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Boar Seminal Plasma Components and Fertilization

By

Jiuming Zhu 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

Department of Agricultural, Food and Nutritional Science

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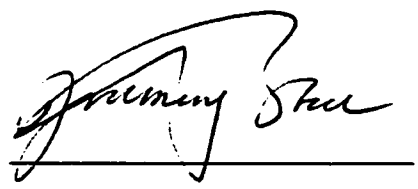

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Boar seminal Plasma Components and Fertilization** in partial fulfillment of the requirements for the degree of **Master of Science.**

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Date Dec 10, 1999

Abstract

Relationships between seminal plasma components, semen quality and boar fertility were studied. An initial experiment using *in vitro* fertilization (IVF) techniques indicated that sperm recovered from the first sperm-rich fraction (F1) of the ejaculate, and coincubated with their original F1 seminal plasma, had higher penetration rates compared to F1 sperm coincubated with seminal plasma from the sperm-depleted fraction (F2). Western blotting techniques showed that total protein ($P<0.01$), Porcine Seminal Plasma Protein-I (PSP-I) ($P<0.01$) and Protein P7 ($P<0.0001$) were more concentrated in F2 than in F1 seminal plasma; there were differences among boars in these proteins ($P<0.01$, $P<0.05$ and $P<0.0001$, respectively) and also for protein P20 ($P<0.05$). Regression analyses indicated that PSP-I and most of other low M_r proteins were negatively associated with IVF outcomes. Collectively, these data suggest that measurement of seminal plasma proteins may be useful for boar semen evaluation.

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Chapter 1

General Introduction

A boar potentially contributes a far greater genetic effect to a given group of breeding stock than a sow. With the continuous improvement of artificial insemination techniques, each stud boar may sire more than 200 sow or gilt litters per year. Successful fertilization is a prerequisite for an animal to exert its genetic potential. Boar fertility is, therefore, of great importance to the pork industry. It would be very valuable to develop an objective method, in addition to routine laboratory examination of motility and normality, to evaluate boar fertility.

Successful *in vitro* maturation and *in vitro* fertilization (IVM/IVF) techniques have been developed in several mammalian species in the four decades since the birth of the first litter of *in vitro* fertilized rabbits was reported in the United States (Chang, 1959), but their application to assessment of semen quality is limited by the huge expenditure and time-consuming technology involved. Progressive advances in molecular biology provide us with a chance to find one or more biochemical indicators that are well correlated with boar fertility.

In vivo, seminal plasma is an essential medium for sperm functions from ejaculation to fertilization. Large volumes of ejaculates with lower densities of sperm are a species characteristic of swine. Seminal plasma has a greater effect on boar sperm function compared with other farm animals. An important physiological function of seminal plasma is to carry spermatozoa through the female reproductive tract. However,

sperm are also nourished in seminal plasma by utilizing carbohydrates. More importantly, seminal plasma is directly involved in sperm capacitation and fertilization.

In seminal plasma, diverse proteins exert different roles at different stages of the fertilization process. Spermadhesins are a predominant family of boar seminal plasma proteins. These low M_r glycoproteins, ranging from 12 to 20 kDa, account for over 90% of total proteins in seminal plasma (Calvete *et al.*, 1997). Porcine Seminal Plasma Protein-I (PSP-I) is one of two major members of the spermadhesin family. This protein is able to form a non-covalent heterodimer with Porcine Seminal Plasma Protein-II (PSP-II), depending on the components of the oligosaccharide moiety of PSP-I. The two proteins, present in equal amounts, represent well over 50% of total proteins in seminal plasma (Rutherfurd *et al.*, 1992). The *zona pellucida* glycoprotein-binding activity of PSP-I/PSP-II suggests a role during gamete interaction. However, PSP-I/PSP-II heterodimers have been found to bind only loosely to the sperm surface, and are absent from *in vitro* capacitated spermatozoa, while heparin-binding PSP-I monomers are present on capacitated sperm (Dostálová *et al.*, 1994). These results suggested that PSP-I and other spermadhesins, as ligands, are involved in the interaction between lectins on sperm surfaces and oligosaccharides of *zona pellucida* glycoproteins on oocytes. However, it remains to be further investigated how these proteins function in sperm fertilization.

Hormones are another constituent of seminal plasma associated with sperm capacitation and fertilization. Interestingly, extraordinarily large amounts of estrogens are excreted by testicular Leydig cells (Claus *et al.*, 1983; Reeves, 1989). In addition to modulating protein synthesis and secretion, these hormones are also involved in

fertilization. Estradiol-17β (E2) and testosterone (T) are two major participants in this process. Acting indirectly through early activation of the luteinizing hormone (LH) surge, seminal plasma estradiol may stimulate ovulation in estrous animals (Claus *et al.*, 1990). The addition of seminal plasma to the semen extender has been reported to result in an increase in the pregnancy rate and litter size at birth (Claus *et al.*, 1989). It is believed that seminal plasma-derived estrogens can promote gamete transportation in the female germinal tract by stimulating secretion of prostaglandin $F_{2\alpha}$ from the endometrium, which in turn induces contraction of the uterus and oviducts (Claus *et al.*, 1990), and thus affects fertilization. In the bull, heparin-bound proteins are secreted in a testosterone-dose-dependent manner. As also shown in the boar (Calvete et la., 1994b; Töpfer-Peterson *et al.*, 1994 and 1998), these proteins are involved in bovine sperm capacitation and sperm-egg interactions (Nass *et al.*, 1990).

On the basis of these findings, a study was designed to investigate whether proteins and steroids in boar seminal plasma are associated with semen quality and sperm fertilizing ability *in vivo* and *in vitro*, with the ultimate goal of selecting one or more biological indicators to evaluate semen quality and boar fertility.

To gain an appreciation of the principles of mammalian fertilization, a literature review is presented at the beginning of this thesis. The focus of this review is on swine research, although data from various other species of mammals are also considered. In this review, the origins, biochemical properties, and physiological functions of the proteins in seminal plasma and on sperm membranes are first described. Their absorption and transfer between seminal plasma and sperm cells is then discussed. Seminal plasma hormones and their roles in fertilization are also introduced. In the final part of the

review, sperm capacitation and gamete fertilization are discussed in more detail. Four chapters detailing the results of two experiments conducted as part of the research program are then presented.

On the basis of earlier findings observed in our laboratory, that the sperm from the first sperm-rich fraction (F1) are superior to the subsequent sperm-depleted fraction (F2) or the second sperm-rich fraction of the ejaculate with respect to penetration rates, monospermy rates, male pronucleus (MPN) formation rates, numbers of sperm penetrating into each oocyte and numbers of sperm attaching to each oocyte, the first trial was established to explore whether differences in IVF outcomes among three fractions of the ejaculate are derived from seminal plasma or from sperm themselves. By co-incubating F1 sperm with three different fractions of seminal plasma separated from the same ejaculates, the study showed that the IVF differences among the three fractions of ejaculates are derived, at least partially, from seminal plasma.

The aim of the second trial was to identify and quantify PSP-I and other low M_r proteins in seminal plasma and analyze possible differences between F1 and F2 seminal plasma and among boars. The results indicated that PSP-I and other low M_r proteins are more concentrated in F2 than F1 seminal plasma. But F1 seminal plasma appears to contain more high M_r proteins (≥20 kDa). As well, boars were shown to differ in the amounts of these proteins.

In the final experimental section, information on concentrations of both testosterone and estradiol-17β in seminal plasma is presented, followed by results of multiple regression analysis of associations among IVF estimates, semen quality characteristics and *in vivo* fertility data. Regression analyses indicated that PSP-I and

most other low M_r proteins are inversely related to outcomes of fertilization and embryo development. P13 and P27 (a new product resulting from N-Glycanase deglycosylation of seminal plasma proteins) appear to be positively associated with *in vitro* embryo production. The data suggested that these proteins, especially PSP-I for which a specific antiserum is available, could be used for the assessment of boar fertility.

Chapter 2

Literature Review

Many studies have indicated that proteins and hormones play a pivotal role in embryonic development from gametogenesis, to implantation and gestation. This chapter will review the roles of these proteins and hormones in oocyte maturation, sperm capacitation and fertilization.

2.1. Proteins in Seminal Plasma

Seminal plasma, the liquid fraction of ejaculated semen, comprises secretions of the epididymis, vas deferens, and accessory sex glands including the seminal vesicles, and ampullary, prostate, bulbo-urethral (Cowper's) and urethral (Littre's) glands. Because the boar has no ampullae, its seminal plasma is derived mainly from the seminal vesicles, prostate and Cowper's gland (Ashdown and Hafez, 1993). Despite its great physiological significance as the carrier of spermatozoa to the female reproductive tract, the functions of the various proteins and polypeptides in seminal plasma are still poorly understood (Ashdown and Hafez, 1993). Proteins or polypeptides are major constituents of seminal plasma. In addition to maintaining osmotic equilibrium and immunological inhibition exerted by seminalplasmin and specific antibodies, some proteins, especially catalytic enzymes, are involved in sperm metabolism (Shivaji *et al.*, 1990). The other proteins play an important role in sperm motility, capacitation, the acrosome reaction and

sperm-egg fusion. Those proteins relevant to fertilization events can be classified into four categories: spermadhesins, sperm motility-stimulating proteins, sperm motility inhibitors and decapacitation factors.

