In vitro phagocytosis of boar spermatozoa by neutrophils from peripheral blood of sows

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A considerable number of spermatozoa are used in each sow in routine artificial insemination. However, within a few hours after insemination, many spermatozoa are phagocytosed by polymorphonuclear leucocytes. Some aspects of sperm transport in the female genital tract in the sow have been thoroughly investigated, whereas little is known about the mechanisms involved in the phagocytosis of spermatozoa, or about which spermatozoa (fresh, capacitated or dead) are the most susceptible to ingestion by polymorphonuclear leucocytes. In this study, phagocytosis was investigated by use of an in vitro phagocytosis assay. Polymorphonuclear leucocytes were challenged with either untreated, cold-shocked or frozen–thawed spermatozoa, or with spermatozoa that had been treated to induce capacitation in vitro. The influence of serum on phagocytosis was also investigated. Treatment of the semen to induce capacitation in vitro considerably reduced the phagocytosis of spermatozoa, whereas crude treatments like cold-shock or freezing and thawing reduced phagocytosis only in the first 15–30 min of incubation with polymorphonuclear leucocytes. Viable spermatozoa were phagocytosed mainly through a pathway that was independent of complement or other serum components (for example, antibodies). Complement had little effect on phagocytosis of spermatozoa, but did cause acrosomal exocytosis and cell death.

Introduction

In modern pig breeding, fertility rates of approximately 90% are common; however, this is only achieved with a sperm dosage of approximately 2.4 billion spermatozoa per insemination. Pig breeders are interested in reducing the number of spermatozoa required per insemination to ensure that the semen of high qualified breeding boars is used as efficiently as possible. For this purpose, it is necessary to obtain a better understanding of the fate of inseminated spermatozoa in the genital tract of the sow.

After insemination, the number of spermatozoa in the female genital tract in the sow decreases to 5–10% within a few hours (First et al., 1968; Pursel et al., 1978; Viring, 1980; Kamerman, 1994). Up to 50% of the spermatozoa are lost due to the back flow of semen in the first few hours after insemination (Baker et al., 1968; Kamerman, 1994; Steverink et al., 1998). Another important cause of the rapid decrease of the number of spermatozoa in the genital tract is phagocytosis of spermatozoa by polymorphonuclear leucocytes (PMN) (Lovell and Getty, 1968; Pursel et al., 1978; Kamerman, 1994). These cells migrate into the uterine lumen directly after insemination. At 2 h after insemination, the number of PMN was found to be about the same as the number of spermatozoa (Lovell and Getty, 1968; Pursel et al., 1978).

Leucocyte recruitment and phagocytosis of spermatozoa can be considered as a normal physiological response to clean the genital tract in preparation for receipt of the embryos. However, phagocytosis of spermatozoa has also been suggested as a mechanism for preferential elimination of senescent or dead spermatozoa, or spermatozoa with a decreased fertilizing ability (Symons, 1967; Moyer et al., 1970; Cohen and Tyler, 1980; Vogelpoel and Verhoef, 1985; D'Cruz et al., 1995). Sperm surface recognition plays an important role in phagocytosis (Toshimori et al., 1991). It is believed that some of the changes in the plasma membrane that occur during capacitation are similar to those during ageing. Therefore, the hypothesis of preferential phagocytosis of ageing and damaged spermatozoa could also pertain to capacitated spermatozoa.

In the genital tract, spermatozoa have to undergo capacitation to become competent to fertilize the ova (Chang, 1951; Austin, 1952; Yanagimachi, 1994). Capacitation can also be induced in vitro. In boar spermatozoa, capacitation in vitro can be achieved by incubation of the spermatozoa at 38°C in an IVF medium in the presence of bicarbonate (Suzuki et al., 1994). These incubation conditions induce specific changes in the plasma membrane, which include changes in the lipid architecture and the release or reorganization of surface components (Dostálöva et al., 1994; Gadella et al., 1994; Ashworth et al., 1995; Harrison et al., 1996). Some of these changes also occur after prolonged storage of ejaculated boar semen (Gadella et al., 1994) or after cold-shock treatment (De Lieve et al., 1990). The latter treatment also produces more...
pronounced changes, ranging from fine to coarse alterations of the acrosomal morphology, and even vesiculation or complete loss of the acrosomal cap (Pursel et al., 1972; Plummer and Watson, 1988) and cell death.

