and the effect of such suppression on the fertility of mares inseminated in the presence of uterine inflammation, was analyzed. Sperm cells were suspended in either SP, semen extender or a mixture of both, and each was mixed with PMN-rich uterine secretions collected at 12 h after artificial insemination (AI). SP reduced binding between spermatozoa and PMNs significantly ( $P < 0.05$ ). Fertile spermatozoa were suspended in SP or semen extender and used to inseminate mares 12 h after the induction of uterine inflammation. The pregnancy rate was normal (77%) when spermatozoa were suspended in SP, but was dramatically reduced to only 5% when spermatozoa were suspended in extender. The proteins from SP, blood plasma (BP) and a skim-milk-based semen extender (skim milk extender, SME) were precipitated by ammonium sulfate, resuspended in PBS and dialyzed. The effect of the precipitated proteins on sperm–PMN binding was compared with fresh, untreated SP. Both fresh SP, and isolated SP proteins reduced sperm–PMN binding ( $P < 0.001$ ). Conversely, proteins isolated from either BP or SME did not reduce sperm–PMN binding. The different concentrations of SP proteins used showed a dose-dependent suppression of sperm–PMN binding. Concentrations of 1 mg/ml SP protein significantly reduced sperm–PMN binding and 6 mg/ml reduced the binding to a level similar to that observed with fresh whole SP ( $P < 0.001$ ). Finally, SP protein digested with proteinase K resulted in the complete loss of SP suppressive activity confirming that the effective component is a proteinaceous substance.

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## Introduction

Breeding-induced endometritis is a transient physiological inflammation that serves to clear excess, dead spermatozoa and other contaminants from the uterus after breeding (Troedsson 1999). The inflammation is caused by spermatozoa, is characterized by a rapid influx of polymorphonuclear neutrophils (PMNs) into the uterine lumen within 1 h after artificial insemination (AI), and lasts for up to 36 h in normal mares (Kotilainen *et al.* 1994, Katila 1995, Troedsson 1999, Troedsson *et al.* 2000, 2001). Since natural breeding and insemination with fresh semen is usually performed every other day, uterine inflammation from a previous insemination may not affect the fertility of spermatozoa from repeated inseminations. However, breeding with preserved semen (frozen and cooled semen) is

commonly repeated at intervals of 6–24 h, with the subsequent inseminations occurring during a time when the uterus contains a large number of PMNs (Kloppe *et al.* 1988, McKinnon & Voss 1993, Katila *et al.* 1996, McKinnon 1996, Shore *et al.* 1998, Troedsson *et al.* 1998, Woods *et al.* 1990, Alghamdi *et al.* 2001). The binding of equine sperm to PMNs with subsequent phagocytosis of spermatozoa has been documented (Troedsson *et al.* 2000, 2001, Alghamdi *et al.* 2001). Incubation of equine spermatozoa in uterine secretions collected at 6, 12 or 24 h after breeding resulted in extensive sperm–PMN binding and reduced sperm motion characteristics (Alghamdi *et al.* 2001). Spermatozoa bind exclusively by the head portion to PMNs, resulting in large cell aggregates, which may interfere with sperm transport to the

oviduct. However, the effect of sperm–PMN binding on fertility of the mare has not been determined.

Seminal plasma has been associated with immunosuppressive activities in many species including the horse (Anderson & Tarter 1982, Schope *et al.* 1984, Bouvet *et al.* 1987, Troedsson *et al.* 1995a,b, 2000, Imade *et al.* 1997, Rozeboom *et al.* 1999, 2000, 2001). We have previously demonstrated that SP significantly reduced the proportion of spermatozoa phagocytosed by PMNs, although the mechanisms were not determined. In order for spermatozoa to undergo phagocytosis by PMNs, sperm cells must first bind to the surface of PMNs, which can be mediated by the opsonization of spermatozoa, or by a specific receptor–ligand binding mechanism. We hypothesize that SP suppresses phagocytosis of spermatozoa by inhibiting sperm–PMN binding. Here we report that SP protein suppresses sperm–PMN binding *in vitro* in a dose-dependent manner, and that SP increases the fertility rate of mares inseminated with fertile spermatozoa 12 h after the induction of uterine inflammation by inoculation of killed spermatozoa.