2.1.1. Spermadhesins

The spermadhesins are produced by the accessory gland(s) of the male reproductive tract, mixed with sperm during ejaculation, and as their name implies, are coated on the sperm surface (Sanz *et al.*, 1992a). The spermadhesins are the most predominant proteins in boar seminal plasma. Each member of the spermadhesin family is usually named after the single-letter codes for the first three amino acid residues at their N-terminus (e. g. AWN has N-terminal sequence of Ala-Trp-Asn), with the exception of PSP-I (Porcine Seminal Plasma Protein-I) and PSP-II (Porcine Seminal Plasma Protein-II) (Calvete *et al.*, 1995a). They are generally derived from seminal vesicle epithelial cells. AWN-1 is, in addition, partially synthesized by tubuli recti and rete testis epithelial cells (Sinowatz *et al.*, 1995). Spermadhesins account for over 90% of total protein in seminal plasma (Calvete *et al.*, 1997). PSP-I and PSP-II are two major spermadhesins in the boar and they represent well over 50% of the total proteins in porcine seminal plasma (Rutherfurd *et al.*, 1992). The complete amino acid sequences of five boar spermadhesins have been determined: AQN-1 (Sanz *et al.*, 1992a), PSP-I (AQN-2) (Rutherfurd *et al.*, 1992; Calvete *et al.*, 1993a), PSP-II (Calvete *et al.*, 1995a), AQN-3 and AWN (1 and 2) (Sanz *et al.*, 1992b). Except for AQN-2 which is 18,000-20,000 Daltons, the other four spermadhesins have molecular weights between 12,000-16,000 Daltons, consist of 109-133 amino acid residues, and share 40-60% primary

structural identity (Calvete *et al.*, 1993a, 1995a). AWN-1 and AWN-2 differ only in that the latter contains an acetylated N-terminal residue. N-glycoforms of AQN-3 (Rutherfurd *et al.*, 1992) and N- and O-glycosylated AWN species (Calvete *et al.*, 1993b & 1994a) have also been identified. Glycosylation not only contributes to the structural diversity of the protein family but also affects the ligand-binding capabilities of the glycosylated spermadhesins, in that it abolishes their *zona pellucida*-binding activity without impairing heparin binding (Calvete *et al.*, 1993a&b, 1994a). With the exception of PSP-II, the other four spermadhesins possess strong heparin-binding ability (Jonakova *et al.*, 1991; Sanz *et al.*, 1992a; Hanqing *et al.*, 1991; Moos *et al.*, 1992; Parry *et al.*, 1992).

Boar spermadhesins represent a novel family of sperm-associated, low-molecular mass lectins, whose major roles in different steps of fertilization have been proposed (Calvete *et la.*, 1994b, Töpfer-Peterson *et al.*, 1994 and 1998). During sperm passage through the epididymis AQN-3 and AWN have been shown to bind tightly to the sperm surface by interaction with the phospholipids of the membrane bilayer. At ejaculation the spermadhesins form a protective coat around the sensitive acrosomal region of the sperm head, possibly preventing a premature acrosome reaction. During *in vitro* capacitation most of these aggregated spermadhesins are lost, with the exception of phospholipid-bound spermadhesins (Töpfer-Peterson *et al.*, 1998). However, approximately 7×10^6 molecules of each spermadhesin (AQN-1, AQN-2, AQN-3, and AWN-1) are still present on a single *in vitro* capacitated sperm, and appear to be sufficient to cover the entire acrosomal cap surface with a several-molecule-thick layer (Dostálová *et al.*, 1994). AWN and AQN-3 may serve as primary receptors for the oocyte *zona pellucida*, thus contributing to initial binding and recognition between sperm and egg (Töpfer-Peterson

et al., 1998). The interaction between lectins of the sperm surface and oligosaccharides of the oocyte's *zona pellucida* glycoproteins (Yanagimachi, 1994) mediates sperm capacitation, the acrosome reaction and gamete recognition (Calvete *et al.*, 1992).

2.1.2. PSP-I

PSP-I is a glycoprotein consisting of 109 amino acid residues. Based on a calculation from its amino acid sequence, its predicted molecular mass is 11,978.6 Daltons (Rutherfurd *et al.*, 1992; Calvete *et al.*, 1993a). It is a unique protein and has no significant sequence homology with other proteins (Rutherfurd *et al.*, 1992). It has a single glycosylation site at the asparagine residue at position 47 (Rutherfurd *et al.*, 1992) and is likely to form various kinds of PSP-I derivatives with different sized oligosaccharide components (Kwok *et al.*, 1993). Glycosylation contributes to the structural diversity of this protein family. The glycosylated PSP-I is able to form a non-covalent heterodimer with certain glycoforms of PSP-II (Romero *et al.*, 1996), which is also a glycoprotein with 116 amino acid residues and a predicted molecular mass of 12,643.5 Da (based on calculation from its amino acid sequence, Calvete *et al.*, 1995a). These two proteins represent well over 50% of the total proteins in boar seminal plasma, and PSP-I and PSP-II are present in equal amounts (Rutherfurd *et al.*, 1992). Two types of PSP-I glycoforms (designated as CM-3 PSP-I and CM-4 PSP-I) have been detected in the non heparin-bound fraction of seminal plasma proteins (Calvete *et al.*, 1993a). PSP-I has also been isolated from the heparin-bound fraction of boar seminal plasma (Calvete *et al.*, 1993a). PSP-I is also derived from AQN-2 treatment with PNGaseF (Calvete *et al.*, 1993a). Although heparin-bound and heparin-unbound PSP-I molecules have identical

amino acid sequences and post-translational modifications, they are differently glycosylated. Thus, although both heparin-bound and heparin-unbound PSP-I glycoforms contain a single N-linked carbohydrate, only the latter contains galactosamine, galactose and sialic acid. Thus, it appears that the type of glycosylation may determine which PSP-I glycoforms are recruited by PSP-II to form heterodimers (Calvete *et al.*, 1995a).

PSP-I is largely hydrophilic, as are several other seminal proteins, including PDC-109 (Esch *et al.*, 1983), BSP-A3 (Seidah *et al.*, 1984), and β-microseminoprotein (Akiyama *et al.*, 1985). From the secondary structural analysis of PSP-I, it appears that the protein is likely to have a significant amount of ordered structure, in particular β-sheets and β-turns. This analysis predicts that PSP-I possesses one segment of α-helix, and six segments of β-sheets; however, these secondary structures are not conserved among the seminal proteins from other species, as expected from their highly divergent primary structures.

Although PSP-I/PSP-II heterodimers are unable to bind heparin, both subunits possess this binding activity. Because of its weak heparin-binding capability, PSP-II is believed to be a major component of the non-heparin-bound fraction of boar seminal plasma (Calvete *et al.*, 1995a). In addition, the PSP-I/PSP-II complex and isolated PSP-II, but not PSP-I, exhibited affinity for soybean trypsin inhibitor (STI) and *zona pellucida* glycoproteins (ZPG). These results indicate that the binding site(s) for STI and ZPG on PSP-I/PSP-II are located on PSP-II, and that dimerization abolishes the heparin-binding capability of both subunits (Calvete *et al.*, 1995a; Salis *et al.*, 1998). The glycosylation at asparagine 47 is found to be the cause of the inability of PSP-I to bind STI and ZPG

(Calvete *et al.*, 1993a & 1994b). It also has an indirect effect on the heparin-binding ability of PSP-I through modulation of its aggregation state (Calvete *et al.*, 1995b)

The *zona pellucida* glycoprotein-binding activity of PSP-I/PSP-II suggests a role during gamete interaction. However, PSP-I/PSP-II heterodimers have been found to bind only loosely to the sperm surface, and are absent from *in vitro* capacitated spermatozoa (Dostálová *et al.*, 1994). Interestingly, heparin-bound PSP-I molecules are present on capacitated sperm, suggesting that either the type of glycosylation or dimerization with PSP-II also influences the sperm coating characteristics of PSP-I (and/or PSP-II) glycoforms. Thus, although the biological function(s) of boar seminal plasma PSP-I/PSP-II remain obscure, it has been shown that heterodimerization of specific glycoforms of PSP-I with PSP-II exerts a profound effect on the ligand-binding characteristics and compartmentalization of these boar spermadhesins (Calvete *et al.*, 1995a).

2.1.3. Sperm Motility Stimulating Proteins

Porcine antithrombin III (AT III) has been identified to be a sperm-motility-stimulating protein and a chemoattractant for boar sperm (Lee and Wei, 1994). Kallikrein has also been shown to stimulate human sperm motility (Deperthes *et al.*, 1995).

2.1.4. *Zona pellucida*-Binding Proteins

A group of low molecular mass (16-23 kDa) proteins on ejaculated boar spermatozoa have been shown to have high affinity for homologous *zona pellucida* glycoproteins (ZPGPs). These ZPGP binding proteins are derived from seminal plasma, as shown by their absence from epididymal spermatozoa, and their presence in seminal

plasma as identified by N-terminal amino acid sequence analysis. They bind to ZPGPs by a polysulphate recognition mechanism similar to that found for proacrosin-ZPGP interactions. The haemagglutination activity of boar seminal plasma is also associated with these low M_r glycoproteins. It is suggested that they play a role in regulating the rate of sperm capacitation and survival in the female reproductive tract (Parry *et al.*, 1992).

A low molecular weight protein of 17 kDa, named as ACR.3, was identified, isolated and purified from boar seminal plasma (Moos *et al.*, 1992). Recent results suggest that ACR.3 protein may mediate the primary sperm-egg *zona pellucida* binding and that it is one of the likely candidates for the primary sperm-ZP binding protein (Capkova and Peknicova, 1997).

2.1.5. Trypsin-like Proteins

A trypsin-like protein present in seminal plasma is found to bind to the sperm head. It plays important roles in the sperm-egg interaction. The trypsin-like protein is also involved in the induction of the acrosome reaction (Lee and Wei, 1994).

2.1.6. Fertilization-promoting Peptide

Fertilization-promoting peptide (FPP), a TRH (thyrotropin-releasing hormone)-like peptide, is present at high concentrations in mammalian prostate and semen. Only low concentrations of FPP were detectable in the bulbourethral glands, and the peptide was undetectable in the testes of the marmoset (Kennedy *et al.*, 1997). It was shown to enhance the fertilization potential of spermatozoa (Siviter and Cockle, 1995).

