Inhibition of neutrophil chemotaxis by pig seminal plasma *in vitro*: a potential method for modulating post-breeding inflammation in sows

K. J. Rozeboom¹, G. Rocha-Chavez² and M. H. T. Troedsson³

¹Department of Animal Science, North Carolina State University, Raleigh, NC 27695, USA;

²Departamento de Produccion Animal, Universidad de Guadalajara, Cd. Guzman, Jal 49000, Mexico; and ³Clinical and Population Sciences, University of Minnesota, St Paul, MN 55108, USA

The aim of this study was to determine the regulatory role of pig seminal plasma in post-breeding uterine inflammation. Polymorphonuclear neutrophil (PMN) chemotaxis of lipopolysaccharide (LPS)-activated blood plasma or heat-inactivated blood plasma plus LPS containing increasing concentrations of seminal plasma was assessed in chemotactic chambers. Seminal plasma was diluted serially with McCoy's medium to concentrations of 50.0, 25.0, 12.5, 6.2 or 3.1% (v/v) and added to normal or heat-inactivated pig blood plasma that was activated with LPS before or after incubation in a 37°C waterbath for 30 min. Chemotaxis was determined using blood-derived PMNs and was expressed as a percentage of the positive control of LPS-activated blood plasma. A linear dose-dependent suppression of chemotaxis by seminal plasma

was observed for blood plasma activated before or after addition of seminal plasma. Compared with the positive control, concentrations of seminal plasma < 6.2% failed to suppress PMN chemotaxis ($P < 0.05$). A dose-dependent suppressive effect of seminal plasma on heat stable chemotactic components of pig blood plasma was also observed ($P < 0.05$). A marked suppression was observed at concentrations of seminal plasma > 12.5% of the sample volume ($P < 0.05$). These results indicate that seminal plasma suppresses chemotactic blood plasma components regardless of formation sequence (pre- or post-activation) or source (normal or heat-inactivated blood plasma). These results indicate that seminal plasma may be necessary in diluted boar semen used for artificial insemination to regulate post-breeding inflammation in sows.

Introduction

Post-breeding inflammation in the form of a rapid migration of polymorphonuclear neutrophilic granulocytes (PMNs) into the uterus is found consistently after insemination in sows and mares (Pursel *et al.*, 1978; Rodriguez-Martinez *et al.*, 1990; Kotilainen *et al.*, 1994; Troedsson, 1995; Rozeboom *et al.*, 1998, 1999). A uterine inflammatory response to spermatozoa appears to be a normal physiological reaction after insemination (Rozeboom *et al.*, 1998; Troedsson, 1998; Troedsson *et al.*, 2000). Pig spermatozoa and seminal plasma appear to have equally important, yet different, modulatory roles for initiating and suppressing this response *in utero* (Rozeboom *et al.*, 1999). Although a transient semen-induced uterine inflammation is probably necessary for the establishment of pregnancy (Engelhardt *et al.*, 1997; Rozeboom *et al.*, 1997), uncontrolled post-mating endometritis has been considered a cause of infertility in mares and pigs (McKinnon and Voss, 1993; Troedsson *et al.*, 1995a,b; Rozeboom *et al.*, 2000). Stallion, bull and boar spermatozoa all induce PMN chemotaxis via complement

activation (Clark and Klebanoff, 1976; Troedsson *et al.*, 1998; Rozeboom *et al.*, in press). Although stallion and bull seminal plasma appear to inhibit the activation of complement, the role of boar seminal plasma has received little attention. The aims of the present study were to investigate the role of pig seminal plasma on PMN chemotaxis *in vitro*.

Materials and Methods

Seminal plasma

Seminal plasma was recovered from eight reproductively mature Hampshire and Yorkshire boars (pool A), and two non-related Hampshire boars from a different farm (pool B). After centrifugation at 400 *g* for 10 min, the seminal plasma from each pool of boars was recovered and centrifuged again at 10 000 *g* for 20 min. The supernatant of each pool was recovered and 3 ml aliquots were prepared, frozen and stored at –20°C until used.

