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Phagocytosis of boar spermatozoa *in vitro* and *in vivo*

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For successful conception, fertilization-competent spermatozoa must be present at the site of fertilization in adequate numbers until ovulation has taken place. In pigs, a large volume of semen is delivered into the uterus. Most, if not all, of the inseminated liquid is voided from the vulva within a few hours after insemination and approximately 45% of the spermatozoa are lost. Large numbers of spermatozoa are also lost due to phagocytosis by polymorphonuclear leukocytes (PMNs). In pigs, the recruitment of PMNs to the uterine lumen appears to be triggered by insemination of a volume of liquid, rather than by specific components of that liquid or by spermatozoa or seminal plasma. However, persistence of large numbers of PMNs in the uterine lumen at > 12 h after insemination appears to depend on the presence of spermatozoa in the inseminate. *In vitro* studies have indicated that damaged, killed or capacitated spermatozoa are not phagocytosed preferentially, but that capacitation treatment strongly reduced phagocytosis of spermatozoa. Recent studies have also shown that PMN recruitment and phagocytosis of spermatozoa *in vivo* can be reduced by addition of caffeine plus $CaCl_2$ to the inseminate, which appeared to have positive consequences for the longer term availability of spermatozoa at the site of fertilization.

Introduction

The purpose of insemination is to establish and maintain an adequate population of spermatozoa at the site of fertilization until ovulation has taken place. The number of spermatozoa used for artificial insemination (AI) of pigs is typically $2.5–3.0 \times 10^9$. There is interest in the pig AI industry to reduce the sperm dosage, but it is feared that this might be at the expense of fertilization, especially when sows ovulate a long time after insemination. The duration of the period between insemination and ovulation can be quite long, as the time of ovulation relative to the onset of oestrous behaviour cannot be predicted exactly and varies greatly among sows (Soede *et al.*, 1995). Studies have shown that at the sperm dosage used at present, the results of fertilization are good when ovulation takes place within 24 h after insemination. When ovulation takes place > 24 h after insemination, the farrowing rate decreases appreciably (Soede *et al.*, 1995). This effect was not prevented by using a sperm dosage as high as 6×10^9 spermatozoa per insemination (Steverink *et al.*, 1997). It is important to understand the dynamics in the sperm populations in the genital tract after

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insemination to optimize the number of inseminations per oestrus and the sperm dosage per insemination.

The dynamics of sperm populations in the uterus and oviducts of pigs have been investigated extensively. One of the striking features is the rapid decrease in the number of spermatozoa in the genital tract after insemination (du Mesnil du Buisson and Dauzier, 1955a; First *et al.*, 1968; Pursel *et al.*, 1978). The large volume of the ejaculate and the large number of spermatozoa inundate the length of the uterine horns with semen to enable a build-up of adequate sperm populations in the uterotubal junctions and oviducts. However, the uterine horns must be cleared for the receipt of the embryos, which may arrive as early as 48 h after ovulation. A number of recent studies have contributed to our understanding of the involvement of polymorphonuclear leukocytes (PMNs) in the clearance of spermatozoa from the uterus of sows. These studies have dealt with subjects including mechanisms of recruitment of PMNs to the uterine lumen, mechanisms of attachment of spermatozoa to PMNs and the possibility of modulating the phagocytosis of boar spermatozoa *in vitro* and *in vivo*, which is discussed in this review.

Sperm transport

Cervix and uterus

In natural mating and artificial insemination of pigs the semen is deposited in the cervix, corpus uteri and uterine horns (Burger, 1952; du Mesnil du Buisson and Dauzier, 1955b). Pressure exerted during insemination as well as contractions of the uterine horns facilitate the flow of the semen into and through the length of the horns (Burger, 1952; Baker and Degen, 1972). Some of the semen may be lost by backflow during insemination. Loss of semen during artificial insemination can be minimized by using an inseminate volume of 100 ml rather than 200 ml (Baker *et al.*, 1968). However, these authors suggested that an inseminate volume of 20 ml may be too small for optimal transport of spermatozoa to the tip of the uterine horn. The large volume of the ejaculate of boars ensures that spermatozoa are washed into the horns; indeed, the entire length of the uterine horns is inundated with semen (Burger, 1952).

No significant amount of bulk fluid can be recovered when the uterine horns are inspected a few hours after insemination (du Mesnil du Buisson and Dauzier, 1955a; Hunter, 1981). Originally, it was believed that much or all of the liquid introduced into the uterus is resorbed by the genital tract (du Mesnil du Buisson and Dauzier, 1955a; First *et al.*, 1968; Engelhardt *et al.*, 1997). However, Blandau and Gaddum-Rosse (1974) stated that most of the semen is discharged through the vulva within 2 h after mating. Viring and Einarsson (1981) collected the backflow using colostomy bags attached around the vulva and found that the backflow of semen during the first few hours after insemination can be quite large. More recent studies have shown that the cumulative volume of backflow is equal to the inseminate volume on average (Kamerman, 1994; Steverink *et al.*, 1998; Matthijs *et al.*, 2000a). This finding indicates that none or very little of the inseminated liquid is resorbed by the uterus. It could be argued that the resorption of liquid by epithelia depends on the availability of the appropriate salts. The extender Beltsville thawing solution (BTS; Johnson *et al.*, 1988), which we have used in our studies, contains glucose as its primary osmotic support. However, comparable volumes of backflow were recovered after insemination with either BTS, Tyrode's *in vitro* fertilization (IVF) medium, phosphate buffered saline (PBS) or human infant oral rehydration solution (A. Matthijs, unpublished); the same appears to be true for seminal plasma after natural mating (Viring and Einarsson, 1981).