2.1.7. Protein C Inhibitor (PCI)

PCI is synthesized by cells throughout the male reproductive tract and is present at high concentrations in seminal plasma. Located on the acrosomal membrane, it may function as a scavenger of prematurely activated acrosin, thereby protecting intact surrounding cells and proteins from possible damage (Zheng *et al.*, 1994).

2.1.8. Sperm Motility Inhibitors

At least two varieties of sperm motility inhibiting factors (SMIFs) have been purified and identified. One was estimated at 50 kDa in molecular weight through the molecular sieving method by Iwamoto *et al.* (1992 and 1993). It is stable at pH 6.0-11.0, but thermolabile. The other weighs 5.7 kDa and is thermostable and this peptide, dominated by acidic amino acid residues, possesses maximal absorbance at 220 nm. It is able to inhibit sperm motility in a non species-specific manner. An antigenic species-specificity of SMIF was demonstrated. Immunofluorescence microscopy confirmed that the peptide was secreted by epithelial cells of the seminal vesicle (Strzeżek *et al.*, 1992). Human seminal plasma motility inhibitor (SPMI) originates from the seminal vesicles as a 52 kDa precursor form that is rapidly degraded by prostatic proteases after ejaculation. It is highly associated with seminal coagulum components as very active forms that may affect sperm motility when not properly processed after ejaculation.

2.1.9. Decapacitation Factors

A variety of decapacitation factors have recently been identified in different species, including the pig (Bonilla *et al.*, 1996). These factors are more than 100 kDa in

molecular weight. Some researchers demonstrated that high speed centrifugation can remove decapacitation factors (Fraser *et al.*, 1990; Bonilla *et al.*, 1996). Some proteinase inhibitors are also decapacitation factors and may play roles through inhibiting some proteinases involved in sperm capacitation (Lee and Wei, 1994). Seminal plasma proteins described above are listed in Table 2-1.

2.2. Proteins on the Sperm Plasma Membrane

According to their functions, major sperm plasma membrane proteins associated with sperm capacitation and fertilization are divided into five categories: spermadhesins, cumulus-dissolving proteins, progesterone receptor, egg-binding proteins and other related proteins.

2.2.1. Spermadhesins

Most spermadhesins have been detected on the boar sperm plasma membrane. All of these proteins, 12-14 kDa in molecular weight, are derived from accessory sex glands. They are bound to the major phospholipids on the plasma membrane of capacitated sperm at approximately 7×10^6 molecules of each spermadhesin per cell. They are considered to be associated with sperm capacitation (Dostálová *et al.*, 1995). Therefore, spermadhesins are also called capacitation proteins.

Table 2-1. Major Seminal Plasma Proteins Associated with Capacitation and Fertilization

Category	Name	Molecule Wt (kDa)	Species Observed	Source	Function	Reference
Spermadhesins	AWN-1	12-14	Porcine	ASG	Capacitation	Calvete *et al.*(1994)
	AWN-3	12-14	Porcine	ASG	Capacitation	Calvete *et al.*(1994)
	AQN-1	12-14	Porcine	ASG	Capacitation	Calvete *et al.*(1994)
	AQN-3	12-14	Porcine	ASG	Capacitation	Calvete *et al.*(1994)
	AQN-2	18-20	Porcine	ASG	Capacitation	Calvete *et al.*(1994)
	PSP-I/ BSP-A1/ HSP-I	13/ 29-30	Porcine/ Bovine/ Stallion	Seminal Vesicle	Prevent premature AR & modify sperm plasma membrane	Calvete *et al.* (1995) /Leblond *et al.* (1993)/ Romero *et al.* (1996)
	PSP-II/ BSP-A2/ HSP-II	13/ 29-30	Porcine/ Bovine/ Stallion	Seminal Vesicle	Prevent premature AR & modify sperm plasma membrane	Calvete *et al.* (1995) /Leblond et al. (1993)/Romero *et al.* (1996)
	BSP-A3		Bovine		Modify sperm plasma membrane	Leblond *et al.*(1993)
	BSP-30kDa		Bovine		Modify sperm plasma membrane	Leblond *et al.*(1993)
Sperm-motility -stimulating proteins	AT-III		Porcine		Stimulate sperm motility	Lee & Wei *et al.* (1994)
	Kallikrein (hK2,hGK-1)		Human	Prostate	Stimulate sperm motility	Deperthes *et al.* (1995)
ZP-binding Proteins	ZPBP	16-23	Porcine		Regulate capacitation	Parry *et al.* (1992)
	ACR.3	17	Porcine	ASG	Mediate the primary sperm-egg ZP binding	Capkova & Peknicova (1997)
	Trypsin-like proteins				Induce AR	Lee & Wei *et al.* (1994)
Fertilization-Promoting Peptide	FPP		Mammal	Prostate & bulbourethral	Promote fertilization Potential	Siviter & Cockle (1995). Kennedy *et al.* (1997)
	MCP(D46)	43	Human		Enhance fertilization	Seya *et al.* (1993)
Protein C Inhibitor	PCI		Human	Male Reprod. Tract	Inhibit Acrosin and other proteinase	Zheng *et al*(1994) Robert & Gaqnon (1995)
Sperm Motility Inhibitors	SPMI	50/	Porcine/ Human	Seminal Vesicle	Inhibit sperm motility	Iwamoto *et al.* (1992)
	SMIF	5.7	Porcine	Seminal Vesicle	Inhibit sperm motility	Strzezek *et al.* (1992)
Decapacitation Factors	DCP	>100	Porcine		Inhibit AR	Bonilla *et al.* (1996)
	Proteinase inhibitors				Inhibit AR and/or Fertilization	Lee & Wei *et al.* (1994)

AR-Acrosome Reaction; ASG-Accessory Sexual Glands; AT-Antithrombin; BSP-Bovine Seminal Protein; DCP-Decapacitation ; FPP-Fertilization-Promoting Peptide; HSP-Horse Seminal Protein; MCP-Membrane Cofactor Protein; PCI-Protein C Inhibitor; PSP-Porcine Seminal Protein; SMIF-Sperm Motility Inhibiting Factor; SPMI-Seminal Plasma Motility Inhibitor; ZPBP-*Zona pellucida* Binding Protein; ZP-*Zona pellucida.*

2.2.2. Cumulus-dissolving Proteins

A sperm membrane protein called PH-20 was recently found to be functionally similar to hyaluronidase and is located not only on the inner acrosomal membrane but also bound to the sperm plasma membrane by a glycosyl phosphatidyl inositol anchor. Because this protein possesses hyaluronidase-like activity, it enables acrosome-intact sperm to pass through the cumulus cell layer of the egg (Hunnicutt *et al.*, 1996). As verified earlier, the hyaluronidase on the inner acrosome membrane is usually released and acts as a cumulus-dissolving enzyme only after the acrosome reaction occurs. The findings of Hunnicutt and colleagues explain why acrosome intact spermatozoa in such species as the mouse (Bleil *et al.*, 1988) arrive around the *zona pellucida*.

2.2.3. Progesterone Receptor

The progesterone receptor is a 50-52 kDa protein. Tesarik *et al.* (1992a) reported that the binding of progesterone to sites on the sperm surface resulted in an acrosome reaction. They found that after binding, progesterone could act via a mechanism of aggregation of the cell surface progesterone receptors (Tesarik *et al.*, 1992b). In fact, the aggregation of progesterone-receptor complexes on the sperm surface was followed by a rapid Ca^{2+} influx and eventually an acrosomal reaction (Sabeur *et al.*, 1996). Since a large amount of progesterone in follicular fluid is bound non-covalently to a protein, forming a complex of ~50 kDa (Osman *et al.*, 1989), it can be postulated that the protein component of this complex plays an active role in aggregating sperm surface progesterone binding sites. Thus, the progesterone receptor actually acts as a primary signal for the acrosome reaction.

2.2.4. Egg-binding Proteins

Two varieties of ZP glycoprotein receptors constitute egg-binding proteins (Wassarman, 1992). Mouse ZP3 receptor is also called sp56 and its molecular weight is 56 kDa (Bleil and Wassarman, 1990). When this receptor is bound to the species-specific ZP3, it can induce an acrosome reaction and subsequently acrosin release (Wassarman, 1992). Acrosin is thought to be the ZP2 receptor(Wassarman, 1992). This serine protease, derived from proacrosin autoactivation, is able to directly dissolve the *zona pellucida* of the egg and enables capacitated sperm to pass through the *zona pellucida* (Wassarman, 1992). The ZP3 and ZP2 receptors are distributed on the sperm plasma membrane, and the inner acrosomal membrane, respectively, and are proposed to be the primary and the secondary egg-binding proteins, respectively (Wassarman, 1992; McLeskey *et al.*, 1998).

2.2.5. Other Related Proteins

Three other kinds of proteins related to the *zona pellucida* reaction and/or sperm:oocyte interactions include P34H found in human (Boué *et al.*, 1996), P26H found in hamster and antigenically and functionally homologous with P34H (Boué *et al.*, 1994), and a 62 kDa pig sperm plasma membrane protein (Ash *et al.*, 1995).

Ion channels, such as the Ca^{2+} and Cl^- channels, participate in sperm capacitation and fertilization. It is known that sperm capacitation is a prerequisite for sperm penetration into matured oocytes. High concentrations of Ca^{2+} are necessary for sperm capacitation and it is the Ca^{2+} channel that carries these Ca^{2+} ions into sperm cells and eventually results in sperm capacitation and/or the acrosome reaction (Fraser, 1992).

Table 2-2 lists the major sperm plasma membrane proteins associated with capacitation and fertilization.