## Materials and Methods

### Mares and management

Normal mares (between 5 and 14 years of age) of mixed breeds were used. All mares had histories of normal reproductive performance, negative uterine culture and cytology, and endometrial biopsies with no or minimal histopathological changes on endometrial biopsies (grade I or IIa according to the grading system of Kenney & Doig 1986). All mares were challenged by inoculation with 30 ml AI dose containing  $1 \times 10^9$  sperm cells with at least 50% progressively motile spermatozoa. Only mares that cleared inflammatory products from their uteri within 48 h of insemination were used. Estrus was determined by clinical observation upon exposure to a stallion, and by transrectal palpation and ultrasonography of the reproductive tract. Once a mare was receptive to the stallion in the presence of at least 35 mm follicle and uterine edema, an endometrial cytology swab was obtained. Only mares with negative cytology results were utilized. Mares were palpated and ultrasonographically scanned daily in estrus and every other day in diestrus.

### Semen collection and isolation of fresh spermatozoa

Semen was collected from stallions of known fertility by the use of a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) equipped with an in-line gel filter (Animal Reproduction Systems, Chino, CA, USA). Semen volume was recorded, and sperm concentration and the percentage of progressively motile spermatozoa were determined. Semen was extended 1:3 with a skim-milk-based extender (SME; Kenney's extender, Har-Vet Inc., Spring Valley, WI, USA) and SP was removed by centrifugation at 400 g for 10 min. The spermatozoa pellet was

washed in semen extender in order to remove all SP, and the sperm cells were resuspended in the corresponding media depending on the experiment. All sperm samples were adjusted to a concentration of  $50 \times 10^6$  sperm cells/ml, and kept at room temperature (23–25 °C). Only sperm cells with progressive motility of  $\geq 50\%$  were utilized.

### Preparation of SP

SP was collected by centrifugation of fresh semen at 400 g for 10 min to remove the bulk of sperm cells. An additional centrifugation at 3000 g for 20 min was performed to eliminate all sperm cells. Equal amounts of SP from each of three stallions were pooled and the antibiotics used in the semen extender were added to the SP pool (amikacin sulfate (500 µg/ml), potassium penicillin G (1500 IU/ml); Brinsko & Varner 1992). A culture swab was taken 20 min after the addition of the antibiotics and SP was then divided into 30 ml aliquots and frozen at –20 °C until used. Only bacteriologically negative SP preparations were used.

### Preparation of killed spermatozoa

Semen was collected from two stallions of normal fertility, evaluated and extended 1:3 with semen extender as described above. Spermatozoa were removed from SP by centrifugation at 400 g for 10 min. An equal number of sperm cells were pooled and the sperm pellet was washed twice with PBS (pH 7.2). Sperm cells were layered over a Percoll gradient (Sigma), and pelleted by centrifugation at 800 g for 30 min. Sperm cells were recovered from the bottom layer, resuspended in PBS and washed twice. All materials and solutions were sterilized and sperm processing was performed under sterile conditions. A bacteriological culture sample was obtained from the pooled spermatozoa and the remainder were divided into 2 ml aliquots each containing  $1 \times 10^9$  spermatozoa. Spermatozoa were killed by snap freezing in liquid nitrogen and were stored frozen at –20 °C until used. Only sperm preparations with negative bacteriological cultures were used in the experiment.

### SP protein preparation

Proteins were precipitated from the pooled SP with ammonium sulfate (33% w/v), collected by centrifugation (2000 g/15 min) and resuspended in a volume of PBS (pH 7.2) equal to the amount of SP used for precipitation so that the protein concentration was similar to that of the original SP. Both the proteinaceous and non-proteinaceous portions of SP were dialyzed against two changes of PBS overnight at 4 °C in a dialysis cassette with 3500 Molecular weight cut off (Slide-A-Lyzer, Pierce, Rockford, IL, USA). Blood plasma and the SME were processed similarly for use as control proteins of non-SP origin. Another SP-protein batch was similarly prepared with the exception that the

protein was resuspended in PBS at a concentration of 40 mg/ml for use in the quantification experiment.

### Preparation of PMNs

At the time of semen collection, blood was collected from a healthy mare and PMNs were isolated as previously described (Troedsson *et al.* 1993). Briefly, heparinized blood was subjected to centrifugation, the buffy coat was layered over a lymphocyte separation medium (Litton Bionetics, Kensington, MD, USA) and the PMNs were recovered from the buffy coat. The PMNs were washed and re-suspended in PBS at a concentration of  $14 \times 10^6$ /ml.