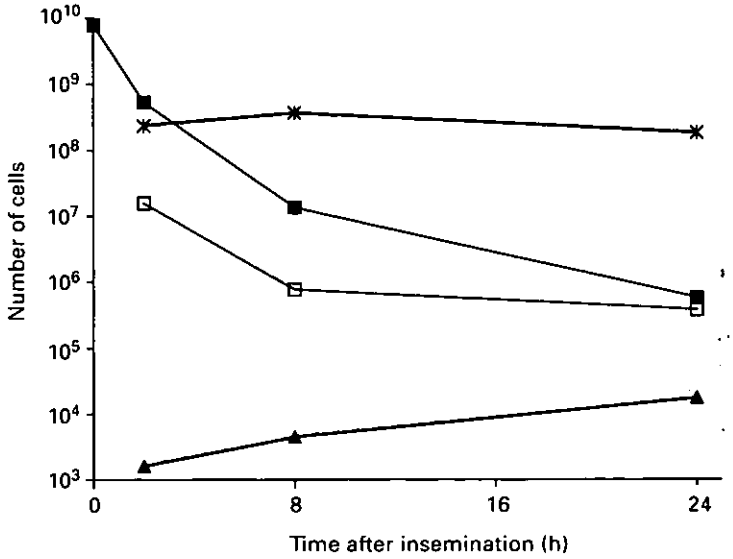

Fig. 1. Number of spermatozoa in uterus (■), uterotubal junction (□) and oviducts (▲), and number of polymorphonuclear leukocytes in the uterus (*), from 0 h to 24 h after insemination of gilts. Note logarithmic scale on *y* axis. Data from Pursel *et al.* (1978).

Although almost the entire volume of inseminate is lost by backflow, it is obvious that not all of the spermatozoa are lost with it. Apparently, many spermatozoa are able to move from the bulk liquid into the mucus lining the epithelium and are retained. Viring and Einarsson (1981) estimated that approximately one-third of the inseminated spermatozoa is lost by backflow of semen after natural mating. After artificial insemination with an inseminate volume of 80 ml, up to 45% of the inseminated spermatozoa are lost within 4 h after insemination (Kamerman, 1994; Steverink *et al.*, 1998; Matthijs *et al.*, 2000a).

The number of spermatozoa in the uterus decreases markedly within a few hours after insemination (du Mesnil du Buisson and Dauzier, 1955a; First *et al.*, 1968; Pursel *et al.*, 1978) (Fig. 1). Initially, the backflow of semen plays a major role in this reduction. Phagocytosis of spermatozoa by PMNs is another important mechanism. Although First *et al.* (1968) did not observe phagocytosis of spermatozoa before 8 h after insemination, there is increasing evidence that phagocytosis of spermatozoa starts soon after insemination and that it is the major mechanism of clearance of spermatozoa from the genital tract (Lovell and Getty, 1968; Pursel *et al.*, 1978; Rozeboom *et al.*, 1998, 1999; Matthijs *et al.*, 2000a).

The oviducts

Spermatozoa appear in the oviducts as early as 5–15 min after insemination (Burger, 1952; Baker and Degen, 1972). Dead spermatozoa are also transported to the oviducts, but less efficiently than are live spermatozoa (First *et al.*, 1968; Baker and Degen, 1972; Viring, 1980). Radioactive molecules of different sizes, including macromolecules, that were added to the inseminate were traced in the oviducts of sows killed at 1 h after insemination (Einarsson *et al.*, 1980; Viring *et al.*, 1980). By interrupting sperm transport to the isthmus at different times after mating, Hunter (1981) demonstrated that early transport of spermatozoa to the isthmus within the first 30 min after mating is rapid enough to allow fertilization of oocytes. The

number of spermatozoa available in the isthmus continued to increase after that time, as after the first 30 min, the number of accessory spermatozoa on the oocytes increased when sperm transport was allowed to continue for longer. Moreover, a prolonged build-up of the isthmus population of spermatozoa for at least 60 min was required to provide a sperm population that could be maintained at a sufficient level for > 24 h in gilts that were inseminated 1 day too early. The sperm population in the oviducts is maintained and increased by ongoing sperm migration from the uterine horns and uterotubal junction during the first 24 h after insemination (Rigby, 1966; Pursel *et al.*, 1978). Baker and Degen (1972) stated that the uterus continues to supply spermatozoa to the oviducts, while at the same time spermatozoa are lost from the oviducts through the infundibulum to the peritoneal cavity. In other studies, the net size of the oviductal sperm population decreases from 2 h after insemination onwards, parallel with a rapid decrease in the number of spermatozoa in the uterus and uterotubal junction (Viring, 1980; Viring and Einarsson, 1981), or stays approximately constant over the first 24 h after insemination (First *et al.*, 1968).