A test for phagocytosis in vitro was developed to study the influence of the above processes on phagocytosis of spermatozoa. Spermatozoa were treated to induce capacitation in vitro or were subjected to cold shock or freezing and thawing, before incubation with PMN. The role of untreated or inactivated serum was also investigated (that is, serum with intact or inactivated complement) to obtain information about the mechanisms involved in phagocytosis.

**Materials and Methods**

Media

Different modifications of Tyrode’s medium (TM) were used: TM medium: described as ‘standard Tyrode’s medium’ in Harkema et al. (1998), but without propidium iodide; and TMs medium: TM without BSA, but supplemented with 15% (v/v) sow serum. Serum that had been treated to heat-inactivate complement or untreated serum was used. Serum was prepared from blood collected from 11 primiparous sows, pooled and frozen in aliquots at –80°C. Before use, two aliquots were thawed, and one of these was treated to heat-inactivate complement at 56°C for 30 min; the other aliquot remained untreated.

Isolation and preparation of PMN

For each experiment, 20 ml peripheral blood from an individual Dutch Landrace sow was collected in heparinized vacutainers (Venoject, Omnilabo, Breda), and subsequently diluted with an equal volume of PBS. A different sow was used in each experiment. A number of screw-capped polystyrene test tubes. In accordance with a procedure for capacitation of spermatozoa for IVF purposes (Suzuki et al., 1994), one tube was left uncapped and was placed in a humidified incubator for 2 h at 38°C with 5% CO₂ in air. The other tube was capped and maintained at room temperature. In parallel treated samples (without H33342 staining), the ability of spermatozoa to bind fluorescein-conjugated solubilized zona pellucida proteins (FITC-sZP) was measured using flow cytometry, as described by Harkema et al. (1998). The inclusion of propidium iodide in this flow cytometric assay permitted the assessment of membrane integrity (propidium iodide exclusion) in these same samples.

For sperm damage experiments, three aliquants (1 ml) of the washed sperm suspension were transferred into 10 ml screw-capped polystyrene test tubes. In accordance with a procedure for capacitation of spermatozoa for IVF purposes (Suzuki et al., 1994), one tube was left uncapped and was placed in a humidified incubator for 2 h at 38°C with 5% CO₂ in air. The other tube was capped and maintained at room temperature. In parallel treated samples (without H33342 staining), the ability of spermatozoa to bind fluorescein-conjugated solubilized zona pellucida proteins (FITC-sZP) was measured using flow cytometry, as described by Harkema et al. (1998). The inclusion of propidium iodide in this flow cytometric assay permitted the assessment of membrane integrity (propidium iodide exclusion) in these same samples.

For sperm damage experiments, three aliquants (1 ml) of the washed sperm suspension were transferred into 10 ml screw-capped test tubes. The spermatozoa in one tube were subjected to cold shock by immersing the tube in a waterbath at 0–4°C for 30 min. The spermatozoa in the second tube were killed by freezing and thawing; spermatozoa were frozen by placing the tube at −20°C for 30 min. The spermatozoa in the third tube were maintained at room temperature and remained untreated.

Sperm viability was evaluated microscopically before and after the treatments (see below).

**Phagocytosis assay**

Aliquants (80 μl) of the PMN suspension in TM or TMs with either intact or inactivated complement were transferred to a 96-well polystyrene microtest plate. Sperm suspension (20 μl) was added to each well and the test plate
was placed in a humidified incubator at 38°C with 5% CO₂ in air. The final concentrations of PMN, spermatozoa and serum were 8 × 10⁸ ml⁻¹, 4 × 10⁸ ml⁻¹ and 12% (v/v), respectively. The samples were incubated while being swirled gently on a test-plate shaker. After 15, 30, 45, 60 or 90 min, samples were transferred quantitatively into tubes containing an equal volume of 40 mg heparin ml⁻¹ (Sigma) in PBS. Heparin facilitates dissociation of agglutinated PMN.