### Evaluation of sperm–PMN binding

Wet mounts of sperm binding to PMNs were evaluated by light microscopy and expressed as the proportion of PMNs that bound to at least one spermatozoon. A drop of the sperm–PMN mix was placed on a glass slide, covered with a cover slip and the number of sperm-bound PMNs was determined using  $\times 400$  power. A minimum of 200 PMNs were counted for each slide. Sperm binding to PMNs from uterine secretions was studied at 1 h intervals for a total of 4 h at room temperature. However, the effect of SP proteins on sperm binding to blood-derived PMNs was determined after spermatozoa were first incubated with SP protein (37°C for 30 min) followed by another incubation with PMNs, under the same conditions.

### Uterine secretions

Five mares were inseminated with  $1 \times 10^9$  spermatozoa with at least 50% progressive motility and uterine secretion samples were collected 12 h later using cotton tampons as previously described (Troedsson *et al.* 1993, Alghamdi *et al.* 2001). The tampon was recovered by the use of a vaginal speculum and placed in a small, sterilized plastic bag to prevent evaporation. Uterine secretions were retrieved from the tampon by the use of an Arbor press within 10 min of collection and the volume was recorded. All samples were transferred to the laboratory for use immediately after collection. Uterine secretions were then mixed with fresh sperm preparations at a 1:2 ratio and sperm–PMN binding was evaluated at 0, 60, 120, 180 and 240 min at room temperature.

### Induction of uterine inflammation, and management and insemination for fertility trials

Eleven mares were used and each mare was utilized in four consecutive estrus cycles in a  $2 \times 2$  factorial experiment. The factors were: (1) challenge (induction of uterine inflammation by either killed spermatozoa suspended in semen extender or semen extender alone); (2) fertile insemination (FI; with fertile spermatozoa suspended in SP or in semen extender). Before the start of the experiment, mares were assigned randomly to these treatments. Once a mare was detected to be in estrus and had a negative

cytology, uterine inflammation was induced with either killed spermatozoa suspended in 30 ml semen extender or 30 ml semen extender alone depending on the assigned treatment. Mares were given 3000 U human chorionic gonadotropin (hCG; InterVet International B.V., Boxmeer, Holland) at the time of killed spermatozoa inoculation in order to shorten and standardize the interval from AI to ovulation. Twelve hours later, mares were inseminated with fertile spermatozoa suspended in either SP or semen extender depending on the assigned order. If a mare did not ovulate within 48 h after fertile AI, she was excluded from the experiment, treated with prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ; Estrumate, Miles, Inc., Shawnee Mission, KS, USA) 5 days after ovulation, and the treatment was repeated during the next estrus. Pregnancy was determined by transrectal ultrasonography at 11 and 15 days after ovulation. When diagnosed pregnant at day 15, an injection of 10 mg  $PGF_{2\alpha}$  was given to all mares to return them to estrus and prepare them for the next treatment.

### SP protein digestion

To determine if the immunosuppressive factor in SP was a proteinaceous compound, 1 ml aliquots of precipitated SP proteins (12 mg/ml) were incubated at 56°C for 3 h with proteinase K (PK; 100 µg/ml; Promega). To control for any effect of PK on the binding assay or motility, a PK inhibitor, phenylmethanesulfonyl fluoride (PMSF; 40 µg/ml; Roche), was included either at the time of PK addition or after the digestion was completed. Serial concentrations of PK and PMSF, and the incubation conditions, were determined in preliminary experiments. The possible effect of PMSF on motility and binding assay was controlled by treating SP protein with PMSF alone. Untreated SP protein controls were either heated similarly to the other treatments or used without heating. Sperm cells were incubated in these preparations for 30 min at 37°C followed by the addition of PMNs as described above and the binding was compared with sperm cells incubated in SME alone. Digestion of SP protein was confirmed by SDS-PAGE.

