Effects of stallion seminal plasma on hydrogen peroxide release by leukocytes exposed to spermatozoa and bacteria

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Summary

The ability of stallion seminal plasma to modify phagocytosis of spermatozoa and Streptococcus zooepidemicus was examined. Phagocytosis was monitored indirectly as the H$_2$O$_2$ produced by peripheral blood leukocytes after addition of spermatozoa or bacteria. Hydrogen peroxide production after addition of ejaculated spermatozoa was greater ($P < 0.01$) than after addition of epididymal sperm. Furthermore, pre-incubation of epididymal sperm with 6.25-50% seminal plasma caused a dose-dependent increase in subsequent H$_2$O$_2$ production by leukocytes ($P < 0.05$). In addition, equine serum was capable of opsonizing epididymal and ejaculated sperm. Seminal plasma also directly stimulated phagocyte function because leukocytes preincubated with 12.5% or 25% seminal plasma released more H$_2$O$_2$ after addition of S. zooepidemicus than control leukocytes ($P < 0.05$). It is suggested that the opsonization of spermatozoa and the direct stimulation of phagocytes by seminal plasma may represent mechanisms for clearing spermatozoa and bacteria from the reproductive tract.

Key words: seminal plasma, spermatozoa, phagocytosis, bacteria

Introduction

Deposition of semen in the reproductive tract causes a local infiltration of leukocytes in many species (McDonald et al., 1952; Austin, 1957; Yanagimachi and Chang, 1963; Mattner, 1968). This invasion of primarily polymorphonuclear leukocytes (PMNs) results in phagocytosis of sperma-
tozoa (Austin, 1957; Bedford, 1965; Phillips and Mahler, 1977), a process that may be important for preventing polyspermy, selecting spermatozoan sub-populations for fertilization or for reducing the probability of inducing anti-sperm immunity (Cohen, 1984; Hancock, 1984). In addition, semen is contaminated with microorganisms (see Mann and Lutwak-Mann, 1981) and phagocytosis by PMNs invading the reproductive tract contributes to prevention of genital tract infection.

Since spermatozoa and bacteria in semen are bathed in seminal plasma, this fluid could potentially regulate the activity of PMNs in the vagina, cervix and uterus. In the experiments reported here, we evaluated whether stallion seminal plasma would opsonize spermatozoa for phagocytosis and directly stimulate phagocyte function. In the mare, PMNs rapidly migrate into the uterine lumen in response to inflammation (Williamson et al., 1984; Cuoto and Hughes, 1985). These uterine phagocytes may be particularly important in the horse since semen is deposited directly in the uterus during natural mating and spermatozoa and bacteria are therefore not reduced in number by a cervical barrier. Also, the uterine mucosa is capable of mounting a local immune response to antigenic material (Widders et al., 1985a, 1986) and phagocytosis may be one means of reducing the development of local anti-sperm antibodies.

Materials and Methods

Materials

Horse blood was collected from the jugular vein of mature Thoroughbred mares of unknown reproductive status and serum was harvested by centrifugation of the clotted blood. The strain of Streptococcus zooepidemicus used was a clinical isolate maintained on tryptic-soy agar plates containing 5% sheep serum (BBL Microbiology Systems, Cockeysville, MD). Sigma Chemical Co. (St. Louis, MO) provided Hank's balanced salt solution without phenol red or bicarbonate (HBSS), horseradish peroxidase type VI (HRP) and phenol red. Dialysis tubing was from Spectrapor (Los Angeles, CA).

Collection of seminal plasma and spermatozoa

Stallions of various breeds, mostly Thoroughbreds and Arabians, were used. Ejaculates were collected from stallions mounting a teaser mare using an artificial vagina fitted with a filter to remove the gelatinous fraction of semen. Semen was centrifuged at 400 × g for 10 min. After aspiration of seminal plasma, spermatozoa were resuspended in HBSS. Both fractions were stored at −20°C until use.

Epididymal spermatozoa were harvested from epididymis recovered soon after slaughter. The tail of each epididymis was opened with a scalpel and
the spermatozoa flushed out with HBSS. Flushings were frozen at \(-20^\circ\text{C}\) and stored until later use.