Samples were mixed thoroughly, left for 15 min and mixed again. Subsamples of 75 µl were fixed by adding 25 µl of 2% (v/v) glutaraldehyde (Fluka, Brunschwig chemie, Amsterdam) in PBS. Heparin facilitates dissociation of agglutinated PMN. Samples were mixed thoroughly, left for 15 min and mixed again. Subsamples of 75 µl were fixed by adding 25 µl of 2% (v/v) glutaraldehyde (Fluka, Brunschwig chemie, Amsterdam) in PBS. ‘Blank’ samples, that is spermatozoa without PMN (80 µl TM mixed with 20 µl sperm suspension), were incubated in parallel to monitor sperm survival during the treatment. In addition, ‘reference’ samples, that is frozen–thawed (killed) semen, were incubated in parallel with the PMN to provide a reference for the phagocytotic activity of the PMN. The frozen–thawed semen was taken from a large stock of semen from one ejaculate, and was washed three times in PBS before use.

Microscopical evaluation of phagocytosis and sperm viability

For evaluation of phagocytosis, wet mounts of the fixed samples were examined using a combination of phase-contrast and fluorescence microscopy (Olympus BH2, Tokyo) at × 400 magnification. These mounts enabled quantitation of fluorescently labelled spermatozoa inside and outside the phagocytes (Fig. 1). By focusing at different levels in the mount, spermatozoa that were located above or below the PMN could be clearly distinguished from those that were phagocytosed. Moreover, in many PMN that had ingested a spermatozoon, the presence of the sperm nucleus or tail caused a conspicuous change in the shape of the PMN (Fig. 1). A total of 200 spermatozoa were evaluated and classified as inside or outside the PMN (phagocytosed or not).

The same fixed mounts were used to assess the acrosome morphology of the non-phagocytosed spermatozoa at the moment of fixation. A total of 100 cells were classified, using phase-contrast microscopy at × 400 magnification, as normal apical ridge, slightly altered normal apical ridge, damaged apical ridge, missing apical ridge and loose acrosomal cap, as described by Pursel et al. (1972). Spermatozoa with a normal or slightly altered apical ridge were considered to be acrosome intact and viable. Spermatozoa with a damaged apical ridge or a vesiculating or absent acrosomal cap were considered to be non-viable or dead. These assessments were also performed in the ‘blank’ samples before incubation, and after 15 and 60 min of incubation. In addition, in a number of unfixed samples taken at various time points during incubation, the percentage of motile spermatozoa was estimated at 38°C using phase-contrast microscopy at × 100 magnification.

Statistical methods

Data were analysed with a generalized linear mixed model (GLMM) using statistical procedures presented by Engel and Keen (1994 and 1996) and Engel and Buist (1996). Percentages (y) were modelled as $y = \frac{P}{100 - P}$, where ε is the error component and P is the ‘true’ percentage, that is, the percentage without error. Because the observations as percentages were limited to the interval 0–100, traditional statistical methodology, for example, ANOVA, was not appropriate. The true percentages were ‘stretched’ by using a logistic link function. The logit-transformed true percentages were modelled, similar to ANOVA, as a sum of the main effects and interactions of the experimental factors: $\logit(P) = \log\left(\frac{P}{100 - P}\right) = \sum$ of main effects and interaction terms. In contrast to ANOVA, the variance of the error term is not constant, but tends to be smaller for more extreme percentages. It is assumed that: $\text{Var}(\phi) = \phi P (100 - P)$, where φ will be estimated from the data and represents residual variation. The sum of effects included additional error terms, for example, random effects for samples or ejaculates, modelling the relationship between repeated observations on the same sample or ejaculate. Additional random effects were assumed to be distributed normally with corresponding components of variance. Since components of variance for experimental effects cannot be estimated with acceptable accuracy, these effects were entered as systematic effects and not as random effects. Consequently, standard errors of the means do not reflect variation between experiments, but variation within experiments only. All calculations were performed with the statistical programming language Genstat 5 (Genstat 5 committee, 1993), using procedures IRREML (Keen and Engel, 1998) for fitting the model, and VWALD (Buist and Engel, 1992) for testing main effects and interactions.
Results

Phagocytosis of spermatozoa

Spermatozoa were rapidly ingested when incubated with PMN. May–Grünwald/Giemsa-stained smears showed that most of the phagocytes were neutrophils (Fig. 2), but occasionally some phagocytosing monocytes were also found. The wet mounts of the fixed samples observed by phase-contrast and fluorescence microscopy showed spermatozoa in different stages of phagocytosis. The attachment of the spermatozoa started either at the tail or the head. The number of spermatozoa per phagocyte, partially or entirely ingested, varied from zero to four. The time course of phagocytosis was biphasic in the untreated semen, in the semen treated to induce capacitation in vitro and in the cold-shocked and frozen–thawed semen (Figs 3 and 4). Phagocytosis was rapid in the first 30–45 min of challenge, followed by a second phase in which phagocytosis proceeded more slowly.