Table 2-2. Major Sperm Plasma Membrane Proteins Associated with Capacitation and Fertilization

Category	Name	Molecular Wt (kDa)	Species Observed	Location	Source	Function	Reference
Spermadhesins	AWN-1	12-14	Pig	Sperm Head	Acces. Sex. Glands	Capacitation	Dostálová *et al.* (1995)
	AQN-1	12-14	Pig	Sperm Head	Acces. Sex. Gland	Capacitation	Dostálová *et al.* (1995)
	AQN-3	12-14	Pig	Sperm Head	Acces. Sex. Glands	Capacitation	Dostálová *et al.* (1995)
	AQN-2	12-14	Pig	Sperm Head	Acces. Sex. Glands	Capacitation	Dostálová *et al.* (1995)
Cumulus-dissolving Proteins	PH-20	64	Guinea Pig	Sperm Plasma & Inner Acrosomal Membrane		Hyaluronidase-like activity	Hunnicutt *et al.* (1996)
	Hyaluronidase		Mammal	Inner Acrosomal Membrane		Dissolve Cumulus Oophorus	Meyers *et al.* (1997)
Progesterone Receptor		50-52	Human	Sperm Plasma Membrane		Induce Ca^{2+} Influx and AR	Sabeur *et al.* (1996)
Egg-binding Proteins	ZP3 Receptor (sp56)	56	Mouse	Sperm Head		Induce AR and Acrosin Release	Wassarman (1992)
	ZP2 Receptor (Acrosin)		Mouse	Inner/Outer Acrosomal Membrane		Dissolve ZP	Wassarman (1992)
Other Related Proteins	P34H	34	Human	Acrosomal Cap	Caput Epididymidis	Involved in Sperm:ZP interaction	Boué *et al.* (1996)
	P26h	26	Hamster			Involved in Sperm:ZP interaction	Boué *et al.* (1994)
	PM protein	62	Pig	Sperm Plasma Membrane		Associated with Sperm Penetration Ability	Ash *et al.* (1995)

2.3. Surface Protein Transformation of Sperm

From ejaculation to fertilization, sperm must undergo a series of biochemical and physiological changes in order to attain the ability to penetrate their homologous oocytes

and this process is referred to as "capacitation". These modifications occur during the interaction between sperm and the environment of the female genital tract fluid. However, seminal plasma factors may affect sperm capacitation and fertilizability. Voglmayr and Sawyer (1986) demonstrated that surface proteins of ram sperm were transformed in uterine, oviduct and cauda epididymal fluids *in vitro*. They found that in freshly collected spermatozoa, two predominant molecules, 97 and 24 kDa, were equally distributed, but the larger protein was selectively removed during incubation in oviduct fluid and after 1 h exposure to uterine fluid, so that by 4 h the 24 kDa protein was predominant. Adsorption of radioiodinated uterine fluid components by unlabelled spermatozoa was highly selective. During incubation in labeled uterine fluid for 1 h, a low molecular weight polypeptide (16 kDa) was consistently enriched on the sperm surface, even though it was present only as a 'minor' component in the fluid. Traces of a 29 kDa proteinwere also detectable on the sperm surface. Absorbed oviduct fluid components were of relatively high molecular weight (140, 95, 78 and 53 kDa). Again certain 'minor' fluid components were preferentially incorporated into the sperm plasma membrane.

Spermatozoa incubated in uterine fluid displayed an exceptionally high motility Voglmayr and Sawyer (1986). All sperm samples incubated in this fluid for 1 h consistently received the highest motility score (5). Closer inspection revealed a directional head-to-head agglutination between 2-4 spermatozoa. The coordinated flagellation of such an array gave the spermatozoa a much higher velocity than would have been the case with single cells. After a further 3-h incubation in oviductal fluid,

following exposure to uterine fluid, the spermatozoa had separated again and had resumed a motility pattern that was comparable to that of freshly collected cells.

In comparison with female reproductive tract fluid, seminal plasma components more directly affect suspended spermatozoa. Metz and colleagues (1990) found that ejaculated sperm from three vasiculectomized boars adsorbed approximately 14 pg of proteins per spermatozoon from the seminal plasma harvested from vasectomized boars per 10-min exposure. After washing, 50 to 70% of the spermatozoa in these ejaculates were progressively motile. In contrast, ejaculated sperm from the same boars but collected during late summer when sperm motility was lower (30 to 50% of the sperm were progressively motile following washing) had reduced ability to adsorb proteins from seminal plasma (3 pg of proteins per spermatozoon per 10 min, $P<0.05$). These results indicate that the ability of post-ejaculation sperm to adsorb proteins from seminal plasma is associated with progressive sperm motility, i.e. the higher the sperm motility, the more proteins the sperm adsorb.

After 30-min incubation, seven proteins were adsorbed to different extents (Metz *et al.*, 1990). Of the adsorbed proteins, most of them (82%) were low M_r (12.7, 14.3, 15.6 and 19.7 kDa). The 12.7 kDa protein was the major seminal plasma protein adsorbed by the sperm. This band contained 63% of the total radioactivity adsorbed by the sperm (Metz *et al.*, 1990).

In bovine seminal plasma, it was found that a protein called caltrin may have dual roles in calcium transport, and another protein called seminalplasmin may affect membrane fluidity; both are low M_r proteins (San Agustin *et al.*, 1987; Shivaji, 1986). A low M_r proteinase inhibitor from mouse seminal plasma binds to the sperm membrane

site implicated in binding to the *zona pellucida* (Poirier *et al.*, 1986). Based on work in many species, these adsorbed proteins possess multiple functions including sperm capacitation and/or interaction of sperm and oocytes.

Table 2-3 presents summary data on major exchanges of proteins between sperm and their surrounding medium.

Table 2-3. Sperm Surface Protein Transformations in Various Kinds of Genital Tract Fluids *in vitro*

Species	Ovine	Ovine	Ovine	Ovine	Porcine
Medium	Uterine Fluids	Oviduct Fluids	Cauda Epididymal Fluids	Synthetic Medium	Seminal Plasma
Duration of Incubation	1 h	3 h	2 h		30 min
Components Incorporated (kDa)	16, 29	140, 95, 78, 53	24, 76	No	12.7 (63%), 14.3, 15.6
Component Lost (kDa)	97	97		No	
Predominant Component (kDa)	24	24			
Reference	Voglmayr and Sawyer, 1986				Metz *et al.*, 1990

2.4. Seminal Plasma Hormones Involved in Fertilization

2.4.1. Sources of Seminal Plasma Hormones

To date, estrogens, progesterone (P4), testosterone (T), androstenedione (A) and prostaglandins (PGE_2 and $PGF_{2\alpha}$) have been found in boar seminal plasma. Interestingly, high concentrations of estrone (E1), estradiol-17β (E2) and estrone-sulphate (E1-S) exist in boar semen (Claus *et al.*, 1983,1985, 1987, 1989 and 1990). Most of these steroids originate from testicular Leydig cells (Claus *et al.*, 1983; Reeves, 1989).

2.4.2. Roles and Mechanisms of Action

2.4.2.1. *In vivo*

2.4.2.1.1. Ovulation, Oocytes and Sperm Transport

The effect of seminal plasma hormones on ovulation and gamete transport is mainly attributed to estrogens and prostaglandins derived from follicular fluid and seminal plasma. High concentrations of E1, E2 and E1-s are present in boar semen with considerable differences in mean concentration and total amount among individual ejaculates (Claus *et al.*, 1983, 1985 and 1987; Schopper *et al.*, 1984). The addition of a physiological amount of estrogens to semen extender used for artificial insemination increased both conception rate and litter size born (Claus *et al.*, 1989). It was shown in a further study that the effects of seminal estrogens resulted from stimulation of ovulation and sperm transport by inducing secretion of $PGF_{2\alpha}$ from the endometrium (Claus *et al.*, 1990).

2.4.2.1.2. Regulating Secretion and Involvement in Fertilization of Seminal Plasma Proteins

In the bull, heparin-binding proteins derived mainly from seminal vesicles bind to cauda epididymal spermatozoa in a testosterone-dose-dependent manner (Nass *et al.*, 1990). Heparin-binding proteins may play a role in fertilization by attaching to sperm surfaces, allowing heparin-like glycosaminoglycans in the female reproductive tract to be involved in sperm capacitation. These results suggest that testosterone may be indirectly involved in fertilization.

Trypsin-acrosin inhibitor (BSTI-I) is a protein identified in boar seminal plasma (Erkens *et al.*, 1996). BSTI-I concentrations were testosterone-dependent, in both the seminal and blood plasma, as shown by the response to castration and testosterone propionate administration (Erkens *et al.*, 1996). After insemination, seminal plasma BSTI-I was found to be absorbed and could be detected in the circulation of inseminated sows, and peak concentrations were reached about 4 h after insemination. Absorption of seminal plasma BSTI-I varied during the period of estrus; however, no relationship between protein concentrations and the fertilization rates and embryo survival rates was demonstrated (Erkens *et al.*, 1996).

2.4.2.2. *In vitro*

Although no difference was found between control and treatment in the absence of soluble *zona pellucida*, sperm incubated in a progesterone-supplemented medium had a higher rate of acrosomal reaction, in the presence of soluble *zona pellucida* or *zona pellucida*-intact oocytes, compared with the *zona*-free control (Barboni *et al.*, 1995).

A more recent study confirmed that progesterone receptors exist on the sperm plasma membrane; they are associated with the fluidity of sperm plasma membrane and subsequently the influx of calcium ions and efflux of chloride ions (Sabeur *et al.*, 1996).

2.5. Capacitation and Fertilization

2.5.1. Sperm Capacitation

It is well known that before penetration, ejaculated spermatozoa must undergo a series of biochemical and physiological changes referred to as "capacitation". These include changes in motility (Cornett and Meizel, 1978), calcium flux (Singh *et al.*, 1978) and lectin binding (Talbot and Franklin, 1978), rearrangement of plasma membrane autoantigens and particles (Friend, 1980), changes in plasma membrane phospholipid composition (Davis *et al.*, 1980), loss of surface-associated inhibitory components (Fraser, 1984), and other surface modifications (O'Rand, 1982). Only capacitated spermatozoa are able to penetrate the oocyte plasma membrane and subsequently fuse with oocyte oolema.