Standard pooled plasma

Peripheral blood was withdrawn, using a vacutainer system (Becton Dickinson, Rutherford, NJ) containing

Email: Kevin_rozeboom@ncsu.edu

sodium heparin as an anticoagulant from the jugular vein of five clinically healthy castrated pigs (mass approximately 105 kg). The blood was centrifuged at 1000 *g* for 10 min and the blood plasma from all pigs was saved and mixed. Aliquots (2 ml) of the pooled blood plasma were prepared and stored at -20°C until used. Untreated plasma served as a control for spontaneous chemotactic activity of blood plasma and blood plasma inactivated by heating in a 59°C waterbath for 40 min served as a negative control for complement dependent chemotactic activity.

Preparation of PMNs

A total of 20 ml of venous blood was withdrawn from the jugular vein of a clinically healthy castrated pig (mass approximately 105 kg) in a vacutainer system containing sodium heparin as an anticoagulant. The blood was centrifuged at 1000 *g* for 10 min and the blood plasma was removed. The buffy coat was recovered with a sterile Pasteur pipette and mixed with an isotonic saline solution (9 g NaCl l⁻¹) layered on lymphocyte separation medium (Organon Teknika, Durham, NC), and centrifuged at 1000 *g* for 20 min. The PMN-rich buffy coat was collected from the pellet underneath the lymphocyte separation medium and re-suspended in a 2.25% (w/v) dextran solution (average *M_r* 250 000; Sigma, St Louis, MO) in saline. After 15 min of sedimentation, the supernatant was collected and the cells were washed twice with saline and counted.

Experiment 1

This experiment was designed to determine the effect of seminal plasma on PMN chemotaxis as it relates to interference on activation of chemotactic substances in blood plasma by bacterial endotoxin (lipopolysaccharide (LPS); Forsberg and Carlson, 1998). A positive control was prepared mixing 40 µg *Escherichia coli* LPS (Sigma) dissolved in 20 µl saline (9 mg NaCl l⁻¹) with 400 µl pooled blood plasma. An additional five samples of LPS were mixed with blood plasma in the presence of 50.0, 25.0, 12.5, 6.2 and 3.1% (v/v) seminal plasma (final seminal plasma concentrations; diluted in McCoy's modified medium (Sigma; with sodium bicarbonate and without L-glutamine)). A negative control was prepared by mixing LPS with 400 µl McCoy's 5a modified medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). Random migration of PMNs towards seminal plasma was assayed using 400 µl undiluted seminal plasma only (100%) and an additional sample of LPS plus heat-inactivated blood plasma (heated at 56°C for 35 min) was used as a control for non-complement related chemotactic factors. After preparation, samples were incubated at 39°C for 30 min to activate chemotactic factors in blood plasma and the process was stopped by repeated heat inactivation at 56°C for 35 min and subsequently dilution with 1.6 ml McCoy's medium. A 48-well chemotaxis chamber (Neuroprobe Inc, Cabin John, MD) was used for all chemotaxis assays. Aliquots (25 µl) of each

sample were placed in corresponding wells of the bottom plate of the chamber and a 25 mm × 80 mm 3 µm pore size polycarbonate filter (Osmonics, Livermore, CA) was placed on top of the plate. After assembly of the chamber, 2.5 × 10⁶ PMNs suspended in 50 µl McCoy's medium were added to each of the wells in the upper plate. The chamber was incubated at 38°C with 5% CO₂ for 80 min to allow PMNs to migrate towards the chemoattractants. After incubation was complete, the chamber was inverted onto a paper towel to allow draining of the remaining cell suspension. The filter was recovered and placed upside down on a microscope slide. The treatment areas located on the filter were labelled with a permanent marker to retain identity during the staining and counting procedures. The filter was secured with clips, fixed with alcohol for 5 min and stained with Harris's haematoxylin and Wright stains (Sigma, St Louis, MO). The number of PMNs from eight consecutive microscopic fields was counted in different areas of the filter and the mean number of neutrophils per treatment was calculated. The chemotactic response was expressed as a percentage of the positive control (LPS-activated blood plasma). Five trials were conducted and each sample was assayed in triplicate. The means and standard error of the means were computed and registered.