Blandau and Gaddum-Rosse (1974) reported that boluses of liquid are moved up the isthmus towards the isthmic–ampullar junction by continuous antiperistaltic contractions in the oviducts. Comparable waves in the ampulla carry the liquid up to the infundibulum. In addition, an upward directed ciliary beat contributes to the upward transport in the oviducts (Gaddum-Rosse and Blandau, 1973). These mechanisms probably contribute to the upward movement of spermatozoa in the isthmus towards the site of fertilization and, ultimately, towards the peritoneal cavity (Baker and Degen, 1972; Blandau and Gaddum-Rosse, 1974). Indeed, spermatozoa are found in all segments of the oviducts (du Mesnil du Buisson and Dauzier, 1955a; Viring, 1980; Viring and Einarsson, 1980, 1981). Hunter (1984) showed that shortly after insemination the number of spermatozoa present in the proximal part of the isthmus was high enough to support fertilization. However, when continuation of the supply of spermatozoa from the lower isthmus was obstructed by ligation, the population of spermatozoa in the proximal isthmus appeared to be depleted within several hours, as it could no longer support fertilization of oocytes a few hours after ligation. This finding indicates that either the higher isthmus sperm population is relatively small or that the spermatozoa are moved away by the upward transport relatively fast, such that this population is depleted quite rapidly when supply from the lower isthmus is obstructed. Indeed, most studies have indicated that more spermatozoa are found in the isthmus than in the ampulla and that more spermatozoa are found in the distal part of the isthmus, which is closest to the uterotubal junction, than in the proximal part of the isthmus (du Mesnil du Buisson and Dauzier, 1955a; Viring, 1980; Viring and Einarsson, 1980, 1981). Thus, it appears that spermatozoa are retarded in the distal isthmus such that a relatively large population of spermatozoa builds up and is maintained for a considerable time, while feeding a smaller sperm population in the higher isthmus, as spermatozoa are gradually moved upwards and are ultimately lost in the peritoneal cavity. The mechanisms of retardation are assumed to comprise physical obstruction (retained in crypts and crevices), binding to epithelium or suppression of motility by other mechanisms (Katz *et al.*, 1989; Hunter, 1990; Suarez *et al.*, 1991).

Recruitment of PMNs

Insemination of pigs triggers a massive influx of PMNs into the lumen of the uterus (Lovell and Getty, 1968; Pursel *et al.*, 1978; Rozeboom *et al.*, 1998, 1999; Matthijs *et al.*, 2000a). Negligible numbers of PMNs are found in the lumen of the genital tract in sows in the luteal phase (Matthijs *et al.*, 2000a). Considerable numbers of PMNs can be found in the lumen of uninseminated oestrous sows, but not as many as in inseminated sows (Matthijs *et al.*, 2000a).

Extensive infiltration of PMNs into the basal region of the uterine epithelium of pigs occurs at pro-oestrus (Bischof *et al.*, 1994; Engelhardt *et al.*, 1997), which is probably generated under the direct or indirect control of sex hormones. Some studies have revealed a hormone-controlled synthesis of cytokines, such as the granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 6 (IL-6) in mice (Robertson and Seamark, 1992), and IL-8 in humans (Arici *et al.*, 1998). These cytokines are thought to be involved in the migration of PMNs into the endometrial stroma.

The number of PMNs recruited to the uterine lumen after insemination of sows is quite large. Matthijs *et al.* (2000a) studied the total number of PMNs recruited and the number of phagocytosed and non-phagocytosed spermatozoa. Colostomy bags were attached around the vulva to collect the backflow of semen and all parts of the genital tract were flushed after the sows were killed at 4 h after artificial insemination with 2.4×10^9 spermatozoa. In the inseminated sows, the total number of PMNs was at least nine times higher ($P < 0.01$) than in uninseminated oestrous sows. The total number of PMNs recovered was approximately $3–5 \times 10^9$, which is more than the number of inseminated spermatozoa. At 4 h after insemination, approximately 30 times more PMNs than non-phagocytosed spermatozoa were found in the uterus of sows. The numbers of PMNs found in the uterus were in accordance with those reported by Pursel *et al.* (1978). The number of PMNs reported by Matthijs *et al.* (2000a) was somewhat higher, but these workers used adult multiparous sows compared with nulliparous sows in the study of Pursel *et al.* (1978).

The recruitment of PMNs to the uterine lumen probably starts immediately after insemination. Lovell and Getty (1968) found no PMNs in the luminal contents of pigs at 10 min after mating, but found large numbers (only four times lower than the number of spermatozoa) at only 30 min after mating. Pursel *et al.* (1978) reported large numbers of PMNs in the uterine lumen at 2 h after insemination. Moreover, the backflow of semen collected in the first hour after insemination contained large numbers of PMNs (Matthijs *et al.*, 2000a).

