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Seminal DNase Frees Spermatozoa Entangled in Neutrophil Extracellular Traps

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ABSTRACT

Insemination always stimulates neutrophil migration into the female reproductive tract (FRT), which eliminates excess spermatozoa and bacterial contaminants introduced by the breeding process. However, the presence of neutrophils in the FRT at the time of semen deposition has been shown to result in sperm-neutrophil binding that reduces motility and fertility. Although the binding and trapping mechanism has not been determined, seminal plasma (SP) was found to include a protein factor or factors that reduced sperm-neutrophil binding and improved fertility of sperm inseminated in the presence of neutrophils. Although DNase has been shown to be present in the SP of different species and has been associated with improved fertility in bulls, the mechanism(s) explaining this association and the paradox of DNA-packed cells being associated with DNase have remained unresolved. We demonstrate that sperm-activated neutrophils extrude their DNA, which in turn traps sperm cells and hinders their motility (and ultimately may hinder sperm transport to the fertilization site). DNase activity present in the SP digests the extruded DNA and frees entangled spermatozoa, which in turn may allow more spermatozoa to reach the oviduct, and explains at least one mechanism by which SP increases the rate of fertility. The ability of SP proteins to suppress neutrophil activation in the presence of spermatozoa did not render neutrophils incapable of combating bacteria, demonstrating that SP proteins are highly selective for suppressing neutrophils activated by spermatozoa, but not by bacteria.

immunology, seminal vesicles, sperm, sperm motility and transport

INTRODUCTION

Neutrophils are recruited into the female reproductive tract (FRT) following insemination in a response resembling classical inflammation, a feature that plays a major role in eliminating excess spermatozoa and microbial contaminants introduced during the breeding process [1–5]. However, neutrophils present in the FRT at the time of insemination have been shown to bind to spermatozoa, forming extensive clusters [6–8] that reduce sperm motility [6] and fertility [9–11], and often remain untreated because of their asymptomatic nature [11–13]. The ability of spermatozoa to stimulate an immune response is moderated by the immunosuppressive activities of seminal plasma (SP) [14–16], which has been demonstrated to suppress neutrophil activation and chemotaxis as well as the binding to and phagocytosis of spermatozoa [9, 10, 17, 18]. In addition, SP has been shown to improve the fertility of equine and swine spermatozoa inseminated in the presence of neutrophils, although the mechanism is still unclear [9, 10]. Human reproductive tract leukocytosis and repeated inseminations in animals are naturally occurring situations whereby spermatozoa are deposited in the presence of neutrophils, resulting in reduced fertility. This is especially important in artificial insemination (AI), because semen processing often results in the dilution or removal of the SP.

We found that SP contains a protein factor or factors that reduce neutrophil binding to spermatozoa in vitro in a dose-dependent fashion [9], and this may result in increased spermatozoa reaching the oviduct, thus providing a possible explanation for improved fertility [9, 10]. Recently, Brinkmann et al. [19] demonstrated a novel mechanism of antimicrobial action whereby activated neutrophils extrude their nuclear DNA and associated proteins (e.g., histones) to form neutrophil extracellular traps (NETs) that ensnare and kill microbes. They showed that bacteria activate neutrophils, resulting in formation of NETs, and that the release of DNA from activated neutrophils is time- and dose-dependent. Furthermore, they showed that formation of NETs is observed from motile neutrophils, is faster than the apoptosis time-course, is not accompanied by cytoplasmic markers, and that these neutrophils exclude vital dyes. Although they show that NETs are not the result of leakage during cellular disintegration, they did not exclude the possibility that formation of NETs is an early step in the program for neutrophil cell death. Whether this mechanism is also operative in situations where neutrophils encounter spermatozoa has not been investigated. A fertility-associated antigen in bovine SP was found to share sequence similarity with DNase I [20], and to show a positive correlation with fertility [21], but its mode of action has remained unclear. Because formation of NETs is DNA-based [19] and because DNase has been found in the SP of several species [20, 22–27], we hypothesized that seminal DNase may play a role in reduced sperm-neutrophil binding and cluster formation. Furthermore, because histones and granule proteins were found to possess bactericidal activities [19] and because neutrophil recruitment into the FRT serves to combat bacterial infection, the physiological immunosuppressive properties of SP have been questioned [8].

Identification of the mechanism of SP immunosuppressive properties will improve our understanding of reproductive physiology, and will further our understanding of reproductive immunology in terms of the FRT's ability to tolerate male antigens. Here we show that neutrophil activation by either bacteria or spermatozoa results in formation of NETs, and that seminal DNase digests these NETs to free entrapped spermatozoa but does not inhibit the bactericidal activities of neutrophils. Our findings also provide a functional role for the presence of seminal DNase and help explain the seemingly paradoxical role of seminal DNase being associated with DNA-packed spermatozoa.

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MATERIALS AND METHODS

All sample collections were carried out in accordance to the Institutional Animal Care and Use Committee, Research Subjects' Protection Program at the University of Minnesota.