Many factors may be involved in sperm capacitation. However, calcium ions and caffeine have been identified as two of the most important factors affecting *in vitro* fertilization (Cheng, 1986).

2.5.1.1. Calcium Ions

According to Cheng (1986), who was the first to successfully achieve the birth of 'test-tube' piglets derived from *in vitro* capacitation and fertilization, a minimum of 4.7 mM Ca^{2+} is essential for *in vitro* capacitation. Boar spermatozoa can capacitate without preincubation if the fertilization medium contains caffeine and a high concentration of Ca^{2+}, but this leads to a high incidence (60-100%) of polyspermy in *in vitro* matured oocytes (Nagai, 1994). Okamura *et al.* (1992) examined the effects of several reagents on

Ca^{2+} uptake and efflux in boar epididymal spermatozoa and suggested the existence of an interaction between a bicarbonate carrier and the Ca^{2+} transport system. Harrison *et al.* (1993) also reported a bicarbonate-mediated Ca^{2+} influx in boar spermatozoa. Bicarbonate was recently confirmed to be beneficial to *in vitro* fertilization as a component of the buffer system for media (Suzuki *et al.*, 1994).

2.5.1.2. Caffeine

Caffeine supplementation of fertilization medium was found to facilitate *in vitro* fertilization (Suzuki *et al.*, 1994). An earlier study indicated that capacitation with caffeine had no influence on fertilizing ability, though it can improve progressive sperm motility (Dacheux and Paquignon, 1983).

2.5.2. Fertilization of Oocytes by Sperm

Sperm-Oocyte interactions can be divided into six steps:

Step1: Spermatozoa Passing through Cumulus Cells surrounding the Oocyte

The cumulus cell layer is regarded as the first obstacle to sperm penetration. As discussed previously, PH-20 was originally identified on the surface of both acrosome-intact and acrosome-reacted guinea pig sperm plasma membrane, and it is the hyaluronidase-like activity of PH-20 that enables acrosome-intact guinea pig sperm to pass though the cumulus cell layers (Hunnicutt *et al.*, 1996). As shown in Table 2-4, acrosome-intact mouse spermatozoa bind to *zona pellucida* (Bleil *et al.*, 1988). It was, therefore, speculated that their penetration of cumulus layers might rely on a PH-20

analogue in the mouse sperm plasma membrane, al though this has not been identified yet (Lin *et al.*, 1994; Snell and White, 1996). In contrast, acrosome-reacted spermatozoa from other species can penetrate the cumulus layers with the help of catalysis of hyaluronidase released from the acrosome following a precocious acrosome reaction.

Step 2: Sperm Binding to the *Zona pellucida*

The *zona pellucida* (ZP) is regarded as the secondary obstacle for sperm fertilization. The mouse *zona pellucida* glycoproteins comprise mZP1 (200 kDa), mZP2 (120 kDa) and mZP3 (83 kDa) (Wassarman, 1992).

ZP3 emerged as the molecule responsible for species-specific binding (Wassarman and Litscher, 1995). ZP3 O-linked oligosaccharides are essential for sperm receptor function (Wassarman, 1992 and 1999). In terms of binding effectiveness, intact ZP is more effective than purified ZP3, and the latter is more effective than simple O-linked oligosaccharides. ZP2 functions as the receptor for acrosin derived from the inner sperm membrane (Snell and White, 1996). The role of ZP1 remains unclear so far.

At least three proteins were reported as candidate ZP3 adhesion molecules on acrosome-intact mouse sperm. They were β(1, 4)-galactosyl transferase (GalTase) (Shur and Bennett, 1979; Miller *et al.*, 1992; Gong *et al.*, 1995), sp56 (Bleil and Wassarman, 1990; Wassarman, 1992) and Tyrosine-phosphorylated Protein p95 (Leyton and Saling, 1989).

Sperm surface GalTase stands out as the candidate adhesion/signaling molecule with the longest history and most extensive experimental support. GalTase was first implicated in ZP adhesion eighteen years ago when it was reported that mouse sperm

carrying certain haplotypes had increased fertilizing ability that correlated with increased levels of surface GalTase activity (Shur and Bennett, 1979).

Step 3: Acrosome Reaction

It is generally believed that the acrosome reaction proceeds in the presence of *zona pellucida* substrates or *zona*-intact oocytes. The acrosomal status of pre-binding sperm is different among species and is summarized in Table 2-4.

Table 2-4. Acrosomal Status Immediately Before Sperm Binding to *Zona pellucida*

Species	Acrosomal Status	Reference
Mouse	Intact	Bleil *et al.*, 1988
Guinea-pig	Reacted	Huang *et al.*, 1981
	Intact/Reacted	Myles *et al.*, 1987
Rabbit	Reacted	Kusan *et al.*, 1984
Human	Intact/Reacted	Morales *et al.*, 1989
Hamster	Intact/Reacted	Cummins *et al.*, 1986
Pig	Partially Reacted	Yonezawa *et al.*, 1995
	Intact	Fazeli *et al.*, 1997

The acrosome reaction is characterized by fusion and shedding of the outer acrosome membrane and sperm plasma membrane, release of acrosomal contents, and exposure of the inner acrosome membrane, and this process is regarded as regulated exocytosis. As discussed previously, progesterone is able to enhance the acrosome reaction *in vitro.*

While the initial interactions on the surface of the ZP essentially go unnoticed by the egg, the acrosome reaction that they initiate is critical for transforming the sperm into a fusogenic cell (Yanagimachi, 1994). In addition to release of hydrolytic enzymes presumably required for sperm to penetrate the ZP, the acrosome reaction brings about

remodeling of the sperm surface, with a portion of the original plasma membrane being replaced by the newly exposed inner acrosomal membrane. Although many of the details of this process remain unclear, membrane proteins undergo changes in location and activity required both for continued adhesion to the ZP and for fusion with the egg plasma membrane (Myles, 1993; Yanagimachi, 1994).

Step 4: Sperm Penetration of the *Zona pellucida*

After the acrosome reaction, sperm must maintain their association with the ZP as they make their way to the egg plasma membrane. In the mouse, ZP2 serves as the secondary binding molecule in the egg ZP (Bleil *et al.*, 1988) for an as yet unidentified mouse sperm surface protein. A likely candidate is a mouse homologue of PH20, a GPI-linked guinea pig sperm surface protein involved in binding of acrosome-reacted sperm to the ZP (Primakoff *et al.*, 1985; Thaler and Carullo, 1995).

Step 5: Sperm Binding to and Fusion with the Oocyte Oolemma

Sperm that have traversed the ZP are poised to bind to and fuse with the egg plasma membrane. Although numerous proteins involved in intracellular fusion reactions have been identified (Rothman, 1994), until recently there were no compelling candidates for proteins that would enact the intercellular fusion reaction. An hypothesis for sperm-egg fusion, based on viral models, is that a protein complex with dual binding and fusion functions would be found on the sperm or egg, and that a receptor(s) for this binding/fusion complex would be found in the plasma membrane of the opposite gamete (White, 1995). Interaction between these sets of complementary proteins would induce a

conformational change in the binding/fusion complex, leading to exposure of a hydrophobic fusion domain that would interact with the target lipid bilayer to consumate fusion (White, 1995).

Antibody inhibition studies have implicated several sperm surface proteins in binding and fusion (Myles, 1993; Allen and Green, 1995). Of these, a complex referred to as fertilin (previously, PH30) has been characterized in the greatest detail. The seminal observation implicating fertilin was the demonstration that an anti-fertilin monoclonal antibody inhibits sperm-egg fusion *in vitro* (Primakoff *et al.*, 1987).

The oolemma (egg plasma membrane) is regarded as the final obstacle for sperm-egg fusion. The equatorial region has been reported to be the first region of the sperm plasma membrane to fuse with the oolemma (Yanagimachi, 1988), though the post-acrosome region may fuse at the same time or shortly thereafter (Oura and Toshimori, 1990). Radiolabeling studies showed that both inner acrosome membrane and sperm plasma membrane are incorporated into the oocyte oolema after sperm fuse with the oolemma.

Sperm surface molecules involved in binding and fusion include PH-30, DE, M29, M37 and OBF13. PH-30, also called fertilin, was identified in guinea pig sperm and is composed of an α (45-49 kDa) and β (25-33 kDa) subunits (Primakoff *et al.*, 1987; Snell and White, 1996). PH-30 β possesses a disintegrin domain but the function of PH-30 α remains unclear. PH-30 is located on the whole head of testicular sperm, but becomes concentrated in the posterior head region during epididymal maturation of the sperm (Phelps *et al.*, 1990). PH-30 protein also undergoes a change in location and PH-30 β proteolytic processing (Phelps *et al.*, 1990). It remains in the posterior head region

after the acrosome reaction, so that at the time of fertilization, it is in an appropriate region to act in sperm-egg fusion (Primakoff *et al.*, 1987).

DE protein is composed of two 37-kDa polypeptides (D and E), that differ in their isoelectric points. Identified in the mouse, DE is thought to originate as a secretion from the epididymal epithelium and become attached to the sperm surface during epididymal transit (Kohane *et al.*, 1980a,b). Immunofluorescence studies show DE is initially associated with the dorsal region of the acrosome, but is relocalized to the equatorial region of the sperm head during capacitation (Rochwerger and Cuasnicu, 1992a). Thus, at the time of sperm fusion with the egg it is also in the correct location to participate in fusion. However, immunological studies suggested that DE didn't affect sperm binding to the egg (Rochwerger *et al.*, 1992b).

M29, M37 (Saling *et al.*, 1985) and OBF13 (Okabe *et al.*, 1988) were all identified in mouse sperm and were observed to associate with sperm binding and/or fusion (Saling *et al.*, 1985; Okabe *et al.*, 1988; Kawai *et al.*, 1989).