In sows evaluated at 4 h after insemination, it was clear that the recruitment of PMNs was triggered by the intromission of liquid into the uterus, rather than by the presence of spermatozoa or seminal plasma (Matthijs *et al.*, 2000a). The number of PMNs recruited in sows inseminated with semen extender (BTS) only was not significantly different (in fact tended to be higher) from that in sows inseminated with extended boar semen or seminal plasma in BTS. The number of PMNs recovered from sows inseminated with Tyrode's medium, PBS or 'oral rehydration solution' was similar to the number of PMNs recovered from sows inseminated with BTS. These data demonstrate that it is not an active substance in BTS that is causing the PMN recruitment, but rather the intromission of a volume of liquid. These results are consistent with those of Rozeboom *et al.* (1998, 1999), who found no difference in the numbers of PMNs in uterine lavages collected at 6 h after insemination of gilts with either extended semen, seminal plasma or extender only. The results of Matthijs *et al.* (2000a) (at 4 h after insemination) indicated that seminal plasma may even have a dampening effect, as the inseminates that contained seminal plasma tended to have a lower PMN response than did the inseminates with little or no seminal plasma, although this difference was not significant. Rozeboom *et al.* (1998, 1999) did not find a suppressing effect of seminal plasma at 6 h after insemination, but did detect a significant effect of pure (undiluted) seminal plasma on the persistence of the PMNs in the uterus at 12–24 h after insemination. In contrast to the earlier PMN recruitment, the continuation of PMN recruitment appeared to be triggered specifically by spermatozoa, as Rozeboom *et al.* (1998, 1999) reported that large numbers of PMNs persisted from 12 h to 36 h after insemination only if the inseminate contained spermatozoa. By this time, most of the spermatozoa have already been cleared from the genital tract. Thus, this second wave of PMN recruitment does not have a role in the initial rapid loss of most of

the spermatozoa, but may be needed for the final stage of clearing of the uterus in preparation for the arrival of the conceptuses.

Phagocytosis of spermatozoa

PMNs are the main phagocytes involved in the clearance of spermatozoa from the genital tract of pigs. Spermatozoa are found attached to and ingested by PMNs in the uterine lumen, whereas the number of free spermatozoa decreases markedly to just a few per cent of the inseminated number of spermatozoa within only a few hours (Lovell and Getty, 1968; First *et al.*, 1968; Pursel *et al.*, 1978; Viring, 1980; Kamerman, 1994) and reaches as low as 1% at 24 h after insemination (First *et al.*, 1968; Pursel *et al.*, 1978). The clearance of spermatozoa from the uterus after insemination has also been observed in other mammalian species. Uterine clearance is a normal physiological process that serves to prepare the uterus for the reception of the embryos, which may arrive as early as 48 h after ovulation in pigs (Oxenreider and Day, 1965).

Several authors have hypothesized that phagocytosis of spermatozoa may also be a mechanism for the selection of superior spermatozoa (for example see Symons, 1967; Cohen and Tyler, 1980; D'Cruz and Haas, 1995). One reason for this suggestion was the apparent redundancy of spermatozoa in most species. However, in terms of biomass or bioenergy the male ejaculate is not very extravagant (Kemp *et al.*, 1990) and competition between males may have been a selection force to favour increased numbers of spermatozoa, at least in promiscuous species (Birkhead and Møller, 1998). Nevertheless, the large number of spermatozoa per ejaculate prompted the hypothesis that only a very small percentage of the spermatozoa are genetically intact (Cohen, 1973). Selection of human spermatozoa on the basis of their chromosomal defects has been reported (Ibrahim and Pedersen, 1988; Van Dyk *et al.*, 2000). However, this selection is probably caused by selection of spermatozoa on the basis of cellular morphological or functional abnormalities that coincide with genetic defects. Such a coincidence can occur when local, temporal or patient-related conditions in the testes are unfavourable. Moreover, the results of IVF and intracytoplasmic sperm injection (ICSI) indicate that it is unlikely that most spermatozoa are genetically defective.

Another hypothesis is that senescent, dead or prematurely capacitated spermatozoa are ingested preferentially by phagocytes (for example see Symons, 1967; Cohen and Tyler, 1980; D'Cruz and Haas, 1995). However, in a recent study of phagocytosis of boar spermatozoa by PMNs *in vitro*, it was shown that damaged or dead spermatozoa are not targeted preferentially and that motile, intact spermatozoa were actually phagocytosed faster than were killed spermatozoa (Matthijs *et al.*, 2000b). Moreover, in regular pig AI almost all the spermatozoa of a fresh inseminate are intact, whereas phagocytosis in sows *in vivo* starts almost immediately after insemination (Lovell and Getty, 1968; Pursel *et al.*, 1978; Rozeboom *et al.*, 1998, 1999; Matthijs *et al.*, 2000a).