Spermatozoa

Fresh semen was collected from a stallion of normal fertility by the use of a Missouri model artificial vagina, and the semen was extended 1:3 with skim milk-based semen extender (Kenney's extender; Har-Vet). Seminal plasma was removed by centrifugation at 400 × *g* for 10 min and the sperm pellet was resuspended in Dulbecco PBS (pH 7.2; Invitrogen Corp.) to a concentration of 100×10^6 sperm/ml. This method allowed reduction of SP to less than 0.05% with the stallion used (average semen volume 25 ml, and average sperm concentration 300×10^6 sperm/ml).

Neutrophils

Neutrophils were isolated from whole blood collected from a healthy mare and isolated as previously described [28]. Neutrophils were washed and resuspended in PBS at 14×10^6 cell/ml, and kept on ice until used.

Seminal Plasma Protein

Fresh semen was collected from three stallions of normal fertility and the bulk of spermatozoa were removed by centrifugation at 400 × g for 10 min. To ensure the removal of all spermatozoa, SP was subjected to centrifugation at 3000 × *g* for 20 min, and the SP was then buffered and treated with phenylmethylsulphonylfluoride (PMSF). Proteins were precipitated by 33% (w/v) ammonium sulfate, collected by centrifugation at 2000 × *g* for 15 min, resuspended in PBS, and dialyzed (3500 MWCO; Slide-A-Lyzer; Pierce) against two changes of PBS at 4°C overnight. The protein was divided into aliquots and frozen at −80°C until used.

Detection and Quantification of Endonuclease Activity in SP Protein

Endonuclease activity of crude SP protein (0.02, 0.07, 0.2, 0.6, 2, and 6 mg ml^{-1}) was compared to purified, RNase-free DNase I (0.3, 0.6, 1.2, 2.5, and 5 U ml^{-1}; Roche), and BSA (6 mg ml^{-1}) using 1 μg of purified plasmid DNA substrate (pIND, 5 kb) at room temperature (24°C) for 20 min. Inactivation of SP and purified DNase I (heating at 70°C for 10 min) was also performed before the addition of plasmid DNA. Treated DNA was then separated by electrophoresis on 2% agarose gels and stained with ethidium bromide, and digital photographs were analyzed semiquantitatively using densitometry. Values obtained with purified DNase I were used to plot a standard curve and endonuclease activities of SP protein were quantified. Results of densitometry were validated using spectrophotometry according to procedures previously described [25]. To determine whether seminal DNase activity varied among stallions, samples of SP protein (1 mg ml^{-1}) from 5 different stallions were compared to each other and to control blood plasma protein (1 mg ml^{-1}) using 1 μg of plasmid DNA. To determine whether seminal DNase activity was enhanced additively or synergistically by the addition of DNase I, SP protein alone (1 mg ml^{-1}) was compared to DNase I alone (5 U ml^{-1}) and in combination at the same concentrations.

Time Lapse Analysis

Time lapse analysis was performed using inverted dual fluorescent/phase contrast microscopy connected to a digital camera and a computer system (Axiovert200, Zeiss). Neutrophils (7×10^6 ml^{-1}) alone, or mixed with spermatozoa (25×10^6 ml^{-1}) or with *Escherichia coli* (25×10^6 ml^{-1}) were treated with Sytox Green (Molecular Probes) at a final concentration of 5μM, placed in a Petri dish, and covered with mineral oil, and time lapse analysis was performed for a total of 60 min. Sytox Green is impermeant to intact cell membranes with a high specificity for DNA, but its permeability to sperm cells has not been determined, although our data indicate that it is permeable to live equine spermatozoa. PBS supplemented with 1% BSA was used for the analysis, and the incubation was performed at room temperature (24°C). Additional experiments for comparing the effects of media (PBS vs. RPMI; Cellgro) and incubation temperature (24°C vs. 37°C) were performed. For the evaluation of neutrophil and sperm viability during NETs formation, a live-dead staining kit (Molecular Probes) was used according to the manufacturer's instructions; membrane-permeant SYBR 14 stains live cells green and membrane-impermeant propidium iodide (PI) stains dead cells red.

Quantification of DNA Release

Neutrophils (7×10^6 ml^{-1}) were incubated in 96-well plates with either *E. coli* (25×10^6 ml^{-1}; positive control) or equine spermatozoa (25×10^6 ml^{-1}) in the absence or presence of 6 mg ml^{-1} SP protein in duplicate. Each well contained Sytox Green at a final concentration of 5 μM. Neutrophils, *E. coli*, and spermatozoa alone were used separately as negative controls. Fluorescence was measured (excitation 485 nm, emission 516 nm) using a fluorescence microplate reader (FL600FA Fluorescence reader; Bio-Tek Instruments, Inc.) immediately after the cells were combined and at 10-min intervals up to 60 min. The fluorescence of spermatozoa and *E. coli* cells alone in the absence of neutrophils was subtracted from the total fluorescence of coincubated cells. PBS supplemented with 1% BSA was used for the analysis, and the incubation was performed at room temperature (24°C). Additional experiments for comparing the effects of media (PBS vs. RPMI; Cellgro) and incubation temperature (24°C vs. 37°C) were performed.