*Hydrogen peroxide production by leukocytes*

Activity of circulating phagocytes (primarily neutrophils) was measured indirectly by measuring \(\text{H}_2\text{O}_2\) produced during phagocytosis of spermatozoa or bacteria. The method is based on the peroxidase-mediated oxidation of phenol red by \(\text{H}_2\text{O}_2\) (Pick and Keisari, 1980; Rajkovic and Williams, 1985). Leukocytes were harvested from heparinized jugular blood of mares by gravity sedimentation in dextran as previously described (Asbury et al., 1982) and resuspended in HBSS at a final concentration of \(8 \times 10^6\) PMNs/ml. The horse radish peroxidase-phenol red solution (HRP-PR) was prepared freshly each day by mixing 0.7 ml HRP solution (343 units/ml in 0.05 M phosphate, pH 7.0), 0.3 ml 1% (w/v) phenol red and 9 ml HBSS. Frozen stallion spermatozoa were thawed, washed with HBSS and adjusted to a concentration of 13 or \(20 \times 10^6\) cells/ml in HBSS. Autoclaved *S. zooepidemicus* were prepared as detailed by Asbury et al. (1982) to give a suspension having an \(A_{525}\) of 75. Prior to introduction in the assay, spermatozoa (0.5 ml) were opsonized with 0.5 ml of horse serum or with HBSS containing 0.1% (w/v) bovine serum albumin (HBSS-BSA) as a negative control. After incubation at 37°C for 30 min, sperm were centrifuged at 10,000 \(\times\) g for 1 min, washed twice with 1 ml HBSS and resuspended in 0.5 ml HBSS. Bacteria were also opsonized with serum or HBSS-BSA as detailed by Brown et al. (1985).

The assay was carried out in 1.5 ml microcentrifuge tubes. After adding 250 \(\mu\)l HRP-PR solution, 125 \(\mu\)l spermatozoa or bacteria and 125 \(\mu\)l HBSS, phagocytosis was initiated by addition of 250 \(\mu\)l leukocytes. Cultures were incubated for 1 h at 37°C on a rocking platform. Reactions were terminated by adding 250 \(\mu\)l 0.25 M glycine–NaOH (pH 12.5) and centrifuging at 10,000 \(\times\) g for 2 min. The quantity of \(\text{H}_2\text{O}_2\) produced was determined by measuring the \(A_{610}\) of the supernatant fractions.

Each assay contained several controls including tubes containing leukocytes without bacteria or spermatozoa (to measure \(\text{H}_2\text{O}_2\) production in the absence of phagocytosis), leukocytes with serum-opsonized bacteria (positive control for phagocytosis and opsonization), leukocytes with HBSS-BSA-opsonized bacteria (negative control for opsonization) and leukocytes with opsonized spermatozoa or bacteria where glycine–NaOH was added at time zero (to determine background absorbance). Determinations were done in triplicate. For all experiments, the amount of \(\text{H}_2\text{O}_2\) produced as a result of phagocytosis was derived by subtracting \(A_{610}\) values for cultures of leukocytes without bacteria or spermatozoa from \(A_{610}\) values for cultures with spermatozoa or bacteria. Data were converted to nmoles
H₂O₂ produced/h per culture by means of a standard curve constructed similarly to that of Pick and Keisari (1980).

Opsonization of epididymal sperm by seminal plasma

Epididymal stallion spermatozoa were incubated with HBSS containing 0, 6.25, 12.5, 25 or 50% seminal plasma (pooled from several stallions) for 30 min at 37°C. After washing once with HBSS, sperm were resuspended in HBSS to their original concentration of 20 × 10⁶ cells/ml. Sperm were then opsonized with either serum or HBSS-BSA as described earlier and added to cultures containing leukocytes and HPR-PR to determine sperm-induced H₂O₂ production. The experiment was done in two replicates, with each replicate being done with spermatozoa from a different stallion. Results were analysed by analysis of variance (Steel and Torrie, 1960) with concentration of seminal plasma and type of opsonin (serum vs. HBSS-BSA) as main effects.