After treatment to induce capacitation in vitro, spermatozoa showed hyperactivated motility and head-to-head agglutination. A subpopulation of the live (propidium iodide-negative) spermatozoa showed clearly an increased ability to bind solubilized zona pellucida proteins. The size of this subpopulation, expressed as a proportion of the total sperm population, was $41 \pm 22.0\%$ (mean $\pm$ sd; $n = 3$). Treatment of semen to induce capacitation in vitro before co-incubation with PMN greatly reduced phagocytosis of spermatozoa. At all time points during the 90 min challenge, the percentage of phagocytosed spermatozoa in this type of treated semen was significantly ($P < 0.05$) lower than in untreated semen (Fig. 3).

There was no significant difference in the percentage of phagocytosed spermatozoa between untreated, cold-shocked or frozen–thawed semen at 45, 60 and 90 min. After 15 min, the percentages of phagocytosed spermatozoa in the cold-shocked and in the frozen–thawed semen were significantly lower ($P < 0.05$) than that in the untreated semen (Fig. 4).

Whether the serum had been treated to inactivate complement had little effect on phagocytosis of spermatozoa in untreated, cold-shocked or frozen–thawed semen. The percentage of phagocytosed spermatozoa tended to be higher in medium with untreated serum than in medium containing serum with inactivated complement (Fig. 4a,b); however, this difference was significant only for frozen–thawed spermatozoa ($P < 0.05$). In semen that had been treated to induce capacitation in vitro, the percentage of phagocytosed spermatozoa was significantly higher in medium with untreated serum than in medium containing serum with inactivated complement (Fig. 3a,b).
In vitro phagocytosis of boar spermatozoa

Effects of several treatments of semen on the phagocytosis of boar spermatozoa during incubation at 38°C with polymorphonuclear leucocytes (PMN) from sows in the presence of either inactivated (a) or intact (b) complement. Untreated semen (■) was compared with cold-shocked (○) or frozen–thawed (△) semen. Mean ± SEM (n = 4). *P < 0.05 compared with both cold-shocked and frozen–thawed semen.

Nevertheless, the percentage of phagocytosed spermatozoa in semen that had been treated to induce capacitation in vitro was still significantly lower (P < 0.05) than that in untreated semen (Fig. 3b).

In TM without serum, very few frozen–thawed spermatozoa were phagocytosed. The average percentage of spermatozoa phagocytosed in reference semen (frozen–thawed, killed spermatozoa) after 60 min of incubation with PMN was 3.9 ± 1.1% in the absence of serum and 68.9 ± 22.5% in the presence of sow serum with inactivated complement (mean ± SD, n = 3) (P < 0.005). Inclusion of serum from other species (cattle, guinea-pig) produced similar results to those in medium without serum, that is, very few frozen–thawed spermatozoa were phagocytosed. Pre-incubation (‘opsonization’) of the frozen–thawed spermatozoa for 30 min in medium containing sow serum and inactivated complement, followed by washing three times in medium without serum, gave similar results to those observed in the presence of sow serum (one experiment, data not shown).

Live spermatozoa (fresh semen) were phagocytosed in the absence of serum. The average percentage of phagocytosed spermatozoa in fresh semen after 60 min of incubation with PMN was 34.4 ± 17.3% in the absence of serum and 51.1 ± 21.8% in the presence of sow serum with inactivated complement (mean ± SD, n = 3).

Sperm viability

In untreated semen and in semen that had been treated to induce capacitation in vitro, the percentage of motile spermatozoa was about 70%. The percentage of spermatozoa with an intact acrosome was not significantly lower in semen treated to induce capacitation in vitro than in untreated semen (Table 1). The percentage of propidium iodide-negative spermatozoa was significantly lower after treatment to induce capacitation in vitro. The average percentages of propidium iodide-negative spermatozoa were 87 ± 6.5 and 62 ± 20.0 for untreated and treated spermatozoa, respectively (mean ± SD, n = 3).