Binding Assay

Equine neutrophils (7×10^6 ml^{-1}) were incubated with spermatozoa (25×10^6 ml^{-1}) in either PBS, PBS plus SP protein (6 mg ml^{-1}), or PBS plus DNase I (30 U ml^{-1}; based on the quantification of SP endonuclease activity compared to DNase I). Binding was evaluated as the percentage of neutrophils that bound to at least one sperm cell as previously described [9]. Briefly, 20 μL of mixed cells were placed on a glass slide, covered with a cover slip, and examined by light microscopy (400× magnification) and sperm-bound and -unbound neutrophils were counted. A minimum of 200 neutrophils were counted from each slide, and the percentage of neutrophils bound to sperm was calculated. Blood plasma protein as well as milk protein were used as controls.

Bacterial Growth Conditions

Escherichia coli (strain DH5α) was grown to log phase at 37°C in Luria Bertani (LB) broth with shaking, and serial dilutions (10^3–10^6) were prepared in PBS. Aliquots were plated on LB agar plates and grown overnight at 37°C; the colony-forming units (CFUs) were counted and bacterial concentrations calculated.

Bactericidal Activity

Equine neutrophils (5×10^6 cells) suspended in PBS were incubated with *E. coli* (5×10^5 cells) in the presence or absence of SP protein (6 mg ml^{-1}) in a total volume of 200 μL at 37°C. A similar number of *E. coli* were incubated in PBS alone as a control. Neutrophils, SP protein, and media that included no *E. coli* were used as controls. After 5 and 10 h of incubation, aliquots were plated onto LB agar plates and incubated overnight at 37°C, and CFUs were counted and expressed as percentage of *E. coli* growth when incubated in PBS alone.

Scanning Electron Microscopy

Equine neutrophils (5×10^6 ml^{-1}) were incubated alone, and in the presence of either lipopolysaccharide (LPS; Sigma; 100 ng ml^{-1}) or spermatozoa (5×10^6 ml^{-1} each) for 20 min. The samples were carefully washed with PBS (pH 7.4) and fixed in 2% glutaraldehyde and osmium tetroxide. Cells were sedimented on poly-L-lysine-coated 12-mm coverslips and dehydrated in graded ethanol followed by absolute acetone. Samples were critical point-dried in a Tousimis Autosomdri 814 critical point drying apparatus (Tousimis Instrument). Preparations were mounted onto aluminum stubs and coated with 30 nm of gold/palladium using a Denton model DV-502 high vacuum evaporator (Denton Vacuum, Inc.). Preparations were viewed using a Hitachi S-3500N scanning electron microscope (Hitachi Instruments). Genomic DNA from calf thymus (Sigma) was processed similarly to elucidate the appearance of DNA when visualized by scanning electron microscopy (SEM).

Statistical Analysis

Data were analyzed using the Statistix program (Analytical Software). General ANOVA was used for analyzing binding, DNase quantification, and bactericidal data, and DNA release data were analyzed by ANOVA for repeated measures. Significance was set at $P < 0.05$. All data were collected from a minimum of 3 independent trials performed on different days.

RESULTS

To determine whether spermatozoa stimulate the formation of NETs, we monitored, over time, a mixture of isolated equine

spermatozoa and neutrophils with a DNA-specific stain (Sytox Green). The coincubation of sperm with neutrophils led to a time-dependent increase in formation of NETs, and both motile and nonmotile spermatozoa were trapped in the extruded DNA and were easily visible using fluorescence microscopy (Fig. 1, A–C, and Supplementary Video 1 [available online at http://www.biolreprod.org]). The formation of NETs and the subsequent entanglement of spermatozoa were confirmed by SEM (Fig. 1D) and the structure of DNA under SEM was confirmed by comparison to genomic DNA prepared and examined similarly (see inset in Fig. 1D). To confirm that the extruded DNA was neutrophil-derived, spermatozoa were incubated separately with Sytox Green for 2 h to stain their DNA, and unbound dye was removed by washing before neutrophil addition. The number of spermatozoa internalizing Sytox Green increased over time, but their DNA remained compacted and was not extruded. Time-lapse analysis showed that sperm DNA was not extruded after the addition of neutrophils, confirming that formation of NETs emanated only from neutrophils (Fig. 1E and Supplementary Video 5 [available online at http://www.biolreprod.org]). Using live-dead stains, neutrophils that extruded their DNA were found to be impermeant to PI and were stained green (SYBR 14), whereas those staining positive for PI did not extrude their DNA (Fig. 1F). To better characterize the permeability of neutrophils and spermatozoa to the dyes used, we incubated these cells individually with the Sytox Green or live-dead stains and evaluated the number of cells internalizing these dyes. Approximately 6% of neutrophils stained positive with Sytox Green and PI, and this percentage remained fairly constant for the 2-h analysis time. However, spermatozoa incorporated Sytox Green in increasing numbers over time, but without apparent deterioration of motility. Further, the hyperactivity of sperm increased over time, indicating progression toward capacitation. However, only approximately 50% of sperm internalizing Sytox Green were positive for PI, indicating cell death.