It is important to note that clearance of spermatozoa from the genital tract by PMNs is not a specific immune response. The female genital tract is fully capable of generating an immune response to foreign material (Hogarth, 1982). Moreover, spermatozoa are highly antigenic when introduced into the peripheral blood circulation (Hancock, 1981; Hogarth, 1982). However, insemination does not elicit a cell-mediated antibody immune response in healthy females (Hancock, 1981; Hogarth, 1982). It is very important that a classical immune response is prevented, as this would lead to the development of sterilizing anti-sperm immunity, which does occur sometimes as a pathological condition (Hogarth, 1982; Hancock, 1984; Haas and Beer, 1986). PMNs enhance their non-immunological receptors while migrating towards the lumen of the uterus (Targowski and Niemialtowski, 1986). In

healthy females, spermatozoa in the female genital tract are not destroyed by the membrane attack complex of the complement system, although complement factors (Bedford and Witkin, 1983; Hasty *et al.*, 1994) and antibodies (Symons and Herbert, 1971; Hussein *et al.*, 1983; Haas and Beer, 1986) are present in the genital tract. Complement and antibody concentrations are low in fertile women and sows (Hussein *et al.*, 1983; Haas and Beer, 1986), although Cohen (1984) reported an increase in antibody concentrations during the massive influx of neutrophils in rabbits after insemination. It has been proposed that the blocking of sperm antigens by the so-called 'natural antibodies' (Hancock, 1981) might help to prevent a classical immune response. Furthermore, it has been reported that seminal plasma can have a suppressive effect on proliferation of lymphocytes (Bouvet *et al.*, 1987; Veselský *et al.*, 1991), antibody response (Dostál *et al.*, 1997), phagocytic activity (Lazarevic *et al.*, 1995) and complement activation (Chowdhury *et al.*, 1996). Moreover, in humans, glycosaminoglycans, such as heparin, which are present in the female genital tract, are also able to inhibit complement activation (Ekre *et al.*, 1992). In pigs, glycosaminoglycans can abolish the toxic effect of intact complement of sow serum on boar spermatozoa *in vitro* (A. Matthijs, unpublished). These mechanisms may help to prevent an immunological reaction of the female and protect the spermatozoa from being destroyed by the membrane attack complex.

In vitro studies: mechanisms of attachment

Opsonin-dependent phagocytosis

The phagocytosis of boar spermatozoa has been studied using an *in vitro* phagocytosis assay (Matthijs *et al.*, 2000b). The phagocytosis of spermatozoa was studied by challenging PMNs isolated from the peripheral blood of sows with boar spermatozoa in a controlled environment. These studies were used to gain insights into the role of opsonins in attachment and phagocytosis of spermatozoa, as well as the phagocytosis of spermatozoa of different health or capacitation status. These studies demonstrated that the presence of serum from sows was essential for phagocytosis of killed spermatozoa and also appeared to stimulate phagocytosis of fresh, intact spermatozoa. In Tyrode's medium containing sow serum with inactivated complement, approximately 70% of the spermatozoa were phagocytosed during 60 min of incubation with PMNs. However, in the absence of serum, the phagocytosis of killed (frozen–thawed) spermatozoa was almost completely absent. Inclusion of serum from other species (cattle and guinea-pigs) produced results comparable to medium without serum (virtual absence of phagocytosis of frozen–thawed spermatozoa). The effect of sow serum was retained, at least in part, after thorough washing of spermatozoa that had been pre-incubated with sow serum. These results demonstrate that species-specific components of sow serum, which are probably antibodies or complement factors, can bind to boar spermatozoa and mediate phagocytosis of spermatozoa by PMNs.

Active complement factors play only a modest role. Phagocytosis of spermatozoa was hardly affected by whether the serum used had been treated to inactivate complement. However, the presence of intact complement rapidly induced cell death and acrosomal vesiculation.

Another class of opsonins could be the so-called 'natural antibodies'. As discussed above, insemination of females does not usually elicit a specific immune response (Hancock, 1981; Hogarth, 1982; Haas and Beer, 1986), such that no specific anti-sperm antibodies are produced. 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 (for example see Symons, 1967; Tung *et al.*, 1976; Hancock, 1979). These antibodies are called

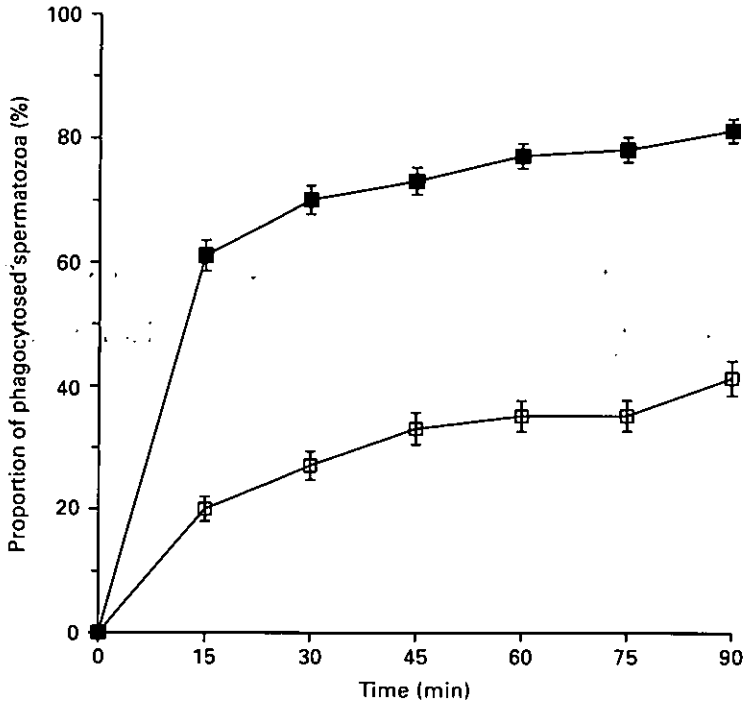

Fig. 2. Effect of *in vitro* capacitation treatment on the phagocytosis of boar spermatozoa by polymorphonuclear leukocytes *in vitro*. Fresh boar semen was obtained from the artificial insemination station at Bunnik, NL. Untreated semen (■) was compared with treated semen (□) (Matthijs *et al.*, 2000b).