On the surface of egg plasma membrane, Integrin has been identified (Almeida *et al.*, 1995; Bronson *et al.*, 1995; Evans *et al.*, 1995), and is composed of two subunit heterodimers: α (120-180 kDa) and β (90-110 kDa). Identification of an integrin on the sperm plasma membrane indicated that it directly combined with PH-30 β on the sperm surface during sperm-egg binding and fusion. This has been hypothesized, but not yet directly proven.

Step 6: Activation of Fertilized Oocytes

The first event of egg activation after PH-30 β binding to an integrin in the egg plasma membrane is tyrosine phosphorylation of an integrin itself. This leads to a decrease in integrin binding activity (Tapley *et al.*, 1989). As a result, a feedback inhibition is initiated to block polyspermy. This is the so called "vitelline" block.

Another possible mechanism for blocking polyspermy is the *zona* reaction. Following sperm fusion with the egg in mice, cortical granules in the activated oocyte released a kind of non-trypsin-like protease that can catalyze a *zona* glycoprotein transformation from ZP2, a 120-kDa glycoprotein to $ZP2_f$, a 90-kDa form (Moller and Wassarman, 1989; Ducibella *et al.*, 1990). Conversion of ZP2 into $ZP2_f$ correlates with the hardening of the *zona* during the fertilization process (Wassarman, 1990).

2.6. Implications

In summary, seminal plasma plays an important role in gamete transport, sperm maturation, capacitation and fertilization in pigs. Of the many varieties of proteins in boar seminal plasma, PSP-I is one of the most predominant spermadhesins and has been proven to be associated with sperm capacitation and gamete recognition and binding. Estradiol-17β and testosterone are two major steroids in boar seminal plasma. They enhance transportation of sperm and oocytes in the female reproductive tract and secretion of heparin-binding proteins from the seminal vesicles. These major macromolecules are thought to be candidate indicators of semen quality and boar fertility. Further study is necessary to discover their associations with semen characteristics, IVF

estimates and field fertility data. Such studies are described in the following three chapters.

2.7. **Bibliography**

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Chapter 3

Effects of Seminal Plasma from Different Fractions of Individual Ejaculates on *in vitro* Fertilization in Pigs

3.1. Introduction

Due to an abundant amount of testicular interstitial tissue (Fawcett *et al.*, 1973), the boar secretes a variety of reproductive hormones including estrogens into semen (Claus and Hoffmann, 1980). Seminal estrogens have been shown to influence ovulation and sperm transport via secretion of $PGF_{2\alpha}$ from the endometrium (Claus *et al.*, 1990) and consequently to improve conception rate and litter size (Claus *et al.*, 1989). Proteins are another major component of seminal plasma and are derived from the epididymis, but mainly from the boar accessory sex glands. Many of the seminal plasma proteins have been purified and characterized; they affect sperm maturation, capacitation/decapacitation, movement and fertilization (Rutherfurd *et al.*, 1992; Strzeżek *et al.*, 1992; Parry *et al.*, 1992; Kwok *et al.*, 1993; Siviter and Cockle, 1995). Spermadhesins are a major family of proteins in boar seminal plasma and account for over 90% of total protein (Calvete *et al.*, 1997). They have been reported to be associated with sperm capacitation and gamete recognition and binding (Sanz *et al.*, 1992a and b, 1993; Calvete *et al.*, 1994; Topfer-Petersen *et al.*, 1994 and 1998). However, more direct evidence is needed to identify how these seminal hormones and proteins affect sperm capacitation and the fertilization process

In vitro fertilization (IVF) in pigs has been widely studied since sperm capacitation was first described in 1951 (Austin, 1951&1952; Chang, 1951). This technique has been successfully applied in evaluating boar sperm fertility (Wang *et al.*, 1991; Ivanova and Mollova, 1993; Martínez *et al.*, 1993) and in our laboratory, more consistent IVF results were obtained by using spermatozoa from a standardized sperm-rich fraction (Xu *et al.*, 1996). Fraction 1 (first sperm-rich fraction) sperm of the ejaculate were superior to either fraction 2 (sperm-depleted fraction) or fraction 3 (second sperm-rich fraction) sperm in terms of penetration rate, male pronucleus (MPN) formation rate, polyspermy rate and average number of sperm penetrating each fertilized oocyte. Whether this variability in fertilization capacity comes from the seminal plasma or from the sperm themselves has not been clarified.

This study, in which three standardized fractions of seminal plasma were separately used to incubate sperm pellets from the first sperm-rich fraction of the same ejaculate, was designed to compare possible influences of seminal plasma on fertilization capability using IVM/IVF techniques.

3.2. Materials and Methods

3.2.1. Preparation and Maturation of Oocytes

Ovaries with mature follicles were obtained from freshly slaughtered prepubertal gilts at a local abattoir (Gainers Co., Edmonton, AB). They were held in a plastic jar and transferred to our laboratory in a polystyrene box within 40 min after recovery to avoid major changes in temperature. After washing with saline, oocyte-cumulus complexes

(OCCs) were aspirated together with follicular fluid from healthy-looking 3-6 mm follicles (clear follicular fluid and well vascularized) using a 10-cc syringe attached to an 18 ½ gauge needle. After removal of supernatant, spontaneously precipitated OCCs were washed three times with Dulbecco's Phosphate Buffered Saline (PBS) supplemented with 1% (v/v) porcine follicular fluid (pFF), which was prepared from 3-6 mm, transparent and grape-like follicles during our preliminary study. Only OCCs containing oocytes with evenly-distributed cytoplasm and multilayered compact cumulus cells were harvested under a dissecting microscope as being satisfactory for maturation culture.

The oocyte maturation medium comprised 85% (v/v) TCM-199 medium (12340-30, Life Technologies), 10% (v/v) pFF and 5% (v/v) additive mixture containing 2 mg ml^{-1} glutamine (BDH, Toronto), 1.4 mg ml^{-1} L-ascorbic acid (BDH, Toronto), 0.7 mg ml^{-1} insulin (I-1882, Sigma) and 1 mg ml^{-1} PVA in TCM-199 medium. Two ml of this solution was added to individual 35×15 mm petri-dishes and 0.1 ml multi-gonadotropin stock containing 5 mg ml^{-1} FSH (USDA-pFSH-B-1, AFP-5600), 0.05 mg ml^{-1} LH (NIADDK-oLH-26, AFP-5551b), 0.4 μg ml^{-1} prolactin (USDA-pprl-B-1, AFP-5000) and 1 mg ml^{-1} PVA in TCM-199 medium, was then added to each dish. All details of this methodology were previously described by Xu *et al.* (1996), except that in the present study, based on the observations by Yoshida *et al.* (1992), the pFF supernatant used in the media was directly filtered with a 0.2 um syringe filter (190-2520, Nalgene) without heat treatment.

Fifty to sixty selected OCCs were transferred to individual petri-dishes and incubated for 44 h under 5% (v/v) CO_2 at 39 °C . Oocytes with many evenly-expanded cumulus cells attached were regarded as matured oocytes and randomly transferred to

individual 4-well dishes with 10 OCCs in each well containing 0.9 ml preincubated fertilization medium.

3.2.2. Collection and Treatment of Semen

Three young, fertile Landrace boars (coded as Boar A, B and C) housed at the Alberta Swine Genetic Cooperation (ASGC) boar stud were selected for experimental use. They were regularly collected twice a week with a gloved-hand method. On all occasions, the whole ejaculate was collected into a series of 15-ml sterile tubes and the sperm concentration in each tube was measured at 540 nm with a Spectronic 301 spectrophotometer immediately following collection. The most concentrated tube of the first sperm-rich fraction of the ejaculate was defined as fraction 1 (F1), the least concentrated tube after the first sperm-rich fraction as fraction 2 (F2) and the most concentrated tube of the second sperm-rich fraction as fraction 3 (F3). Only the ejaculates possessing 80% or higher sperm motility measured immediately after collection in F1 sperm, were used in IVF and delivered to our laboratory before 6:00 pm on the day of collection.

The semen was centrifuged at 550×g for 10 min at 25 °C and 0.5 ml precipitated sperm from F1 was then pipetted into 2.0 ml seminal plasma collected from F1, F2 and F3 of the ejaculate, respectively. The recombined semen was gently mixed and then kept in a polystyrene box for 14-16 h at 25 °C.

3.2.3. Capacitation and Insemination of Semen

After co-incubation, semen was washed twice with sperm washing medium (9 mg ml^{-1} NaCl, 1 mg ml^{-1} BSA, 0.1 mg ml^{-1} Kanamycin) and a third time with sperm

preincubation medium (0.55 mg ml^{-1} D-Glucose, 0.9 mg ml^{-1} Calcium lactate, 0.1 mg ml^{-1} Sodium pyruvate, and 0.1 mg ml^{-1} Kanamycin in M-199 medium, 4 mg ml^{-1} BSA added just before use), using centrifugation at 550×g for 5 min for the first wash, and at 500×g for 5 min for the second and third washes. Sperm concentration was then adjusted to 4×10^8 sperm ml^{-1} with preincubation medium before incubation at 39 °C under 5% (v/v) CO_2.

After 1.5 h of preincubation, sperm agglutination was scored for 20 aggregates of sperm on a central screen of semen specimens under a microscope with a 5-point system. Grade 1, 2, 3, 4 and 5 represent 1-3, 4-6, 7-9, 10-12 and 13-15 sperm, respectively, in each aggregate. One hundred microliter of further diluted semen at a concentration of 2×10^7 sperm ml^{-1} in fertilization medium (sperm preincubation medium containing 0.3884 mg ml^{-1} caffeine-sodium benzoate (50:50, w/w, mixture) (Sigma, St. Louis, MO) was subsequently inseminated into each culture well containing expanded OCCs. The final sperm concentration was 2×10^6 sperm ml^{-1} and the sperm to oocyte ratio was 2×10^5:1 during fertilization.