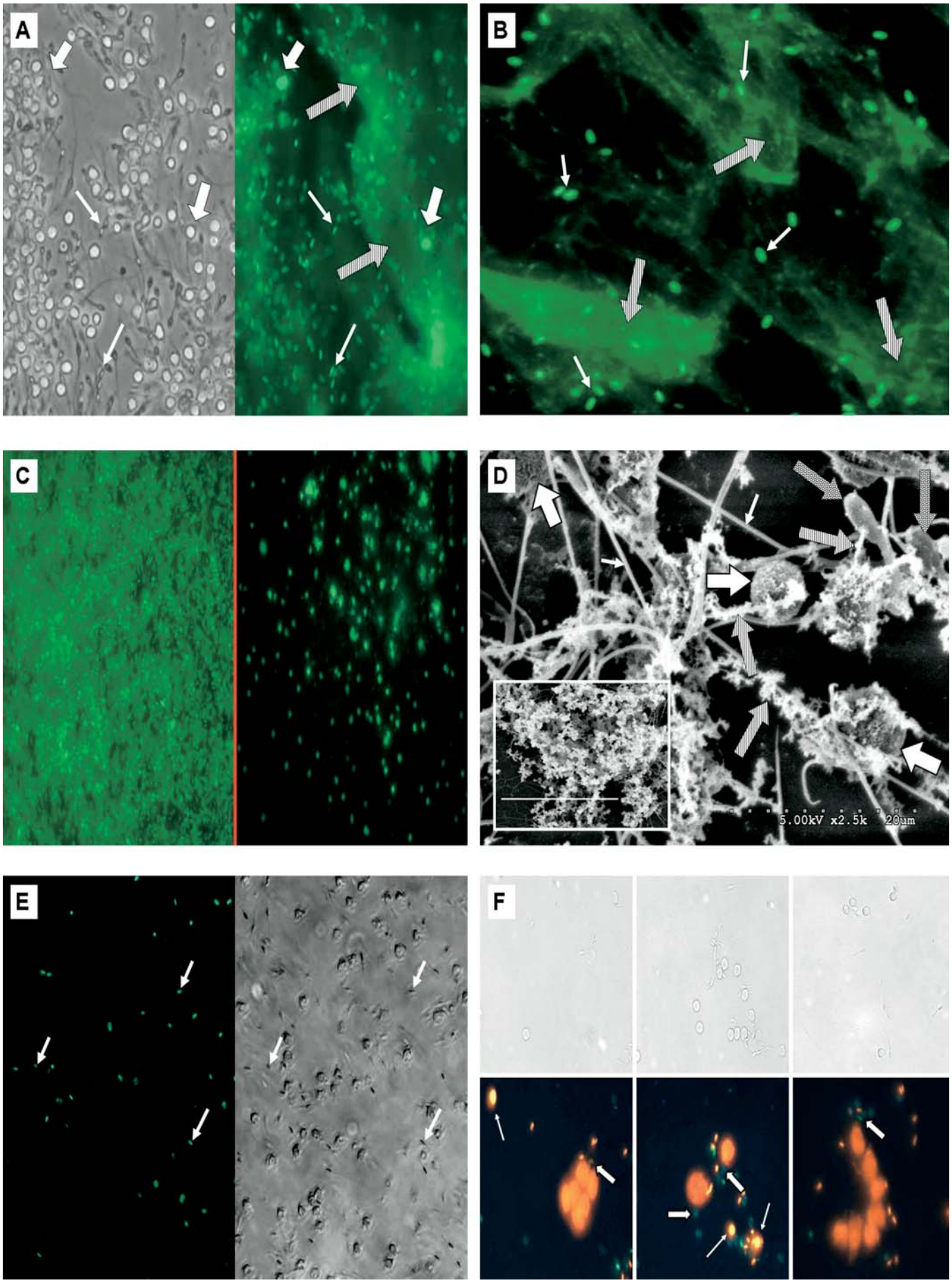
Plasmid DNA (pIND) was treated with either SP protein, purified DNase I (Roche), or BSA (control) and separated by agarose gel electrophoresis. Both DNase I and SP protein showed dose-dependent endonuclease activities that were inhibited by heating at 70°C for 10 min (Fig. 2A). Ethidium bromide-stained materials on the gel were confirmed as degraded plasmid-specific DNA fragments by Southern transfer of the gel and hybridization to a DIG-labeled (Roche) plasmid probe (data not shown). To estimate endonuclease activity of SP protein, different concentrations of crude SP protein (0.02–6 mg/ml) were compared to different concentrations of DNase I (0.3–5 U ml^{-1}; Fig. 2A). Using densitometry, we determined that 1 mg of precipitated crude SP protein contains approximately 5 U of DNase enzymatic activity. Similar methods were used with swine and human SP and gave comparable DNase activity in U mg^{-1} of crude protein (data not shown).