Phagocytosis of epididymal and ejaculated spermatozoa

Epididymal (n = 3) and ejaculated spermatozoa (n = 5) were collected from individual stallions, washed and resuspended in HBSS to a final concentration of 13 × 10⁶ spermatozoa/ml. For sperm from each stallion, separate aliquots were opsonized with serum or HBSS-BSA. Spermatozoa were then tested for ability to stimulate H₂O₂ production by leukocytes. Differences between ejaculated and epididymal spermatozoa were analysed by Wilcoxon’s test (Steel and Torrie, 1960) while differences between HBSS-BSA and serum within sperm type were determined by t-test.

Direct effect of seminal plasma on leukocytes

Seminal plasma was pooled from several stallions and dialysed extensively against 10 mM Tris-buffered saline (pH 7.5, TBS) using dialysis tubing with a molecular weight exclusion limit of 6000–8000. Leukocytes (8 × 10⁶ PMNs/ml in HBSS) were incubated with equal volumes of dialysed seminal plasma diluted in TBS containing 0.1% BSA to give final seminal plasma concentrations of 0, 3.125, 6.25, 12.5, 25 and 50% (v/v). After 30 min at 37°C, cells were washed once in HBSS and resuspended in HBSS to their original concentration. The leukocytes were then tested for ability to release H₂O₂ in response to serum-opsonized S. zooepidemicus. The experiment was replicated five times with each replicate using leukocytes from a different mare. Data were analysed by Dunnett’s t-test (Steel and Torrie, 1960) to determine which treatments differed from control leukocytes (0% seminal plasma).
Results

**Opsonization of epididymal spermatozoa by seminal plasma**

Epididymal spermatozoa were incubated sequentially with seminal plasma and either serum of HBSS-BSA (Fig. 1). The $\text{H}_2\text{O}_2$ released by leukocytes after addition of spermatozoa was greater ($P < 0.01$) for sperm opsonized with serum than for sperm incubated with HBSS-BSA. Regardless of whether sperm were opsonized with serum or HBSS-BSA, $\text{H}_2\text{O}_2$ production increased as a function of the concentration of seminal plasma used for pre-incubation ($P < 0.05$), indicating that seminal plasma opsonized epididymal spermatozoa.

**Phagocytosis of epididymal and ejaculated spermatozoa**

We reasoned that if seminal plasma opsonizes spermatozoa, the phagocytosis of ejaculated sperm should be greater than the phagocytosis of epididymal sperm. This, indeed, was the case (Fig. 2). Addition of ejaculated spermatozoa to cultures of leukocytes increased $\text{H}_2\text{O}_2$ production $50\%$ above $\text{H}_2\text{O}_2$ produced by leukocytes cultured without spermatozoa. The stimulation of $\text{H}_2\text{O}_2$ production was greater ($P < 0.05$) for serum-opsonized sperm.

![Fig. 1. Opsonization of epididymal spermatozoa by seminal plasma and serum. Spermatozoa were incubated sequentially with 0–50% seminal plasma and with either serum of HBSS-BSA. Phagocytosis of the treated sperm was evaluated as $\text{H}_2\text{O}_2$ production by leukocytes. Production of $\text{H}_2\text{O}_2$ was significantly affected by concentration of seminal plasma and type of opsonin (serum vs. HBSS-BSA).](image-url)
Fig. 2. Phagocytosis of epididymal and ejaculated sperm pre-incubated with serum or HBSS-BSA. Phagocytosis was evaluated as H$_2$O$_2$ production by leukocytes. Points represent observations for individual samples of spermatozoa while the bars represent group means. Lines connect data for spermatozoa from the same stallion. Production of H$_2$O$_2$ was significantly affected by type of sperm and, for ejaculated sperm, by type of opsonin.

than for sperm incubated with HBSS-BSA. In contrast, addition of epididymal spermatozoa did not increase H$_2$O$_2$ release above controls, resulting in a difference (P < 0.01) between ejaculated and epididymal spermatozoa. This difference was not due to release of H$_2$O$_2$ by the spermatozoa themselves because sperm cultured without leukocytes did not produce any H$_2$O$_2$ (results not shown).

Direct effects of seminal plasma on leukocytes

Pre-incubation of leukocytes with from 3.125% to 25% dialysed seminal plasma increased their ability to release H$_2$O$_2$ in response to serum-opsonized S. zooepidemicus (Fig. 3). The difference between control and treated leukocytes was significant (P < 0.05) at 12.5% and 25% seminal plasma. Leukocytes pre-incubated with 50% seminal plasma tended to produce less H$_2$O$_2$ in response to bacteria than leukocytes pre-incubated with other concentrations of seminal plasma, suggesting that higher concentrations of seminal plasma may be non-stimulatory.