'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 juveniles.

Opsonin-independent phagocytosis of fresh spermatozoa

In contrast to killed spermatozoa, intact (untreated) spermatozoa were phagocytosed in the absence of serum (Matthijs *et al.*, 2000b). This finding indicates that ligands for PMN attachment are already present on the surface of spermatozoa. 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 (Ofec and Sharon, 1988). The complement receptor Cr3 (CD11b/CD18 β-integrin) on the surface of PMNs can act as a receptor for lectins (Gbarah *et al.*, 1991), as well as for specific carbohydrates (Thornton *et al.*, 1996). Other carbohydrate-recognizing lectin-like molecules have also been described (Weir *et al.*, 1981). There is a high incidence of glycosylated proteins and glycolipids, as well as carbohydrate-binding proteins, on the surface of mammalian spermatozoa (for example see Klint *et al.*, 1987; Macek and Shur, 1988; Dostàlovà *et al.*, 1994; Gadella *et al.*, 1994).

Influence of capacitation treatment

In the *in vitro* model, it was found that treatment of semen to induce capacitation *in vitro* resulted in a substantial reduction of sperm phagocytosis (Fig. 2; Matthijs *et al.*, 2000b). Thus,

this treatment appears to induce a decreased number of binding sites for PMN attachment on the sperm surface. *In vitro* capacitation treatment of boar spermatozoa induces a great number of changes on the surface of the sperm membrane (Töpfer-Petersen *et al.*, 1990; Dostàlovà *et al.*, 1994; Gadella *et al.*, 1994; Ashworth *et al.*, 1995).

The conditions necessary for *in vitro* capacitation of boar spermatozoa have been well described (Yanagimachi, 1994; Harrison *et al.*, 1996). *In vivo* the uterine environment provides these conditions. Spermatozoa can be capacitated fully in the uterus without ascending to the oviducts (Imai *et al.*, 1979; Rath, 1992; Yanagimachi, 1994). Therefore, Matthijs *et al.* (2000b) suggested that the reduction of phagocytosis, as observed after *in vitro* capacitation treatment, could also be produced *in vivo* in the uterus. Thus, spermatozoa could acquire protection against phagocytosis while in the uterus, which would increase their chances of reaching the isthmus and taking part in fertilization.

Modulation of phagocytosis and recruitment

As discussed above, the clearance of spermatozoa from the uterus by phagocytosis of spermatozoa is a normal and necessary process. Nevertheless, it is an attractive proposition to inhibit or postpone the phagocytosis to some extent, so that perhaps successful artificial insemination could be achieved with a reduced dose of spermatozoa. For this reason we have been interested in substances that could affect phagocytosis of boar spermatozoa by PMNs.

In vitro *studies*

Several studies have shown that divalent cations such as Ca^{2+} and Mg^{2+} play an important role in the mechanism of adherence and ingestion of particles by PMNs (Cox and Karnovsky, 1973; Carrasco *et al.*, 1997). This is also true for boar spermatozoa; using an *in vitro* model we found complete inhibition of the ingestion of boar spermatozoa by PMNs in the presence of calcium chelator EDTA (data not shown; H. Woelders and A. Matthijs, unpublished).

EDTA inhibits phagocytosis through its effect on the phagocytotic activity of PMNs, whereas other classes of compound reduce the rate of phagocytosis by a direct effect on the spermatozoa. For example, glycosaminoglycans, notably heparin, reduced phagocytosis of boar spermatozoa by PMNs *in vitro* markedly (data not shown). However, the effect of glycosaminoglycans was lost when the cells were washed in a glycosaminoglycan-free medium. Caffeine is also able to reduce sperm phagocytosis by PMNs. In the *in vitro* phagocytosis model, 1 mmol caffeine l^{-1} reduced the rate of phagocytosis of boar spermatozoa (Fig. 3). The effect was observed immediately, but was stronger after the spermatozoa had been pre-incubated at 38°C in BTS in the presence of caffeine for 30 min. The pre-incubation was more effective when $CaCl_2$ was added (Fig. 3). The reduction of phagocytosis is due to an effect on the spermatozoa, rather than on the PMNs, as the reduction was obtained after the spermatozoa had been pre-incubated with caffeine. Treatment of the semen to induce capacitation also causes a strong reduction of phagocytosis *in vitro* (Matthijs *et al.*, 2000b). Caffeine may accelerate some of the changes related to sperm capacitation (Harrison *et al.*, 1993, 1996; Fraser, 1995). However, caffeine alone cannot induce capacitation. Moreover, capacitation cannot proceed in the presence of EDTA without added calcium (Fraser, 1992, 1995; Harkema *et al.*, 1998) and is also inhibited by the presence of seminal plasma (Nagai *et al.*, 1984; Fraser, 1992). The effect of caffeine on phagocytosis of spermatozoa was observed when boar spermatozoa were incubated in normal BTS in the presence of seminal plasma, although the effect of caffeine was more pronounced when $CaCl_2$ was added (Fig. 3). This finding indicates that capacitation is not a necessary