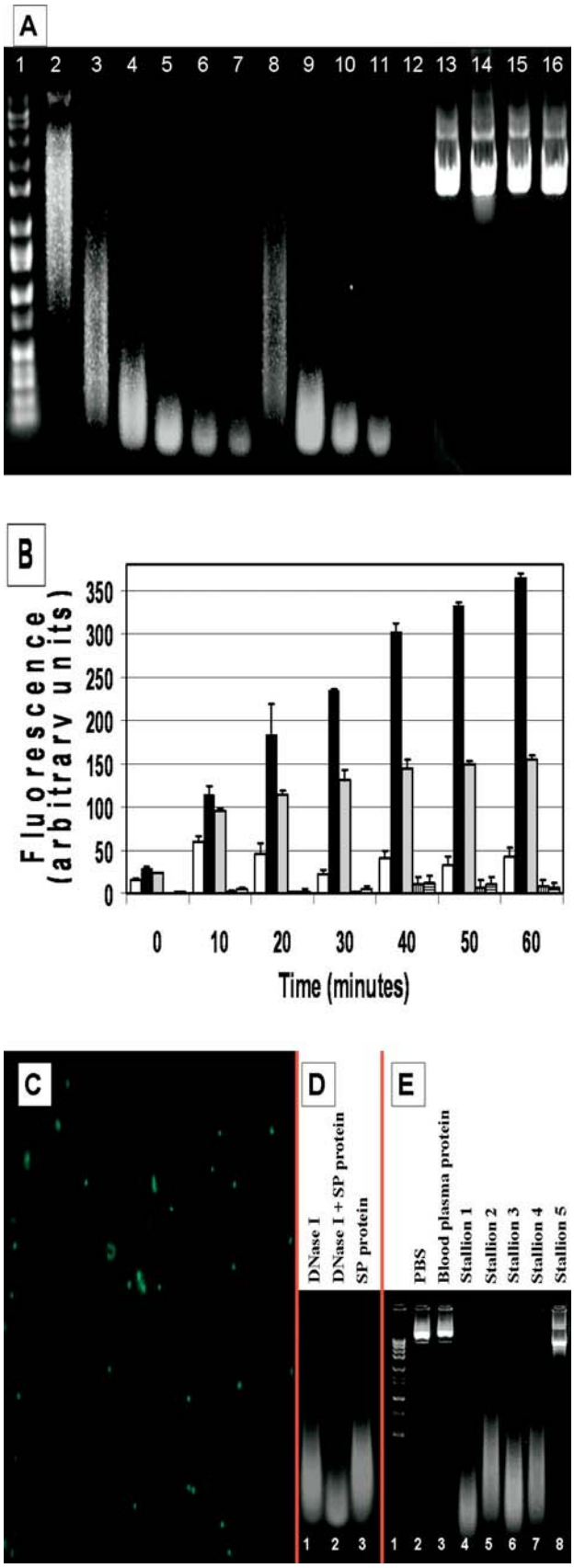
The relative amount of extruded DNA was measured using a fluorescence microplate reader for neutrophils alone and for either sperm-activated or *E. coli*-activated neutrophils (Fig. 2B). Fluorescence values for neutrophils alone did not change markedly for up to 1 h of incubation (Fig. 2, B and C, and Supplementary Video 2 [available online at http://www.biolreprod.org]). However, when spermatozoa were incubated with neutrophils, fluorescence increased to greater than 8-fold and 12-fold following 30 and 60 min incubation periods, respectively (Figs. 1C and 2B and Supplementary Video 1 [available online at http://www.biolreprod.org]). Escherichia coli stimulated neutrophil fluorescence increased more than 5- and 6-fold after 30 and 60 min, respectively (Figs. 1C and 2B and Supplementary Video 1 [available online at http://www.biolreprod.org]). The increased fluorescence from stimulated neutrophils was because of the extrusion of their DNA and was not because of cell death for the following reasons: 1) unstimulated neutrophils did not show any increase in fluorescence; 2) the live-dead stains showed that neutrophils stained positive with PI (red; dead) remained compacted and did not extrude their DNA, whereas those that extruded their DNA stained positive with SYBR 14 (green; live); and 3) neutrophils that were killed with formaldehyde or by leaving them at room temperature overnight failed to extrude their DNA or form NETs. This was in a complete agreement with findings reported by Brinkmann et al. [19] who provided many lines of evidence that NETs were formed by living neutrophils.

To determine whether DNase I combined with SP protein would result in an additive or synergistic enhancement of DNase activity, we compared the equivalent enzymatic activity of SP protein alone to DNase I alone and in combination with SP protein. Results showed that the effect was additive; the combination of DNase I and SP protein resulted in approximately twice as much enzymatic activity as DNase I alone or SP protein alone (Fig. 2D). To determine whether the DNase activity fluctuated among stallions, we compared 1 mg ml^{-1} SP from each of 5 stallions using plasmid DNA degradation analysis. Four stallions had significant DNase activity (with some variation) and one stallion had almost no DNase activity (Fig 2E).