### Statistical analysis

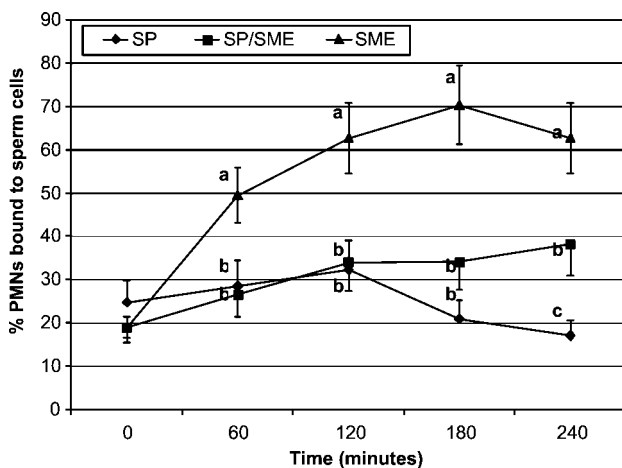
Data for sperm–PMN binding in uterine secretion were analyzed using a general linear model (GLM) for repeated measures with mares, ejaculates and concentrations of SP included in the model as independent variables. Following GLM, the means were further analyzed by the least-square mean procedure. Fertility data were analyzed by the logistic regression procedure. The challenge and the FI were included in the model as independent variables. The fit of the model was examined by the deviance and its *P* value and was further checked with the Wilk–Shapiro/Rankit plot. The 95% confidence interval for the odds ratio was requested after determination of the logistic regression to determine how likely it is that each factor influences the pregnancy outcome. Sperm- and blood-derived

PMN binding data were analyzed using general ANOVA, and stallions and ejaculates were included in the model as independent variables. Following ANOVA analysis, the means were compared using the Bonferroni procedure. For all data analysis, statistical significance was set at  $P \leq 0.05$ .

## Results

The average volume of uterine secretions collected from each mare was  $10.9 \pm 2.1$  ml and the average number of PMNs was  $16.2 \pm 5.4 \times 10^6$ /ml. Sperm-PMN binding was initially similar between samples (0 h), but increased with incubation time in the samples without SP. In contrast, prolonged incubation time did not result in increased binding in the presence of SP (Fig. 1). The presence of SP significantly reduced the binding of spermatozoa to PMNs ( $P < 0.05$ ) compared with sperm cells suspended in extender alone (Fig. 1). No significant difference in sperm-PMN binding was detected between sperm samples suspended in 100% SP and those suspended in a 1:1 mixture of SP and SME.

Ovulation occurred within 48 h of fertile insemination in 42 of the 44 cycles. Two mares (one cycle each) did not ovulate until after 50 h. These mares were given PGF<sub>2</sub> $\alpha$  and were reused during the subsequent estrus. There was no significant difference in pregnancy rates in mares inoculated with dead spermatozoa (9/22) or semen extender alone (9/22). Therefore, these groups were combined for further statistical analysis to determine the effect of SP on pregnancy. The pregnancy rate at 15 days after ovulation was significantly ( $P < 0.05$ ) higher in mares inseminated with fertile sperm cells suspended in SP (17/22) compared with mares inseminated with fertile sperm cells suspended in semen extender (1/22; Fig. 2).

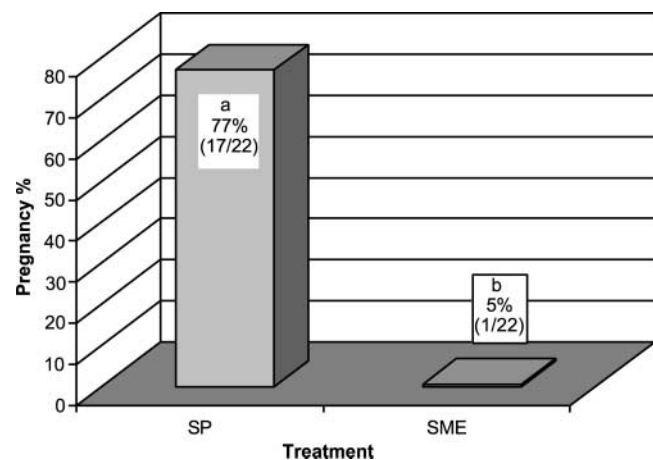


**Figure 1** Binding scores of sperm cells incubated for 4 h at room temperature in uterine secretions collected 12 h after AI. Sperm cells (pooled from two stallions) were suspended in SP alone, in a 1:1 mixture of SP and SME (SP/SME), or in SME alone and binding was determined every 60 min for 4 h. Different superscripts within each time point indicate a significant difference,  $P < 0.05$ .