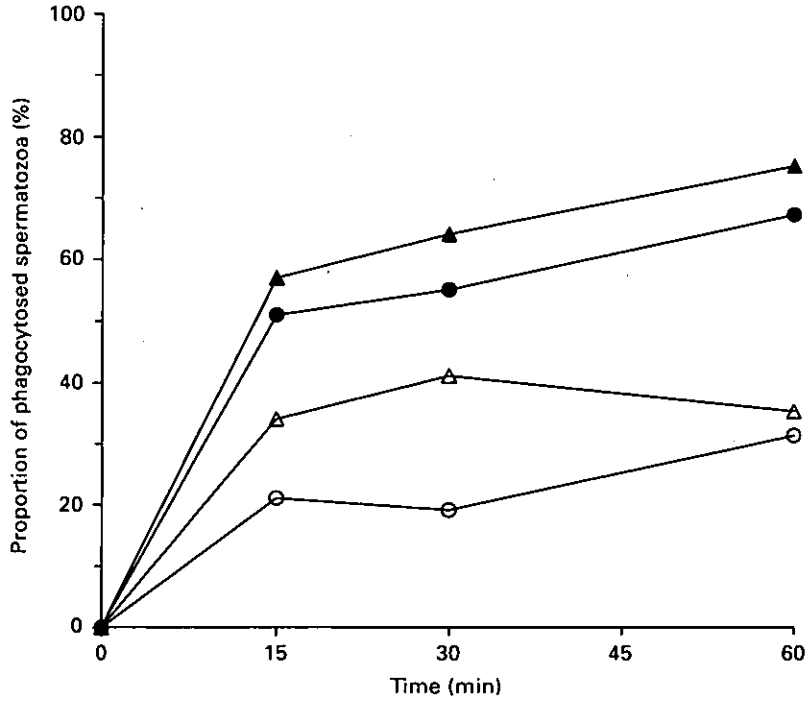

Fig. 3. Effect of pretreatment of boar spermatozoa with caffeine plus $CaCl_2$ on phagocytosis by polymorphonuclear leukocytes (PMNs) *in vitro*. Fresh boar semen was obtained from the artificial insemination station at Bunnik, NL. The spermatozoa were incubated for 30 min at 38°C in regular Beltsville thawing solution (BTS; ▲), or in BTS plus 6 mmol $CaCl_2$ l^{-1} (●), BTS plus 6 mmol $CaCl_2$ plus 1 mmol caffeine l^{-1} (○) or BTS with 1 mmol caffeine l^{-1} (△). The spermatozoa were challenged with PMNs in Tyrode's medium as described by Matthijs *et al.* (2000b).

precondition for the effect of caffeine on phagocytosis. However, it seems likely that there is overlap in the cell biological and biochemical mechanisms that lead to reduction of phagocytosis and capacitation, respectively. Caffeine is a phosphodiesterase inhibitor. Addition of dibutyryl cAMP, a membrane permeable analogue of cAMP, also resulted in a reduction of phagocytosis of boar spermatozoa by PMNs *in vitro* (A. Matthijs, unpublished). Therefore, it is likely that the effect of caffeine is mediated by an increase in the intracellular concentration of cyclic adenosine 3',5'-monophosphate (cAMP).

In vivo *studies: effect on number of spermatozoa and PMN recruitment*

The possibility of reducing the phagocytosis of spermatozoa *in vivo* was tested in several insemination experiments. EDTA, which is effective *in vitro*, is a normal component of the boar semen extender BTS. Nevertheless, phagocytosis of spermatozoa appears to start shortly after insemination. This finding indicates that after much of the liquid of the inseminate has been voided, the free calcium concentration in the remaining uterine fluid is increased to concentrations that permit phagocytosis. In an attempt to over-ride this effect, we attempted to inhibit phagocytosis *in vivo* by using a high concentration of EDTA (25 mmol l^{-1}) in adapted BTS extender. At 4 h after insemination the number of non-phagocytosed spermatozoa found

in the genital tract was not different from that in sows inseminated with extended semen in normal BTS. As brightly fluorescent spermatozoa (stained with Hoechst 33342) were used, the nuclei of phagocytosed spermatozoa could be recognized inside PMNs unless they were digested completely. In the *in vitro* model, spermatozoa were digested inside the PMNs within 1 h after phagocytosis. In the sows that received an inseminate containing 25 mmol EDTA l^{-1}, the number of non-phagocytosed spermatozoa found in the genital tract at 4 h after insemination was not different from that in the control group. However, the number of spermatozoa that could be recognized inside PMNs was much higher. These results indicate that the onset of phagocytosis was delayed initially by the extra EDTA, but that, once started, the rate of phagocytosis in the EDTA group was higher than that in the control group.