In the presence of SP protein, differences in fluorescence patterns between sperm- and bacterial-activated neutrophils were observed using time-lapse fluorescence microscopy (Fig 3C and Supplementary Video 3 [available online at http://www.biolreprod.org]). In the presence of spermatozoa, SP protein appeared not only to degrade the extruded neutrophil DNA, but also to prevent additional neutrophils from extruding their DNA. However, in the presence of *E. coli*, it appeared that SP protein did not prevent neutrophils from forming small clusters of NETs after incubation for 1 h (Supplementary Video 3 [available online at http://www.biolreprod.org]). Because incubation of spermatozoa and neutrophils resulted in the visible formation of clusters (not observable with *E. coli* because of its small size compared to spermatozoa), we examined the effect of SP protein and purified DNase I on

FIG. 1. Sperm-activated neutrophils form NETs that trap spermatozoa. **A**) Phase contrast (left) and fluorescent (right) micrographs of equine neutrophils (large, solid arrows) incubated with equine spermatozoa (small arrows) in the presence of Sytox Green. Note the extruded DNA (hatched arrows). **B**) Micrograph at larger magnification showing spermatozoa (small arrows) trapped in NETs (hatched arrows). **C**) Neutrophils incubated with spermatozoa (25×10^6 ml^{-1}; left side) or *E. coli* (25×10^6 ml^{-1}; right side) resulted in the extrusion of neutrophil DNA forming NETs; see Supplementary video 1 (available online at http://www.biolreprod.org). **D**) SEM of spermatozoa (head; dotted arrows, and tail; small arrows) incubated with neutrophils (large, solid arrows). Note the NETs (hatched arrows) and their entanglement of spermatozoa. Control purified genomic DNA is shown in inset. **E**) Fluorescent (left) and phase contrast (right) microscopy of spermatozoa stained with Sytox Green and washed before adding neutrophils to show that formation of NETs originated from neutrophils and not from spermatozoa. Arrows indicates the same sperm cells in both micrographs. **F**) phase-contrast (upper panels) and fluorescent (lower panels) microscopy of extruded DNA as observed with the live-dead staining kit. Note that the DNA-extruding neutrophils are impermeant to PI (large arrows), whereas neutrophils that took up PI remained compacted and did not extrude their DNA (small arrows). Original magnification **A** ×400; **B** ×800; **C** ×100; **D** ×2500; **E** and **F** ×200. Bar = 20 μm for inset in **D**.





neutrophil-spermatozoa clusters that had already been formed. Coincubation of spermatozoa and neutrophils for 1 h led to an extensive formation of NETs. Addition of SP protein or DNase I to these clusters led to their dispersion (Fig. 3D and Supplementary Video 4 [available online at http://www.biolreprod.org]).

Because we have previously shown that neutrophil-spermatozoa binding is reduced dramatically by the addition of the proteinaceous portion of SP [9], we compared SP protein to the enzymatic effects of DNase I (Roche) on sperm-neutrophil binding. Purified DNase I led to a 50% decrease in sperm-neutrophil binding compared to SP protein (Fig. 3A), suggesting that neutrophil binding to spermatozoa was mediated in part by NETs. However, the increased efficiency of SP protein compared to DNase I alone suggests the involvement of additional protein(s).

To determine whether the immunosuppressive properties of SP interfered with the ability of neutrophils to combat microbial infection/contamination, neutrophils were incubated with *E. coli* at 37°C for 5 and 10 h, aliquots were plated and CFUs counted. Neutrophil bactericidal activity was similar in the presence or absence of SP protein and was greater after 10 h compared to 5 h of incubation (Fig. 3B). After 5 h, CFUs were reduced to 60% of the PBS control, but the differences were not significant ($P > 0.05$). However, after 10 h, CFUs were significantly reduced ($P < 0.001$) to less than 5% compared to PBS alone, demonstrating that SP protein suppresses the neutrophil reaction to spermatozoa without interfering with the ability of neutrophils to combat bacteria.

DISCUSSION

The data presented show that spermatozoa activate neutrophils in a manner similar to that of bacteria, leading to formation of NETs and extensive sperm entrapment. The formation of NETs visualized by time-lapse analysis after activation with bacteria or spermatozoa was time dependent, and was in agreement with the time-dependent DNA release analyzed using a microplate reader. The time-dependent DNA release and NETs formation were also in agreement with findings reported by Brinkmann et al. [19]. The formation of NETs was partially responsible for sperm binding and reduced motility, because the use of DNase resulted in lowered binding and improved motility. However, the greater suppression of

FIG. 2. Detection and quantification of seminal DNase and DNA extrusion. **A**) Agarose gel analysis of plasmid DNA treated with different concentrations of SP protein (lanes 2–7), different concentrations of DNase I (lanes 8–12), BSA (lane 13), inactivated SP protein (lane 14) and inactivated DNase I (lane 15). Molecular weight markers (lane 1) and untreated plasmid DNA (lane 16) are shown. **B**) Time-dependent extrusion of DNA (mean ± *SEM*) from neutrophils incubated alone (□), with spermatozoa (■), or with *E. coli* (■). Addition of SP protein to neutrophils in the presence of spermatozoa (■) and *E. coli* (■) was also measured. Fluorescence was read (EX/EM 485/516 nm) at 10-min intervals up to 60 min. Increased DNA extrusion resulted in the increased fluorescence of DNA-specific dye and was observed only for neutrophils stimulated by bacteria or spermatozoa. **C**) Incubation of neutrophils alone did not result in DNA extrusion and NETs formation (original magnification ×100). **D**) DNase I addition to SP protein showed additive enhancement of seminal DNase. Approximately equal amounts of enzymatic activity were used to degrade 1 μg of plasmid DNA for DNase I (lane 1) and SP protein (lane 3). Combining DNase I and SP protein showed approximately twice the nuclease activity (lane 2). **E**) Different stallions (lanes 4–8) showed different DNase activity using 1 mg SP protein, whereas PBS (lane 2) and blood plasma protein (lane 3) controls had no DNase activity. Lane 1 is a MW marker.