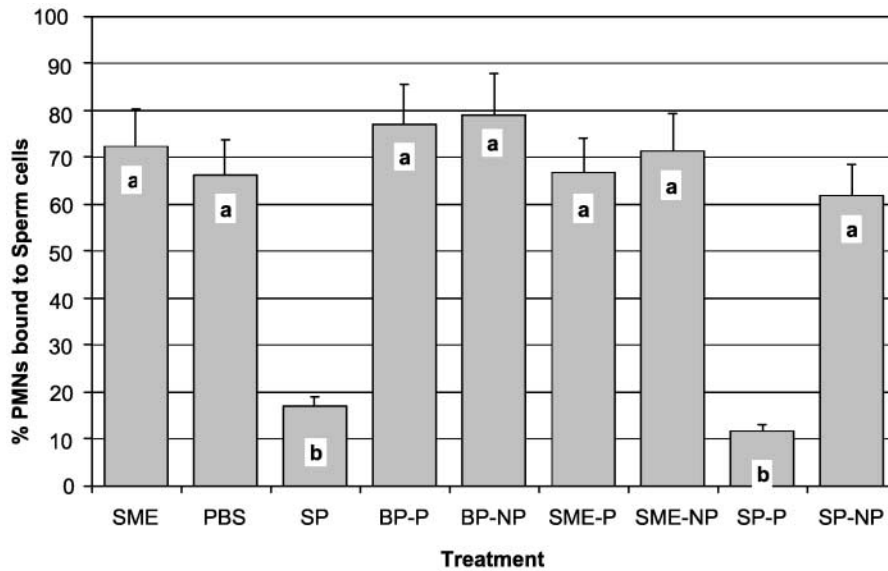
Binding between spermatozoa and PMNs was significantly reduced by the addition of fresh SP and SP protein compared with all other treatments ( $P < 0.001$ ). Sperm-PMN binding was similar when sperm samples were suspended in fresh SP compared with sperm samples suspended in seminal plasma protein ( $P = 0.3$ ). No difference was found between any of the other treatments (Fig. 3), and proteins from blood plasma (BP) and SME contained no suppressive activity for sperm-PMN binding, as was the case with SP protein.

The protein concentration of fresh SP collected from three stallions (three ejaculates each) ranged from 15 to 38 mg/ml. There was a dose-dependent response of the increasing concentrations of SP protein on sperm binding to PMNs. Compared with semen extender, concentrations as low as 1 mg/ml SP protein significantly ( $P < 0.001$ ) reduced sperm-PMN binding. When 6 mg/ml or more SP protein was used, sperm-PMN binding was similar to fresh SP samples (Fig. 4).

As shown in Fig. 5, most of the SP proteins were precipitated by ammonium sulfate (33% w/v). Treatment of precipitated SP protein with PK resulted in essentially complete protein digestion as shown by SDS-PAGE. Compared with unprecipitated SP, the precipitated SP protein appeared to exclude a few protein bands of approximately 83 kDa or greater. Incubation of PK in the presence of PMSF inhibited PK action and preserved the SP proteins. Neither PK nor PMSF altered the binding assay or sperm motility under the conditions of this study. Digestion of the SP protein removed the suppressive activities of SP and resulted in sperm-PMN binding similar to sperm incubated in semen extender alone. However, non-digested SP proteins, including samples treated with PMSF at the time of adding PK, all prevented sperm-PMN binding in a similar way to untreated SP protein (Fig. 6).



**Figure 2** Pregnancy rates of mares 15 days after ovulation. Uterine inflammation was induced by inoculation with killed spermatozoa, and 12 h later the mares were inseminated with fertile sperm cells suspended in SP or in SME. Sperm cells for all fertile inseminations were from one stallion with normal fertility. Different superscripts indicate a significant difference,  $P < 0.05$ .