Experiment 2

This experiment was designed to assess the effect of seminal plasma on PMN chemotaxis in the presence of complement-activated blood plasma. Six samples were prepared by mixing 40 µg *E. coli* LPS (Sigma) dissolved in 20 µl saline (9 mg NaCl ml⁻¹), with 400 µl pooled blood plasma. All samples were incubated at 37°C for 30 min, heat inactivated at 56°C for 35 min and diluted with seminal plasma to concentrations of 50.0, 25.0, 12.5, 6.2, 3.1 or 0% (positive control) (v/v) seminal plasma. A negative control was prepared mixing LPS with 400 µl McCoy's 5a modified medium supplemented with 10% heat-inactivated fetal bovine serum. Random migration of PMNs towards seminal plasma was assayed using 400 µl undiluted seminal plasma only (100%) and an additional sample of LPS + heat-inactivated blood plasma (56°C for 30 min) was used as a control for non-complement related chemotactic factors. All samples were diluted with 1.6 ml of McCoy's medium before being placed in the chemotactic chamber as described in Expt 1.

Experiment 3

This experiment was designed to assess the ability of seminal plasma to regulate migration of heat stable chemotactic components in pig blood plasma. Seminal plasma, blood plasma and neutrophil isolation were prepared as described earlier. However, for this experiment the blood was centrifuged for 10 min at 1000 *g* and the blood plasma from all pigs was removed, mixed and heat inactivated at 59°C for 35 min. Aliquots (2 ml) of the pooled blood plasma were prepared and stored at -20°C until used.

Six test tubes were prepared by mixing 40 µg *E. coli* LPS dissolved in 20 µl saline (9 mg NaCl ml⁻¹) with 400 µl heat-inactivated blood plasma. Samples were incubated at 39°C for 30 min to activate chemotactic factors in blood plasma and the process was stopped by repeated heat inactivation at 56°C for 35 min. Samples were assigned to the following treatments: addition of 50.0, 25.0, 12.5, 6.2, 3.1 and 0.0% (positive control) seminal plasma (final concentrations of seminal plasma). An additional sample was prepared as a negative control by mixing LPS with 400 µl of McCoy's 5a modified medium supplemented with 10% heat-inactivated fetal bovine serum. Random migration of PMNs towards seminal plasma was assayed using 400 µl undiluted seminal plasma only (100%). All samples were diluted with 1.6 ml McCoy's medium before being transferred to a 48-well chemotaxis chamber for the chemotaxis assays. The assay was conducted as described earlier.

Statistical analysis

For each assay, the PMNs of eight consecutive microscopic fields were counted in two different areas of the filter and the mean number of neutrophils per filter was calculated. Triplicate assays were performed for each sample, and the means and standard errors of the mean were computed. The chemotactic response of each seminal component was expressed as a percentage of the positive controls (endotoxin-activated plasma).

Five replicate experiments were performed for the chemotactic assays with the exception of Expt 1, in which two additional replicates using seminal plasma from pool B were analysed separately. The mean and standard variations were expressed as a percentage of the positive control and compared by ANOVA in the linear procedures of the computerized program Statistix (1997). Bonferroni's comparison of group means was used to determine variance between groups. $P < 0.05$ was considered significant.

Pearson's correlation coefficient was used to determine the relationship between concentrations of seminal plasma and its effects on chemotaxis. Logarithmic and square root transformations were used to obtain the best linear fit in the chemotaxis assays and the simple linear regression analysis was conducted to obtain the regression equation and to determine the model adequacy. The level of significance was $P < 0.05$.