The presence of inactivated serum had little effect on the intactness of the acrosome of both untreated spermatozoa and spermatozoa that had been treated to induce capacitation in vitro. During incubation of spermatozoa under identical conditions as used for the phagocytosis assay, but in the absence of PMN (blank samples), the percentage of acrosome-intact spermatozoa decreased only slightly in untreated semen and in semen treated to induce capacitation in vitro (Table 1). The spermatozoa displayed considerable head-to-head agglutination. In contrast, when spermatozoa were mixed with TMs with intact complement, a rapid and complete arrest in motility (0% motile spermatozoa) was observed and almost all spermatozoa had a damaged (vesiculating or absent) acrosomal cap at 15 min after the addition of TMs (Table 1).

The presence of active phagocytosing PMN did not have an adverse effect on sperm viability. After 60 min of incubation with PMN in TMs containing inactivated complement, motility was observed in non-phagocytosed spermatozoa, and in spermatozoa that were attached to the PMN. Spermatozoa ingested by their tail were not motile, and spermatozoa that had been treated to induce capacitation in vitro were not significantly lower in semen treated to induce capacitation in vitro (Table 1).

The cold-shock treatment and the freezing and thawing treatment caused considerable damage to the spermatozoa. After the cold-shock treatment, 65 ± 9.4% of the spermatozoa had not been phagocytosed, of which 68 ± 13.2% were still acrosome intact (44 ± 9.9% of the total number of spermatozoa at time 0) (mean ± SD, n = 3).

The cold-shock treatment and the freezing and thawing treatment caused considerable damage to the spermatozoa. After the cold-shock treatment, 65 ± 29.4% (mean ± SD, n = 4) of the spermatozoa had damaged acrosomes, most of which were vesiculated or absent. After freezing and thawing, the acrosomes of all spermatozoa were vesiculated or were absent.
Table 1. Acrosomal intactness of untreated boar spermatozoa or boar spermatozoa treated to induce capacitation in vitro, before and after addition of modified Tyrode’s medium without polymorphonuclear leucocytes followed by incubation at 38°C

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>Complement</th>
<th>Untreated (n = 7)</th>
<th>Treated (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>95 (3.0)</td>
<td>92 (8.7)</td>
</tr>
<tr>
<td>15</td>
<td>Inactivated</td>
<td>92 (5.4)</td>
<td>89 (7.9)</td>
</tr>
<tr>
<td>60</td>
<td>Intact</td>
<td>81 (8.0)</td>
<td>81 (10.8)</td>
</tr>
<tr>
<td>15</td>
<td>Intact</td>
<td>5 (4.1)</td>
<td>11 (9.5)</td>
</tr>
<tr>
<td>60</td>
<td>Intact</td>
<td>2 (1.9)</td>
<td>3 (1.5)</td>
</tr>
</tbody>
</table>

Values are mean (± so).

Modified Tyrode’s medium was supplemented with 15% (v/v) sow serum; one volume spermatozoa:four volumes medium.

*Acrosome intact spermatozoa:spermatozoa with acrosomes with normal apical ridge.

†Just before addition of TM.

‡Inactivation of complement by incubation of serum for 30 min at 56°C.

Discussion

In this study, the influence of a number of treatments of boar spermatozoa on phagocytosis of the spermatozoa by PMN was investigated. An in vitro phagocytosis assay was used to compare untreated spermatozoa with spermatozoa that had been treated to induce capacitation in vitro and with cold-shocked or frozen–thawed spermatozoa. The role of untreated serum or serum that had been treated to inactivate complement was also studied.

Serum from sows was essential for phagocytosis of frozen–thawed spermatozoa, and it also stimulated phagocytosis of intact untreated spermatozoa. Serum from other species did not have the same effect as sow serum. The effect of sow serum was retained, at least in part, after thorough washing of spermatozoa that had been incubated with sow serum. This finding indicates that species-specific components of sow serum can bind to boar spermatozoa and mediate phagocytosis of spermatozoa by PMN. These components are thought to be antibodies and complement factors.

Complement factors were found to play a modest role. Whether the serum had been treated to inactivate complement had little effect on phagocytosis of spermatozoa. Although intact complement stimulated phagocytosis of spermatozoa that had been treated to induce capacitation in vitro, the presence of intact complement rapidly induced cell death and acrosomal vesiculation. Consequently, the spermatozoa could no longer be considered as ‘viable’ but were comparable to the killed frozen–thawed spermatozoa.