At 6 h after insemination, fertilized oocytes were rinsed with preincubated Whitten's Medium and then transferred to preincubated Whitten's Medium for a further 8-h sperm-free incubation.

3.2.4. Evaluation of Fertilized Oocytes

After completion of fertilization, oocytes were denuded of cumulus cells, mounted on glass-slides with 10-20 oocytes on each slide, and fixed in acetic acid-ethanol (1:3, v/v) solution. After 48 h, oocytes were stained with 0.75% (w/v) lacmoid in

45% (v/v) acetic acid and subsequently rinsed with glycerin-acetic acid-ethanol (1:3:1, v/v/v) solution until their backgrounds were clear.

Stained oocytes were observed at 200- or 400-fold magnification under a phase-contrast microscope. Oocytes containing one or more unswollen and/or swollen sperm head(s), and/or MPN with one or more detached sperm tails were considered as penetrated. Monospermic fertilized oocytes were defined as oocytes containing only one sperm or MPN. Oocytes classified as polyspermic possessed two or more sperm and/or MPN. The number of sperm penetrating each fertilized oocyte included the total number of sperm and MPN. The above observation criteria have been previously described by Ding and Foxcroft (1994) and Xu *et al.* (1996). In the present study, only sperm with their heads attached to the oocyte membrane were counted as "attached". Any unfertilized or recently-penetrated oocyte without a visible polar body (pb) was regarded as an immature oocyte (MI or earlier oocyte), and was not included in the total number of scored oocytes shown in Table 3-2. The experiment was repeated three times.

3.2.5. Statistical Analysis of Data

The experiment was analyzed as a split-plot (Steel and Torrie, 1980) design using the General Linear Model procedures of the Statistical Analysis System (version 6.08, SAS Institute Inc., Cary, NC 27512-8000). Boar was the main plot and the effects of boar were tested against the replicate (block) by boar (main plot) interaction. The effects of seminal plasma (sub-plot) were tested against the boar by fraction interaction, and the interaction between boar and fraction was tested against the replicate by boar by fraction interaction. Where appropriate, multiple comparisons were carried out among individual

means using the Student-Newman-Keul test. All of the differences were evaluated at the level of $P<0.05$.

3.3. Results

For three consecutive replicates, three distinct fractions per ejaculate were obtained from Boar A and B, whereas only the first two fractions were regularly collected from Boar C. The characteristics of the ejaculates collected for study are presented in Table 3-1.

The post-capacitation sperm motility and agglutination are presented in Table 3-2. In relation to the main factor under investigation, the fraction of seminal plasma used for *in vitro* fertilization affected penetration rate ($P<0.001$) but no other measure of oocyte penetration or development. F1 sperm co-incubated with F1 seminal plasma had a higher penetration rate than F1 sperm co-incubated with F2 seminal plasma (Figure 3-1). The results also indicated that the boars differed in MPN formation ($P<0.05$) (Figure 3-2). There was no significant interaction between boar and seminal plasma fraction for any of the *in vitro* characteristics measured.

3.4. Discussion

The use of F1 seminal plasma for co-incubation with F1 sperm resulted in a higher penetration rate than the use of F2 seminal plasma. No significant interaction was found between boar and seminal plasma fraction in our study. As recombined semen was

kept overnight under the same circumstances and then handled in the same IVF procedure, it is assumed that the variability in penetration rate observed was related to the characteristics of the different fractions of seminal plasma used.

Like other plasma, seminal plasma consists of many components, some of which are thought to influence sperm motility, capacitation/decapacitation and fertilization. High concentrations of estrone (E1), estradiol-17β (E2) and estrone-sulfate (E1-s) are present in boar semen with considerable differences in mean concentration and total amount among individual ejaculates (Claus *et al.*, 1983, 1985a, 1985b, 1987; Schopper *et al.*, 1984). Claus and colleagues further demonstrated that the addition of a physiological amount of estrogens to semen extender used for artificial insemination could increase both conception rate and litter size born (Claus *et al.*, 1989).

Seminal plasma proteins are also major components of seminal plasma. In addition to maintenance of semen osmolarity and nourishment for sperm, they are involved in sperm capacitation and gamete recognition and binding (Sanz *et al.*, 1992 a and b, 1993; Calvete *et al.*, 1994; Topfer-Petersen *et al.*, 1994 and 1998). Porcine Seminal Plasma Protein I and II (PSP-I and PSP-II) were identified as glycoproteins with 50% amino acid sequence homology with a family of *zona pellucida*-binding proteins (Rutherfurd *et al.*, 1992; Kwok *et al.*, 1993) and could prevent premature acrosome reaction and immunosupression (Kwok *et al.*, 1993). Fertilization-promoting peptide (FPP) was shown to be present in mammalian prostate at a high concentration and enhanced fertilization potential of sperm (Siviter and Cockle, 1995). At least two kinds of sperm motility inhibiting factors (SMIFs) were purified and identified from boar semen (Iwamoto *et al.*, 1991; Strzeżek *et al.*, 1992). Strzeżek *et al.*(1992) reported that SMIF

was able to inhibit sperm motility in a non species-specific manner. A group of *zona pellucida* binding proteins (ZPBP, 16-23 kDa) has also been described in boar ejaculates. They were derived from seminal plasma, as shown by their absence on epididymal spermatozoa and their presence in seminal plasma as detected by N-terminal amino acid sequence analysis, and could regulate the rate of sperm capacitation and survival in the female genital tract (Parry *et al.*, 1992). More than one decapacitation factor was reported in boar seminal plasma by Bonilla *et al.* (1996) and these factors were greater than 100 kDa in molecular weight.

The above observations indicate that many proteins and hormones in seminal plasma are related to sperm function. Masuda *et al.* (1979) observed differences in the concentrations of protein, as well as citric acid and zinc, which were positively correlated with the agglutinating activity in seminal plasma, either among different fractions of seminal plasma or among different seasons. It can be reasonably inferred that during the 14 to 16 h pre-capacitation sperm culture with substituted seminal plasma, certain qualitative and quantitative changes took place in the proteins and hormones coating the sperm surface, and that this eventually induced functional alterations in the capacitated sperm. Interestingly, about half of the total amount of seminal estrogens was bound to the sperm membrane (Claus *et al.*, 1985a). Whichever hormones or proteins might be involved, the net effect of exposure of standard aliquots of sperm from fraction 1 semen with F1 seminal plasma was more beneficial than co-incubation with F2 seminal plasma during capacitation and fertilization. Whether this is due to the binding of beneficial molecules or hormones, or the removal of factors inhibitory to sperm capacitation is unknown.

At the same sperm:oocyte ratios used for IVF in this study, there was no difference in penetration rate among the three boars tested. Although high and equivalent sperm motility (≥80%) was recorded for each replicate among the three boars when semen was evaluated immediately after collection of the ejaculates, a dramatic post-centrifugation decrease (53%) and then an obvious post-capacitation rebound (70, 63 and 63% respectively for F1, F2 and F3) was observed in Boar B, while Boar C exhibited a gradual drop in sperm motility, along with the poorest post-capacitation sperm agglutination (Table 3-1 and 3-2). These distinctly coincubation-induced effects contributed to sperm penetration rates. Although there was no significant difference in sperm motility among the three boars, the sperm co-incubated with F1 seminal plasma possessed a higher motility and more obvious post-capacitation agglutination than sperm co-incubated with F2 or F3 seminal plasma after capacitation, as shown in Table 3-1 and 3-2.

A high penetration rate was always accompanied by a relatively low monospermy rate (80.47% vs. 27.43%, 89.23% vs. 17.37%, respectively for Boar A and B in F1), consistent with the previous results from our laboratory using similar sperm:oocyte ratios (Xu *et al.*, 1996). Although lower sperm:oocyte ratios could have been used in the present study to better describe the effects of F1 seminal plasma, preliminary results indicated that a further reduction in sperm:oocyte ratio led to a dramatic drop in penetration rates with F2 seminal plasma and it was, therefore, difficult for the three treatments to be properly compared.

In conclusion, three fractions of boar seminal plasma within one ejaculate had different effects on *in vitro* penetration rate of *in vitro* matured porcine oocytes when they

were separately used for co-incubation with the same F1 sperm. Boars were different in *in vitro* developmental ability of MPN. These results suggest that seminal plasma components such as proteins and steroids may contribute to seminal plasma effects on the fertilization process.

3.5. Bibliography

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Table 3-1. Physiological Characteristics of the Three Ejaculates from Each of Three Boars Used in IVF (Mean±SEM)*

Boar	Post-ejaculation Motility†(%)	Post-centrifugation Motility(%)	Ejaculate Volume(ml)	Sperm Concentration ($\times 10^6$/ml)‡		
				Fraction 1	Fraction 2	Fraction 3
A	≥80	80±6	250±5[a]	828±70	<7	248±71
B	≥80	53±15	155±5[b]	1148±150	<7	216±139
C	≥80	67±15	150±15[b]	953±173	<7	N/A

*Within columns, different superscripts indicate significant differences ($P<0.05$).

†This motility was evaluated at ASGC.

‡Measured at 540 nm with a Spectronic 301 spectrophotometer.

Table 3-2. Sperm Motility and Agglutination Estimated after *in vitro* Capacitation (Mean±SEM)*

Boar	Fraction	Post-capacitation Motility (%)	Post-capacitation Agglutination†
Boar A	F1	77±3	3.7±0.3
	F2	70±0	2.3±0.3
	F3	63±9	2.3±0.3
Boar B	F1	70±0	3.7±0.3
	F2	63±7	2.3±0.3
	F3	63±3	2.3±0.3
Boar C	F1	53±13	2.7±0.3
	F2	47±12	2.0±0

*Mean values represent means of three replicates, with 4-8 wells per replicate. † Sperm agglutination was scored for 20 aggregates of sperm on a central screen of semen specimens under a microscope with a 5-point system. Grade 1, 2, 3, 4 and 5 represent 1-3, 4-6, 7-9, 10-12 and 13-15 sperm, respectively, in each aggregate.