In the same experiment, a group of sows was included that were inseminated with inseminates containing caffeine plus $CaCl_2$ (Woelders *et al.*, 2000). In the caffeine plus $CaCl_2$ group, the number of non-phagocytosed spermatozoa present in the uterus at 4 h after insemination was 12 times higher ($P < 0.001$) than in the control group. It is possible that the phagocytosis of spermatozoa was reduced by the direct effect of caffeine plus $CaCl_2$ on spermatozoa, as observed in the *in vitro* phagocytosis model. In addition, the presence of $CaCl_2$ plus caffeine could potentially accelerate sperm capacitation in the uterus and thus influence sperm phagocytosis (compare with Fig. 2). However, a striking effect, which is likely to have contributed to the reduced loss of spermatozoa, was that the addition of caffeine plus $CaCl_2$ strongly reduced the recruitment of PMNs. The total number of PMNs found in animals at 4 h after insemination was three times lower ($P < 0.05$) in the caffeine plus $CaCl_2$ group than in the control group.

The PMNs are thought to be recruited from a large population in the basal region of the uterine epithelium (Bischof *et al.*, 1994; Engelhardt *et al.*, 1997). It is not known whether the reduction of recruitment is due to a direct effect of caffeine on the PMNs or to an effect of caffeine on the endometrium. It has been reported that phosphodiesterase inhibitors are able to restrict mobility and chemotactic activity of PMNs *in vitro* by increasing the intracellular, or perhaps also the extracellular, cAMP concentration (Hill *et al.*, 1975; Rivkin *et al.*, 1975; Carrasco *et al.*, 1997). *In vivo*, such a mechanism might result in restriction of the movement of PMNs from the endometrium into the uterine lumen. However, it does not necessarily inhibit the ability of PMNs to phagocytose once recruited. In fact, caffeine stimulates phagocytotic activity of PMNs, possibly by its ability to stimulate an increase in the intracellular concentration of free calcium in the PMNs (Carrasco *et al.*, 1997).

Although addition of caffeine plus $CaCl_2$ to the inseminate resulted in a much larger number of non-phagocytosed spermatozoa at 4 h after insemination, the number of spermatozoa recovered from the oviducts was not significantly different. However, it has been reported that the uterine sperm population can continue to play a role in replenishment of oviduct sperm populations for up to 24 h after insemination. Thus, the effect of caffeine plus $CaCl_2$ could be beneficial for increasing the likelihood of fertilization, especially when ovulation takes place a long time after insemination.

In vivo *studies: effects on fertilization*

In a subsequent insemination experiment, sows were inseminated with a reduced sperm dosage of only 0.5×10^9 spermatozoa either 26 h before ovulation or at 4 h after ovulation, as determined by transrectal ultrasonography (Woelders *et al.*, 2000). The sows received either inseminates containing caffeine plus $CaCl_2$, which was added just before insemination, or control inseminates ($n = 15$ or 16 per group). The sows were killed on day 5 after ovulation. The observed percentage of sows with > 80% fertilized oocytes (morphologically intact

embryos as a percentage of total recovered oocytes plus embryos) in the caffeine plus $CaCl_2$ group was considerably higher than that in the control group, but the difference was not significant. The observed mean number of cells per embryo also appeared to be higher in the caffeine plus $CaCl_2$ group, but again, the difference was not significant. However, caffeine plus $CaCl_2$ resulted in a significantly greater number of accessory spermatozoa in the zonae pellucidae of oocytes of sows inseminated 26 h before ovulation. It was concluded from both insemination experiments that addition of caffeine plus $CaCl_2$ reduces the rate at which spermatozoa are eliminated from the genital tract. The greater number of accessory spermatozoa per embryo at 26 h after AI indicates that the number of spermatozoa in the oviducts remains sufficiently high for longer, which could have consequences for field fertility in pig AI and could potentially enable usage of a reduced sperm dosage.

Conclusion

PMNs play an important role in the clearance of spermatozoa from the uterine lumen. The phagocytosis of spermatozoa is not aimed specifically at removing dead, damaged or ageing spermatozoa. Fresh, intact spermatozoa are phagocytosed very rapidly *in vitro* as well as *in vivo*. However, incubation under capacitating conditions renders (a subpopulation of) the spermatozoa less vulnerable to phagocytosis.

Establishment of populations of spermatozoa in the uterotubal junctions and oviducts starts almost immediately after insemination. However, replenishment of these populations by ongoing migration of spermatozoa from the uterus can take place up to 24 h after insemination. This may be relevant for the likelihood of fertilization, especially when ovulation takes place a long time after insemination. It is possible to modulate PMN recruitment and phagocytosis of boar spermatozoa by addition of substances to the semen, for example, caffeine plus $CaCl_2$. An insemination study comparing inseminations with and without caffeine plus $CaCl_2$ indicated that the use of caffeine plus $CaCl_2$ could improve the fertility results or could enable the use of a reduced dosage of spermatozoa.

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