Results

Experiment 1

In all four experiments, the chemotactic response to seminal plasma only was similar to the negative control of McCoy's medium supplemented with 10% fetal calf serum (Figs 1–4). Heat inactivation did not significantly suppress PMN chemotaxis in Expt 1 (Fig. 3). Seminal plasma from either pool of seminal plasma caused a dose dependent suppression of chemotaxis when it was added to the samples before LPS activation of blood plasma (Figs 1

Fig. 1. Chemotaxis properties of lipopolysaccharide (LPS) plus blood plasma diluted serially with seminal plasma (SP) from boar pool A to concentrations of 50.0, 25.0, 12.5, 6.2 and 3.1% (v/v) before incubation at 37°C for 30 min (pre-activation; Expt 1). Seminal plasma caused a dose-dependent suppression of chemotaxis when it was added to the samples before LPS activation of blood plasma. Compared with the positive control containing LPS + blood plasma only, a marked suppression of chemotaxis occurred when seminal plasma was added at 6.2% of the sample volume, whereas maximum suppression of chemotaxis occurred when seminal plasma was added at 50.0% of sample volume. NC: negative control of McCoy's medium + fetal calf serum. 100% SP: undiluted seminal plasma used to assay random migration of PMNs towards seminal plasma. Data are expressed as percentages of the positive control. ^{abcde}Values with different letters are significantly different ($P < 0.01$). *Value is significantly different from positive control ($P < 0.05$).

and 2; $P < 0.05$), and a strong negative relationship ($r = -0.96$, $P < 0.01$) was found between the concentration of seminal plasma in the treatment and the percentage of chemotaxis observed. The equation of the regression line was $y = (9.55 - 0.058x)^2$ and the coefficient of determination was $r^2 = 0.97$ ($P < 0.01$).

Experiment 2

Increasing concentrations of seminal plasma caused a reduction in chemotactic activity of LPS-activated blood plasma when seminal plasma was added after activation (Fig. 4; $P < 0.05$). The correlation was also significant ($r = -0.92$, $P < 0.01$) and the equation of the regression line was $y = (8.45 - 0.052x)^2$, with a coefficient of determination of $r^2 = 0.90$ ($P < 0.01$).

Experiment 3

In this experiment, a dose-dependent suppression of chemotaxis was also observed for LPS plus heat-inactivated blood plasma in the presence of increasing concentrations of seminal plasma ($P < 0.05$; Fig. 5). Samples containing $\geq 12.5\%$ (v/v) seminal plasma showed a significantly

Fig. 2. Chemotaxis properties of lipopolysaccharide (LPS) plus blood plasma diluted serially with seminal plasma (SP) from boar pool B to concentrations of 50.0, 25.0, 12.5, 6.2 and 3.1% (v/v) before incubation at 37°C for 30 min (pre-activation). Addition of seminal plasma caused a dose-dependent suppression of chemotaxis when it was added to the samples before LPS activation of blood plasma. Compared with the positive control of LPS plus blood plasma, a marked suppression occurred when seminal plasma was added at 12.5% of sample volume, whereas maximum suppression of chemotaxis occurred when seminal plasma was added at 50.0% of sample volume. Migration of PMNs toward undiluted seminal plasma (SP), LPS and blood plasma (BP) was similar to that of the negative control (NC) of McCoy's medium + fetal calf serum. Data is expressed as a percentage of the positive control. ^{abcde}Values with different letters are significantly different ($P < 0.05$). *Value is significantly different from positive control ($P < 0.05$).

greater suppression of chemotaxis compared with the positive control ($P < 0.01$). The sample containing heat-inactivated blood plasma caused only mild chemotaxis compared with the LPS + heat-inactivated blood plasma sample (positive control). The equation $y = 78.71 + 0.81x$ represents the regression of seminal plasma concentration on the sample (y) and the amount of chemotaxis observed (x). A strong negative relationship was found between the parameters ($r = -0.92$, $P < 0.001$).