sperm-neutrophil binding with SP clearly suggests that other SP proteins are involved. The effect of endonuclease activities found in SP is evidenced by both the degradation of DNA and the dispersion of sperm-neutrophil clusters.

Only approximately 6% of the neutrophils stained positive with Sytox Green immediately after addition of the dye, which is in agreement with the expected number of nonviable neutrophils for the isolation protocol that was used [28]. It is important to note that when neutrophils were not stimulated (by sperm, bacteria, or LPS), this number remained constant for up to 2 h. However, the stimulation of neutrophils resulted in increased DNA fluorescence, but did not increase the approximately 6% of neutrophils that incorporated the dye without extruding their DNA. Because Sytox Green has not been validated as a stain for the viability of cells with dynamic cell membranes, especially for spermatozoa, we used a live-dead staining kit to determine cell viability and whether or not DNA is extruded by cell death. We found that neutrophils stained with PI remained compacted throughout the analysis, whereas those that extruded their DNA were resistant to PI. In addition, neutrophils killed (by treatment with 17% formaldehyde or by leaving them in room temperature overnight) took up Sytox Green but failed to form NETs or extrude their DNA for more than 6 h.

As for spermatozoa, the use of Sytox Green is unreliable for the evaluation of sperm viability (at least with the concentration used here), because 1) large portions of sperm that took up Sytox Green were motile and showed hyperactivation (which is suggestive of capacitation), and 2) approximately 50% of the sperm staining positive with Sytox Green did not stain positive for PI (a more reliable stain for dead sperm). It is likely that as sperm capacitation progresses, sperm membrane permeability to Sytox Green increases. This is supported by the fact that increased membrane fluidity and permeability are among the well-known changes associated with sperm capacitation [29, 30]. In addition, it has been shown that capacitated equine sperm increase from 7% to 60% with the mere removal of SP, and that the resuspension of sperm in SP reverses this phenomena [31]. In the current study, this is supported by the fact that the addition of SP prevented sperm from taking up Sytox Green.

These observations, together with our previous findings that the presence of neutrophils in the reproductive tract at the time of insemination reduces fertility rates, may account for some of the unexplained infertility observed in many species. For example, leukocytosis of the human cervix and vagina has been reported to reduce fertility [11–13]. Similarly, equine and swine uterine neutrophilia at the time of insemination reduces fertility rates significantly [9, 10]. Therefore, in cases of