The effect of seminal plasma was not due to assay interference from residual amounts of seminal plasma present after leukocyte washing because seminal plasma did not alter standard curves of H$_2$O$_2$ (results not shown).
Fig. 3. Effect of pre-incubation of leukocytes with seminal plasma on phagocytosis of serum-opsonized *S. zooepidemicus*. Phagocytosis was monitored as $H_2O_2$ produced by leukocytes. Pre-incubation of leukocytes with 12.5% or 25% seminal plasma increased ($P < 0.05$) subsequent $H_2O_2$ production relative to leukocytes pre-incubated with 0% seminal plasma.

Similarly, effects were not mediated by altering cell viability since, as determined by trypan blue exclusion, viability of leukocytes after incubation with seminal plasma was unaffected by concentration of seminal plasma and averaged $87 \pm 2\%$ ($\bar{x} \pm S.E.M.$).

**Discussion**

Results indicate that seminal plasma can modify phagocytosis of spermatozoa and bacteria by peripheral leukocytes. Specifically, seminal plasma opsonized epididymal spermatozoa for phagocytosis and directly stimulated leukocyte response to serum-opsonized *S. zooepidemicus*. The measure of phagocytic activity used was $H_2O_2$ production by leukocytes. Hydrogen peroxide is produced by phagocytic cells as part of the “respiratory burst” occurring in the early stages of phagocytosis (Trush et al., 1978). Since the major phagocytic cell in both the equine uterine lumen and peripheral blood is the PMN (Kenney, 1978), our results should be relevant to phagocytosis in the uterine environment.
In other species, it has been shown that spermatozoa absorb proteins and antigens from seminal plasma onto their cell surfaces (Hunter and Hafs, 1964; Roberts and Boettner, 1969; Moore and Hibbitt, 1976) and our results suggest that some of these substances increase the affinity of spermatozoa for PMNs. Opsonization of sperm seems sufficient to explain the observed differences in phagocytosis of epididymal and ejaculated spermatozoa, though other changes in the sperm surface after ejaculation could also increase its affinity for PMNs. The factor in seminal plasma that directly stimulated leukocyte response to bacteria is not known. It is probably a macromolecule since it was retained in dialysis tubing with an exclusion limit of 6000–8000 daltons. It is possible that the lower-molecular-weight components of semen also affect phagocyte function but this was not tested. Schopf et al. (1984) have reported that human seminal plasma contains a potent, low-molecular-weight inhibitor of PMNs.

Serum was also capable of opsonizing ejaculated spermatozoa but was not very effective in increasing phagocytosis of epididymal spermatozoa unless the spermatozoa were first exposed to seminal plasma. The major opsonins in serum are usually considered to be IgG and complement components C3b and C4b (Smith and Lumsden, 1983; Asbury et al., 1984). Complement C3b is present in equine uterine secretions but only as proteolytic breakdown products (Asbury et al., 1984). Uterine secretions of the mare contain IgG derived from blood and local production (Widders et al., 1984, 1985a,b) and, at least in other species, spermatozoa in the reproductive tract can be coated with IgG (Cohen and Werrett, 1975). There is also an increase in transudation of serum proteins (Williamson et al., 1984) and opsonin levels (Brown et al., 1985) in uterine secretions of the mare during the uterine inflammatory response. Therefore, it is possible that serum-derived opsonins are involved in clearing spermatozoa from the uterus.

In conclusion, seminal plasma contributes to the removal of spermatozoa and bacteria from the reproductive tract by opsonization and stimulation of leukocyte phagocytic activity. This removal provides means of preventing establishment of venereal disease, reducing the probability of polyspermy and limiting the exposure of spermatozoa to antigen-presenting cells and lymphocytes. Thus, enhancement of non-specific destruction of sperm by PMNs can be seen as another mechanism by which seminal plasma prevents development of anti-sperm immunity, along with the well-recognized role of seminal plasma in inhibiting lymphocyte reactivity (Anderson and Tarter, 1982; Thomas and Erickson, 1984; Martin-du-Pan et al., 1984; Fahmi et al., 1985).

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References