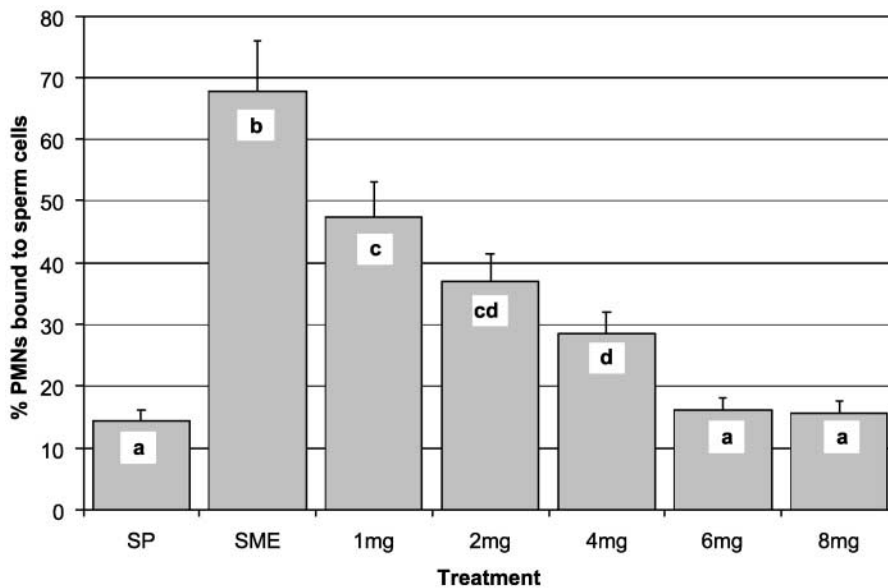


**Figure 3** Percentage of PMNs bound to at least one sperm cell after spermatozoa were incubated at 37°C for 30 min in SME, PBS, SP, BP protein (BP-P), BP non-protein (BP-NP), SME protein (SME-P), SME non-protein (SME-NP), SP protein (SP-P) or SP non-protein (SP-NP), followed by another incubation using similar conditions with a PMN suspension. Spermatozoa were obtained from three ejaculates from each of three stallions. Different superscripts indicate a significant difference,  $P < 0.001$ .

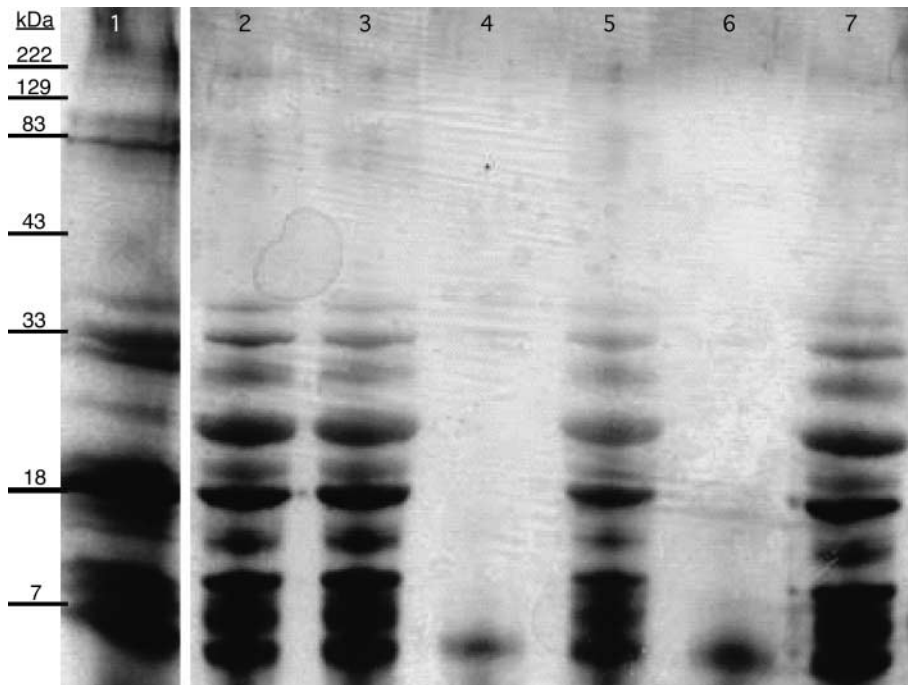
## Discussion

The results from this study provide additional evidence and information about the immunosuppressive/regulatory properties of SP as reported in many species including the horse (Anderson & Tarter 1982, Schope *et al.* 1984, Bouvet *et al.* 1987, Troedsson *et al.* 1995*a,b*, 2000, Imade *et al.* 1997, Rozeboom *et al.* 2001). Seminal plasma appears to protect spermatozoa from being phagocytosed by PMNs *in vitro* (Troedsson *et al.* 2000). Our data suggest that this

protection is at least, in part, mediated through the reduction of sperm–PMN binding. The protective function of SP may be specific to certain subpopulation(s) of spermatozoa, and/or only occur during a temporary window of time, allowing sperm cells to reach the oviduct in order to fertilize an ovum. Our *in vitro* data suggest that this window may last for at least 4 h after insemination (Fig. 1). It is thought that abnormal and apoptotic spermatozoa are more likely to be phagocytosed by leukocytes (Tomlinson *et al.* 1992, Ricci *et al.* 2002); however, our fertility trial



**Figure 4** Percentage of PMNs bound to at least one sperm cell after spermatozoa were incubated at 37°C for 30 min with SP, SME, or in SP protein at 1, 2, 4, 6 or 8 mg followed by incubation using the same conditions with a PMN suspension. Spermatozoa were obtained from three ejaculates from each of three stallions. Different superscripts indicate a significant difference,  $P < 0.001$ .