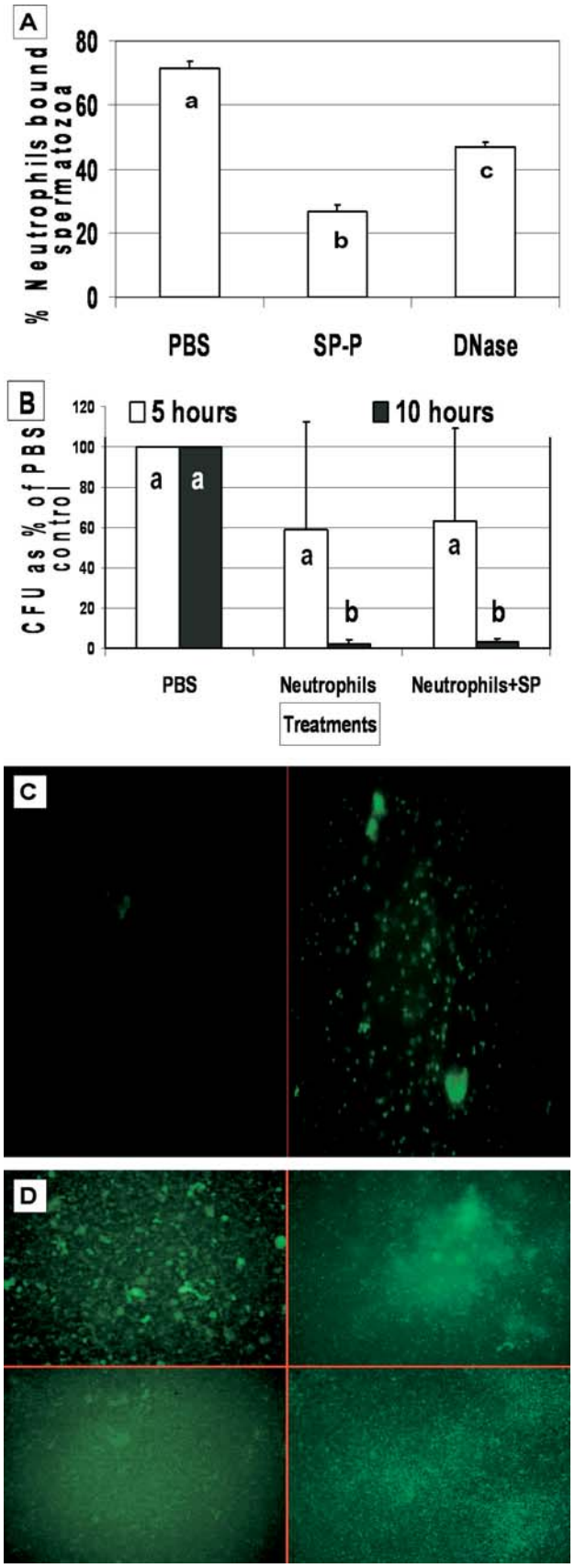


FIG. 3. Immunosuppressive activity of SP reduces sperm-neutrophil binding, but does not abolish neutrophil bactericidal properties. **A**) The percentage (mean ± *SEM*) of sperm-bound neutrophils incubated in the presence of DNase I was reduced to approximately 50% of that observed with SP protein. [abc]Different superscripts indicate a significant difference ($P < 0.001$; n = 4 independent experiments). **B**) *E. coli* were incubated in PBS alone, with neutrophils, or with neutrophils and SP at 37°C. Aliquots were plated onto LB agar after 5 and 10 h. CFU were determined as a percentage (mean ± *SEM*) of bacteria incubated in PBS alone. SP did not abolish the ability of neutrophils to kill *E. coli*. [ab]Different superscripts indicate a significant difference ($P < 0.001$) compared to the PBS control (n = 4 independent experiments). **C**) Seminal plasma negated formation of NETs when neutrophils were activated by sperm (left side) but not when they were activated by bacteria (right side). **D**) Neutrophil-sperm clusters either before treatment (top panels) or after treatment (bottom panels) with SP (left, bottom panel) and DNase I (right, bottom panel). Note dispersed clusters after treatments (bottom panels). Original magnification **C** ×100; **D** ×50.

repeated inseminations and/or subclinical/asymptomatic cases of reproductive tract inflammation, the presence of neutrophils at the time of semen deposition could interfere with sperm transport. This is especially important in AI because the processing of spermatozoa often leads to the removal or dilution of SP. Because DNase present in SP reduces sperm entrapment by NETs, it may serve to assist sperm transport in such cases of neutrophilia. This is supported by the fact that the stallion with no DNase activity is a known carrier of severe combined immunodeficiency, and his fertility with repeated inseminations per estrus is 10% lower than a single insemination. Because the semen content of DNase differs among stallions, as shown here and as previously reported in bulls [20], screening of males for DNase deficiencies may eliminate the need for more expensive reproductive procedures.

Although DNase has been found in the SP of several species [22–27], and has been associated with higher fertility in bulls [20, 21], its mode of action has remained unclear. DNase was among the heparin binding proteins (HBPs) found in bull SP, and these proteins have been reported to regulate capacitation in the presence of heparin [32], suggesting the probable involvement in capacitation and/or sperm cell degradation [20]. Because the HBPs were a mixture of different molecules, this hypothesis has not yet been confirmed. Because spermatozoa have been shown to take up naked DNA present in the surrounding medium [33–35], the presence of DNase in SP has been suggested to be a defense mechanism to prevent incorporation of foreign DNA into spermatozoa [26]. Although this is a possible function for seminal DNase, the low rate of such incorporation may not make this a major reason for the presence of DNase in SP. However, the common encounter of spermatozoa and neutrophils during breeding and insemination and the recent discovery of NETs formation [19] as well as data presented here provide a more robust explanation.

Although DNase I was similar to SP (at equivalent enzymatic activity) in dispersing sperm-neutrophil clusters, it was only 50% as effective in reducing sperm-neutrophil binding between cell membranes of the two cell types. This shows that SP includes other molecules involved in reducing sperm-neutrophil binding. This is supported by the fact that SP contains factors that abrogate DNA extrusion and formation of NETs only when neutrophils are activated by spermatozoa, and not when they are activated by bacteria. It appears that bacteria and spermatozoa activate neutrophils by different mechanisms. The selectivity of SP suppressive properties on neutrophils is also demonstrated by the bactericidal activities of these cells. The ability of bull SP to protect spermatozoa from phagocytosis led Gilbert and Fales [8] to question whether this would impair neutrophil effectiveness in combating bacteria. However, our data suggest that SP did not reduce the bactericidal activities of neutrophils, which provides more evidence for SP selective suppression of neutrophils. We conclude that spermatozoa activate neutrophils, resulting in the extrusion of their DNA and the formation of NETs that trap spermatozoa and hinder their motility, and that seminal DNase degrades these NETs and frees entangled sperm cells. Furthermore, SP proteins suppress the neutrophil reaction to spermatozoa without interfering with the bactericidal activities of neutrophils.

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