Another class of opsonins could be the so-called natural antibodies. In mammals, insemination of the female does not normally elicit a specific immune response (Hancock, 1984; Hogarth, 1982; Haas and Beer, 1986), that is, no specific anti-sperm antibodies are produced. However, in women, in very rare cases, a specific immune response is observed. This response is a pathological condition, which leads to reduced fertility (Hogarth, 1982; Hancock, 1984; Haas and Beer, 1986).

However, antibodies reactive with sperm proteins have been described in blood serum of normal healthy fertile males and females in a number of mammalian species (rabbits, mice, guinea-pigs and humans) (Symons, 1967; Johnson, 1968; Tung et al., 1976; Hancock, 1979a,b; Padma, 1979; Rodman et al., 1985). These antibodies are called ‘natural antibodies’ to indicate that they are present in the blood of all animals, even when the animals have had no contact with the antigen, for example, in juvenile individuals (Tung et al., 1976; Rodman et al., 1985). These antibodies can agglutinate homologous spermatozoa by cross-linking (Padma, 1979) and are also able to fix complement (Johnson 1968; Hancock, 1979a) and to cause cytolysis of spermatozoa in vitro (Hancock, 1979b). The latter is mediated by the membrane attack complex, formed after activation of complement by spermatozoa opsonized with antibodies (D’Cruz et al., 1991). If natural antibodies are present in sow serum and do exert such actions, this could explain observations of head-to-head agglutination of spermatozoa in the presence of serum with inactivated complement, and acrosomal exocytosis and cell death in the presence of serum with intact complement.

Intact (untreated) spermatozoa could also be phagocytosed in the absence of serum. This finding indicates that ligands for attachment are already present on the surface of the spermatozoa and on the PMN. A possible mechanism of phagocyte attachment in the absence of added opsonins could be lectin–carbohydrate interactions, as has been described for the serum-independent phagocytosis of bacteria by neutrophils or macrophages (Ofeck and Sharon, 1988). The complement receptor Cr3 (CD11b/CD18 β-integrin) is present on the cell surface of PMN and can act as a receptor for lectins (Gbarah et al., 1991) and carbohydrates (Thornton et al., 1996). Other carbohydrate-recognizing lectin-like molecules have also been described (Weir et al., 1981). On the surface of mammalian spermatozoa, there is a high incidence of glycosylated proteins and glycolipids (Klint et al., 1987; Tulsiani et al., 1993; Gadella et al., 1994; Nieto et al., 1997), as well as carbohydrate-binding proteins.
(Macek and Shur, 1988; Veselský et al., 1992; Dostálová et al., 1994). Therefore, a mechanism comparable to that of lectinophagocytosis of bacteria could be involved in the serum-independent phagocytosis of spermatozoa observed in the present study.

In the present study, treatment of semen to induce capacitation in vitro resulted in a substantial reduction in phagocytosis of spermatozoa. The in vitro capacitation treatment used induces a number of specific changes in boar spermatozoa (Yanagimachi, 1994; Harrison, 1996). Most importantly, these changes include acquisition of the ability to fertilize in vitro (Suzuki et al., 1994). Studies focusing on specific parts of the process have revealed acquisition of the ability to bind solubilized zona pellucida proteins (Harkema et al., 1998) and to bind intact oocytes (Clarke and Harrison, 1993). In the present study, treatment to induce capacitation in vitro resulted in the development of a subpopulation of propidium iodide-negative spermatozoa with increased ability to bind solubilized zona pellucida proteins. The percentage of spermatozoa that showed increased binding to solubilized zona pellucida proteins was in the same range as that reported by Harkema et al. (1998). Reduction of phagocytosis of spermatozoa after treatment to reduce capacitation in vitro indicates that this treatment induces a reduction in the number of binding sites at the sperm surface for PMN attachment. Treatment of boar spermatozoa to induce capacitation in vitro causes a number of changes in the sperm membrane. These changes include reorganization of membrane lipids (Cadella et al., 1994) and proteins (Saxena et al., 1986; Töpf-Petersen, 1990) as well as the release of some of the sperm associated (glyco) proteins (Dostálová et al., 1994; Ashworth et al., 1995). Therefore, such changes may also induce a reduction in the number of binding sites involved in the serum-dependent as well as the serum-independent phagocytic pathway.