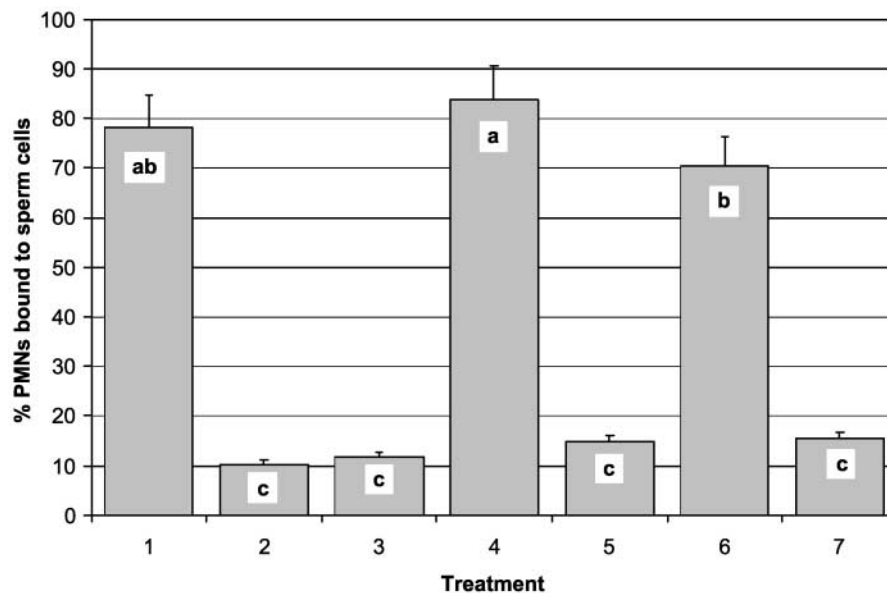


**Figure 5** SDS-PAGE of equine SP proteins (12 µg/lane) before and after precipitation and digestion with PK. Lane 1, whole SP; lane 2, SP protein not treated with PK or heating; lane 3, SP protein not treated with PK but heated similarly to PK-treated protein (56 °C for 3 h); lane 4, PK-treated SP protein without PMSF; lane 5, SP protein treated with PK and PMSF together; lane 6, SP protein digested with PK for 3 h before addition of PMSF; lane 7, SP protein treated with PMSF alone. Relative molecular weight standard of proteins (in kDa) are marked to the left of the figure. Note the few protein bands of whole SP (lane 1) of approximately 83 kDa that were absent from the precipitated SP protein.

showed that even viable spermatozoa capable of fertilization were eliminated in the absence of SP. It is not known whether SP works equally at preventing dead, defective and normal fertile spermatozoa from binding to PMN and being subsequently phagocytosed, or if the protection of SP is specific to viable and fertile spermatozoa.

The reduced sperm motion characteristics of spermatozoa incubated in uterine secretions containing PMNs may

suggest that fewer or no sperm cells will reach the oviduct in the presence of PMNs. However, some sperm cells seemed to be resistant to PMN binding, suggesting heterologous sperm populations with two or more subpopulations (Alghamdi *et al.* 2001). Insemination with fertile spermatozoa 12 h after inducing uterine inflammation, and in the absence of SP, resulted in only one pregnancy (5%). The suppressive properties of SP on sperm–PMN



**Figure 6** The effect of SP protein digestion on sperm–PMN binding. Percentage of PMNs bound to at least one sperm cell after spermatozoa were incubated at 37 °C for 30 min with: 1, skim milk extender; 2, SP protein not heated or treated with PK; 3, SP protein heated (56 °C for 3 h) but without PK; 4, SP protein incubated with PK alone; 5, SP protein incubated with both PK and its inhibitor (PMSF); 6, SP protein digested with PK for 3 h before addition of PMSF; and 7, SP protein treated with PMSF alone. Spermatozoa were obtained from two ejaculates from each of two stallions. Different superscripts indicate significant differences,  $P < 0.001$ .