Discussion

Extensive dilution of semen is used routinely for artificial insemination (Crabo and Dial, 1992). Previous data from our laboratory indicate that the physiological clearing processes of semen and bacterial contamination from the female reproductive tract are altered during these practices. Rozeboom *et al.* (1998) characterized post-breeding uterine neutrophil migration in gilts and showed that this inflammatory response (as measured by uterine neutrophil migration) reaches its magnitude between 6 and 12 h after artificial insemination and persists for as long as 24 h after

Fig. 3. Polymorphonuclear neutrophil (PMN) chemotactic properties of seminal plasma and heat-inactivated blood plasma plus lipopolysaccharide (HIBP + LPS). Chemotaxis is expressed as a percentage of positive control (LPS activated blood plasma). Migration of PMNs toward undiluted seminal plasma (SP) was similar to that of the negative control (NC) of McCoy's medium plus fetal calf serum and markedly lower than that of HIBP plus LPS and the positive control of LPS-activated blood plasma. ^{ab}Values with different letters are significantly different ($P < 0.01$). *Value is significantly different from positive control ($P < 0.05$).

artificial insemination. More recent studies supported this, but also suggested that the components of semen, spermatozoa and seminal plasma have different roles in the initiation, suppression and clearance of uterine neutrophil migration (Rozeboom *et al.*, 1999). Although PMN influxes into the uterus after insemination can persist for as long as 36 h, both the magnitude and clearance time is highly dependent on the presence or absence of seminal plasma in the dose used for artificial insemination (Rozeboom *et al.*, 1999).

The modulatory role of seminal plasma is perhaps most evident in current semen dilution practices when doses of semen that are free of seminal plasma are used for artificial insemination. Rozeboom *et al.* (2000) showed that if artificial insemination doses free from seminal plasma are deposited into a neutrophil-enriched uterine environment (sperm- or endotoxin-initiated), the likelihood of a successful pregnancy is lowered significantly. These results indicate that, in addition to controlling uterine PMN migration, seminal plasma may have a protective role in sperm transport. The mechanism of the protective role of seminal plasma on sperm survival in an inflamed uterine environment *in vivo* is not understood. The results of the present study indicate that pig seminal plasma can interfere with complement activation and suppress the activity of blood components causing chemotaxis. However, it is not completely clear from these data whether seminal plasma inhibits the cleavage of these components (probably C3a and C5a) or simply suppresses their activity after activation. These data indicate that seminal plasma suppresses neutrophil chemotaxis in a dose-dependent fashion *in vitro*.

Fig. 4. Chemotaxis properties of lipopolysaccharide (LPS) plus blood plasma diluted serially with seminal plasma (SP) to concentrations of 50.0, 25.0, 12.5, 6.2 and 3.1% (v/v) after incubation at 37°C for 30 min (post-activation; Expt 2). Addition of seminal plasma caused a dose-dependent suppression of chemotaxis when it was added to the samples before LPS activation of blood plasma. Compared with the positive control of LPS plus blood plasma, a marked suppression of chemotaxis occurred when seminal plasma was added at 6.2% of sample volume, whereas maximum suppression of chemotaxis occurred at 50.0% seminal plasma inclusion rates. NC: negative control of McCoy's medium + fetal calf serum. Data are expressed as a percentage of the positive control. ^{abcd}Values with different letters are significantly different ($P < 0.01$). *Value is significantly different from positive control ($P < 0.05$).

A strong negative relationship was found between seminal plasma concentrations in the treatment and the percentage of chemotaxis observed when seminal plasma was added to blood plasma before complement activation. When seminal plasma was added after complement activation, a slightly less significant, but similar, relationship was found. Increasing concentrations of seminal plasma in heat-inactivated blood plasma also caused reduction of PMN chemotaxis in a dose-dependent way. Normally, LPS activates complement via the alternative pathway in the absence of antibodies (Forsberg and Carlson, 1998), which has been a consistent *in vitro* response observed in our laboratory using horse serum (Troedsson *et al.*, 1999). However, in the present study it was observed that a mixture of LPS and heat-inactivated pig blood plasma also caused a strong chemotactic response, whereas heat-inactivated blood plasma provoked only mild chemotaxis. These results discard complement components as the sole chemotactic factor in pigs and indicate that LPS can activate other factors in blood plasma that stimulate a strong