Apart from the influence of in vitro capacitation treatment, this study investigated the effects of two crude treatments that greatly affect the membrane surface of the spermatozoa, that is, cold shock and freezing and thawing. After 15 min of incubation with PMN, significantly more intact spermatozoa were ingested than cold-shocked or frozen–thawed spermatozoa. However, these two treatments had little effect on the percentage of phagocytosed spermatozoa at the end of incubation. This finding indicates that the sperm damage induced by the cold-shock and the freezing and thawing treatments had only little effect on sperm phagocytosis. However, freezing and thawing resulted in an almost complete abolition of serum-independent phagocytosis. The loss of this phagocytic pathway may explain why the percentage of phagocytosed frozen–thawed spermatozoa after 15 min of incubation with PMN was lower than that of untreated spermatozoa, as the action of serum may require some time for opsonization by serum components. Most of the cold-shocked and all of the frozen–thawed spermatozoa had a vesiculated or absent acrosomal cap. This damage implies a marked alteration in the surface of the sperm head. Parts of the cell membrane are lost and the acrosomal contents and the surface of the inner acrosomal membrane become exposed (Plummer and Watson, 1988; Yanagimachi, 1994). The fact that freezing and thawing resulted in almost complete abolition of serum-independent phagocytosis indicates that the PMN binding sites involved in the serum-independent pathway may be lost during shedding of the acrosomal cap. Furthermore, new antigens are revealed that could be involved in antibody mediated phagocytosis.

In vivo, the narrow uterotubal junction forms an obstacle to the passage of spermatozoa into the oviducts. After insemination, the number of spermatozoa in the uterus must be high to allow build up of a sufficient oviductal sperm population. However, the uterus must be clean and ready to receive the young embryos as early as 48 h after ovulation (Oxenreider and Day, 1965). PMN are recruited in large numbers to the pig uterine lumen shortly after insemination (Lovell and Getty, 1968; Pursel et al., 1978) and rapidly start reducing the number of spermatozoa in the uterus by phagocytosis (Lovell and Getty, 1968; Pursel et al., 1978; Kamerman, 1994). It has been suggested that ageing, damaged or dead spermatozoa are the first targets of phagocytes (Symons, 1967; Moyer et al., 1970; Vogelpoel and Verhoef, 1985; D’Cruz et al., 1995). However, the results from the present study show that, in vitro, intact spermatozoa are phagocytosed to the same extent as, and initially more rapidly than, damaged or dead spermatozoa.

Results from the current study also indicate that the surface of viable spermatozoa provides PMN attachment sites that enable immediate phagocytosis of spermatozoa by PMN without opsonization, which could have two beneficial effects. Firstly, the rapid ingestion of spermatozoa results in a rapid reduction in the concentration of sperm antigen in the female genital tract in the sow, reducing the risk of eliciting a harmful immune reaction of the female against spermatozoa. Secondly, most spermatozoa would be eliminated while still alive and intact, which could minimize the antibody and complement dependent phagocytosis of dead spermatozoa. Human spermatozoa, in particular dead spermatozoa opsonized with serum components, stimulate PMN to produce reactive oxygen species (ROS) (Vogelpoel and Verhoef, 1985). Thus, rapid phagocytosis of intact spermatozoa would help to prevent ROS production, thus reducing damage to the remaining spermatozoa and the uterine environment.

Phagocytosis of spermatozoa in vitro was substantially reduced after treatment to induce capacitation in vitro. The minimum conditions necessary for in vitro capacitation of boar spermatozoa are well described (Yanagimachi, 1994; Harrison, 1996). The uterine environment in vivo also provides conditions for capacitation. Spermatozoa can be fully capacitated in the uterus without moving to the oviducts (Imai et al., 1979; Rath, 1992; Yanagimachi, 1994). Therefore, the reduction in phagocytosis observed after treatment to induce capacitation in vitro may also occur in vivo in the uterus. Thus, spermatozoa would acquire protection against phagocytosis while in the uterus, which would increase their chance to reach the isthmus and take part in fertilization.

The authors would like to thank the Union of Co-operative Pig AI stations, Netherlands, for their financial support, and E. D. Ekkel, C. E. Hack and A. J. Verhoeven for useful information and helpful discussions.
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