binding effectively protected more spermatozoa from binding to PMNs, and being subsequently phagocytosed, before reaching the oviduct, since pregnancy rates were higher in this group (77%). In rabbits, double mating resulted in pregnancies from the second mating despite the need for the second deposit of spermatozoa to traverse the inflamed cervix and uterus (Taylor 1982). When the second mating of rabbits was performed within 1 h of the first, the fertility of the buck determined the pregnancy rates. However, when 4 h elapsed, pregnancy rates were always lower from the second mating regardless of the buck's fertility (Taylor 1982).

The results from the breeding trial are supported by a similar study in sows, in which SP significantly improved conception and farrowing rates in sows inseminated with fertile spermatozoa in the presence of an ongoing uterine inflammation (Rozeboom *et al.* 2000). The higher pregnancy rate in mares inseminated with fertile spermatozoa suspended in SP may also explain the high pregnancy rates that have been observed in wild horses, despite their mating behavior with frequent breedings during estrus (Bristol 1987). Since man-controlled natural breeding and AI with fresh semen is commonly repeated at intervals of 48 h, uterine clearance in normal mares would have been completed by the time of the second breeding. However, the shorter life span of preserved spermatozoa, and the difficult task of accurately predicting the time of ovulation, often forces practitioners and breeders to inseminate mares repeatedly at intervals that can be as short as 6 or 12 h until ovulation. Although this breeding management assures the presence of newly inseminated spermatozoa in the uterus, it does not guarantee that spermatozoa will survive the inflammatory uterine environment and reach the oviduct at the time of ovulation. The normal pregnancy rate of mares inseminated with fertile spermatozoa suspended in SP despite the presence of uterine inflammation strongly suggests an important role of SP in fertility. This is especially relevant in situations of repeated breedings and may be important in mares suffering from delayed uterine clearance where retained PMNs may interfere with sperm transport.

Our results show that the immunosuppressive component of SP is a proteinaceous substance, which could be a peptide(s), a protein(s), a lipoprotein(s), a glycoprotein(s) or a proteoglycan(s). Ammonium sulfate precipitation, dialysis and freezing did not inactivate the effective substance in the SP. In addition, heating for 3 h at 56 °C still did not inactivate the effective component in SP, as was expected based on the previous finding that this immunosuppressive component was not inactivated unless heated at 95 °C for 45 min (Dahmes & Troedsson 2002). A concentration of 6 mg/ml was sufficient to reduce sperm-PMN binding to levels similar to that achieved with whole SP. The dose-dependent reduction of sperm-PMN binding in relation to the amount of SP protein is in agreement with the dose-dependent reduction of phagocytosis and chemotaxis by the addition of SP in the mare, and chemotaxis in

the sow (Troedsson *et al.* 2000, Rozeboom *et al.* 2001). The finding that proteins precipitated from blood plasma and semen extender did not reduce sperm-PMN binding demonstrates that this suppression of binding was specific to SP. Whether there are one or more SP molecule(s) responsible for suppression of sperm-PMN binding is not known, and should be determined.

Ammonium sulfate precipitation (33% w/v) was effective in collecting the immunosuppressive component but, as seen in Fig. 5, some proteins of approximately 83 kDa or greater were not precipitated. This is consistent with a series of preliminary experiments where ammonium sulfate at between 5 and 25% essentially precipitated no SP proteins, while the majority of proteins were precipitated by concentrations of 30–35% (w/v). Once the majority of SP proteins were precipitated using 33% ammonium sulfate, increasing the concentration to 50% resulted in a negligible increase in precipitated proteins. PK digested SP protein completely and destroyed the immunosuppressive action of SP protein. This is a strong indication that the effective molecule is a proteinaceous component(s). In addition, this immunosuppressant SP protein(s) appears to be resistant to heating for up to 3 h at 56 °C.

In conclusion, a proteinaceous substance(s) from equine SP reduces sperm-PMN binding *in vitro*, and SP improves fertility of fresh spermatozoa inseminated into inflamed uteri. This substance(s) can be precipitated from SP, heated for up to 3 h at 56 °C and stored frozen without losing its biological properties. A total of 6 mg/ml crude precipitated SP protein prevented sperm-PMN binding to a level similar to that achieved with fresh whole SP.

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