Fig. 5. Chemotaxis properties of lipopolysaccharide (LPS) plus heat inactivated blood plasma (HIBP; 59°C for 30 min) diluted serially with seminal plasma to concentrations of 50.0, 25.0, 12.5, 6.2 and 3.1% (v/v) after incubation at 37°C for 30 min and heat treatment of 59°C for 35 min (Expt 3). Seminal plasma (SP) caused a dose-dependent suppression of chemotaxis when it was added to the samples after LPS stimulation of heat stable chemotactic blood plasma components. Compared with the positive control of LPS plus HIBP, a marked suppression of chemotaxis occurred when seminal plasma was added at 12.5% of sample volume, whereas maximum suppression of chemotaxis occurred when seminal plasma was added at 50.0% of sample volume. NC: negative control of McCoy's medium + fetal calf serum. 100% SP: undiluted seminal plasma used to assay random migration of polymorphonuclear neutrophils towards seminal plasma. Data are expressed as a percentage of the positive control. ^{abcd}Values with different letters are significantly different ($P < 0.01$). *Value is significantly different from positive control ($P < 0.05$).

chemotactic response, as LPS alone does not elicit a PMN chemotactic response (K. J. Rozeboom, unpublished). A potential explanation for this event is that isolation of neutrophils was never accomplished at 100% purity and that other blood cells such as monocytes (12%) and some lymphocytes (15%) were present in the solution. Blood-derived PMNs rather than uterine derived PMNs were used for all chemotaxis experiments. Endometritis would have to be induced experimentally to obtain uterine derived PMNs, as very few PMNs are normally present in a healthy non-challenged uterus. PMNs obtained from an *in vivo* inflammation represent a heterogeneous sample of freshly recruited already phagocytosing and degenerating cells. In contrast, blood-derived PMNs can be stimulated *in vitro* and, therefore, are more suitable for a standardized experimental protocol in a function assay. LPS may stimulate monocytes to produce cytokines that may interact with blood plasma proteins or act directly as neutrophil activators or chemotactic agents (Abbas *et al.*, 1997).

These studies are the first to demonstrate the ability of seminal plasma to suppress neutrophil chemotaxis in pigs *in vitro*. Previous investigations have reported both inhibitory and stimulatory chemotactic responses from

human, guinea-pig, bovine and equine seminal plasma in the presence of blood plasma (Maroni *et al.*, 1972; Clark and Klebanoff, 1976; Troedsson *et al.*, 1998). Most of these investigations support our findings, with the exception of Clark and Klebanoff (1976), who found that bovine seminal plasma induces chemotactic activity when incubated with serum. It is difficult to speculate on variations in immuno-suppressive activity between species, males within the same species or ejaculates from the same male, as the aims of the present study were to elucidate the general chemotactic properties of pig seminal plasma. Further research is needed to identify potential individual variation with respect to individual boars or ejaculates from the same boar, as it may explain some of the variability in reproductive performance when homogeneous matings are used.

The specific factors or components of seminal plasma that are responsible for reduction of chemotaxis in sows are unclear at this point; however, a protein or group of proteins may be involved in these beneficial effects. Troedsson *et al.* (1999) suggested that a protein fraction in stallion seminal plasma between 50 kDa and 100 kDa molecular mass is probably responsible for suppressing PMN chemotaxis, but more studies are needed before the potential to modulate this response artificially becomes apparent.

In conclusion, the results of the present study demonstrate an important role of seminal plasma in modulating two important events of post-breeding inflammation in sows. The results indicate that seminal plasma is needed in the uterine lumen after mating to ensure a suitable environment for spermatozoa and pre-implantation when multiple inseminations are used for artificial insemination of pigs.

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