Figure 3-1. Effects of seminal plasma fraction on penetration rate (PR), monospermy rate (MR), male pronucleus formation rate (MPNR), number of sperm penetrating each fertilized oocyte (NSI), and number of sperm attached to each matured oocyte (NSO). Three boars were tested with three replicates. A total of 69-116 matured oocytes were scored for each sample. The same letters (a, b and c) on histogram bars indicated no difference in the effect of fraction on the IVF characteristic measured ($P>0.05$).

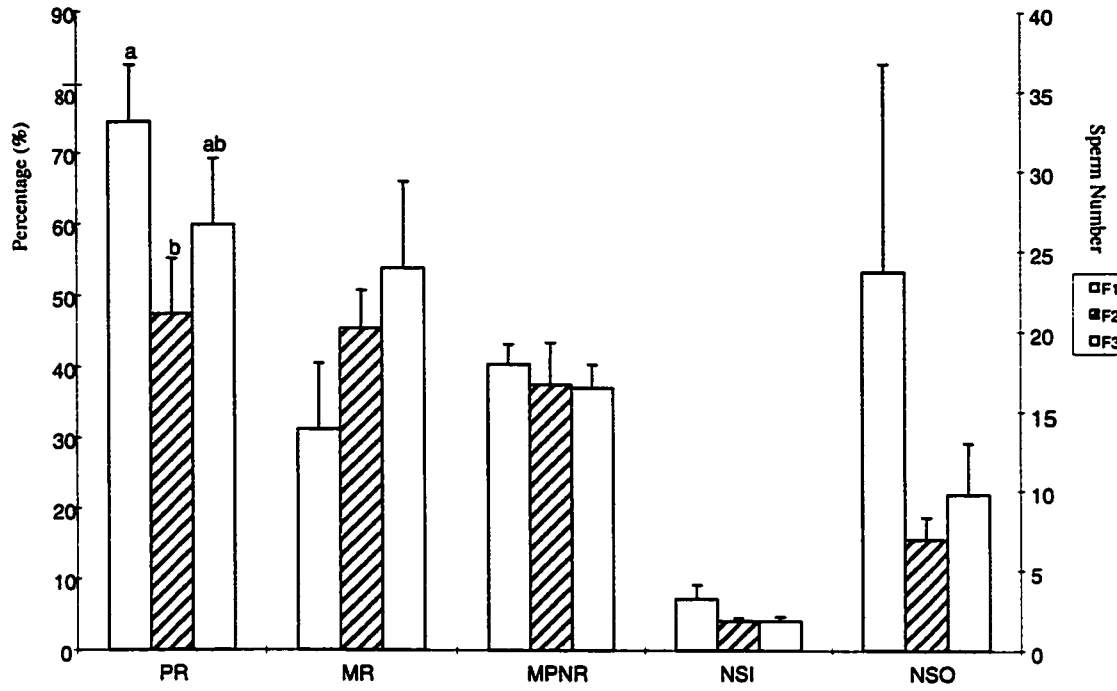
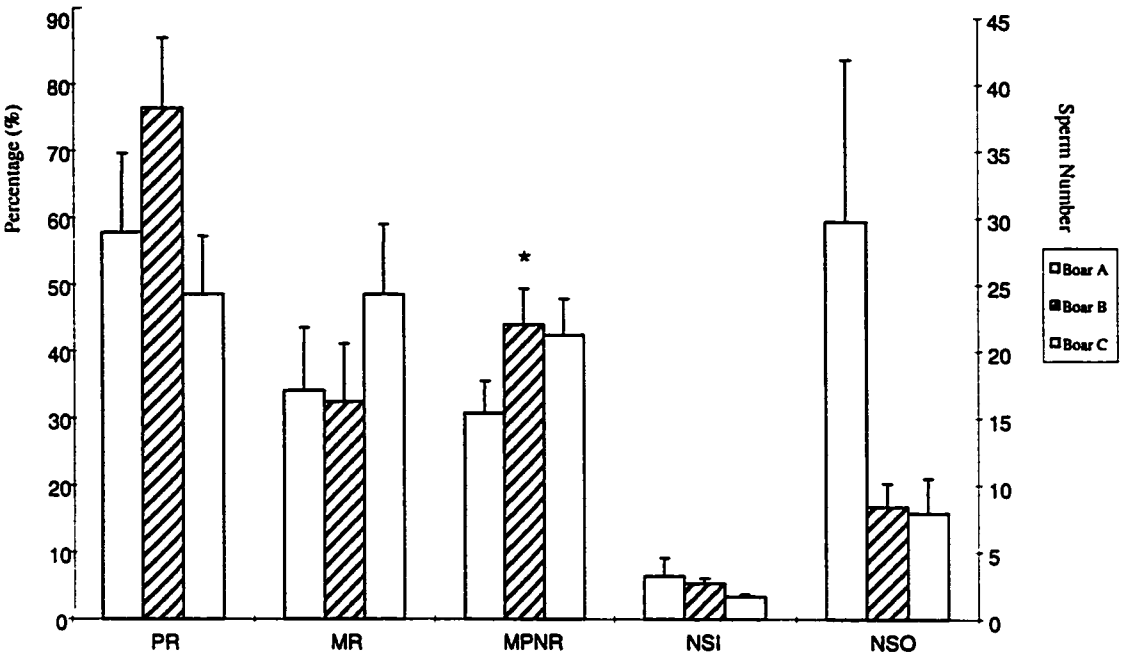

Percentage (%)
Sperm Number
a
b
ab
F1
F2
F3
PR
MR
MPNR
NSI
NSO

Figure 3-2. Effects of boar on IVF. The parameters evaluated include penetration rate (PR), monospermy rate (MR), male pronucleus formation rate (MPNR), number of sperm penetrating each fertilized oocyte (NSI), and number of sperm attached to each matured oocyte (NSO). Means represent average values of three fractions (Boar C has two fractions) with three replicates. A total of 69 to116 matured oocytes were scored for each sample. * Boars were different in MPN formation rates ($P<0.05$).


Percentage (%)
90
80
70
60
50
40
30
20
10
0
45
40
35
30
25
20
15
10
5
0
Sperm Number
Boar A
Boar B
Boar C
*
PR
MR
MPNR
NSI
NSO

Chapter 4

Seminal Plasma Proteins and Boar Fertility: Part 1, Assessment of Porcine Seminal Plasma Protein-I (PSP-I) and Other Low Molecular Proteins in Boar Seminal Plasma

4.1. Introduction

In previous IVF studies in our laboratory, spermatozoa recovered from the first sperm-rich fraction (F1) of the boar ejaculate were superior to those from either the subsequent sperm-depleted fraction (F2) or the second sperm-rich fraction (F3) in terms of penetration rate, male pronucleus (MPN) formation rate, polyspermy rate and average number of sperm penetrating each fertilized oocyte (Xu *et al.*, 1996). Further studies, presented in Chapter 3, indicated that F1 spermatozoa co-incubated with their original F1 seminal plasma had a higher penetration rate than F1 sperm co-incubated with F2 or F3 fractions of seminal plasma. These findings suggest that components of seminal plasma might play an important role in fertilization and the outcome of IVF.

As major components of seminal plasma, proteins not only preserve seminal plasma osmotic status, but also nourish spermatozoa and affect their fertility. Spermadhesins are the most predominant proteins in boar seminal plasma and account for over 90% of total proteins (Calvete *et al.*, 1997). These low molecular mass (12,000-20,000 Daltons) lectins, derived mainly from seminal vesicle epithelial cells (Sinowatz *et al.*, 1995), are mixed with sperm during ejaculation and coat the sperm surface (Sanz *et*

al., 1992b). With the exception of Porcine Seminal Plasma Protein-II (PSP-II), these proteins possess strong heparin-binding ability (Jonakova *et al.*, 1991; Sanz *et al.*, 1992a; Hanqing *et al.*, 1991 Moos *et al.*, 1992; Parry *et al.*, 1992). Glycosylation directly contributes to their structural diversity and affects their *zona pellucida*-binding activity (Calvete *et al.*, 1993a & b, 1994a). Biologically, spermadhesins are thought to play a major role in the process of sperm capacitation and gamete recognition and binding (Calvete *et al.*, 1994b; Topfer-Petersen *et al.*, 1994 and 1998). Of the seven spermadhesins identified to date, PSP-I represents over 25% of total protein in seminal plasma (Rutherfurd *et al.*, 1992) and binds to a number of proteins including endo-β-galactosidase-digested ZP3, soybean trypsin inhibitor, IgA, IgG and α-casein (Kwok *et al.*, 1993). This 109 amino acid residue glycoprotein is able to form a non-covalent heterodimer with certain glycoforms of PSP-II (Romero *et al.*, 1996), resulting in the loss of their pre-heterodimerization heparin-binding ability (Salis *et al.*, 1998). As a multifunctional protein, PSP-I is a potentially useful marker for assessment of semen quality in pigs.

The study described below established techniques for the qualitative and quantitative assessment of total proteins, PSP-I and other low molecular weight (MW) proteins in boar seminal plasma. This methodology was then used to determine differences between two fractions of seminal plasma and among boars.

4.2. Materials and methods

4.2.1. Collection of Boar Seminal Plasma

Two fractions, the first sperm-rich fraction (F1) and the subsequent sperm-depleted fraction (F2), of seminal plasma harvested earlier from six fertile boars were used in this study. The samples were collected before (January 31-February 14, 1996), during (April 10-24, 1996) and after (July 17, 1996) a period when semen from the same boars was evaluated *in vivo* using field artificial insemination. Also, F1 sperm from the same ejaculates used in the present study for determination of seminal plasma proteins, were previously evaluated by IVF techniques and standard laboratory assessments of these semen samples were also made. These earlier studies and the collection and subsequent treatment of semen were described in detail previously (Xu *et al.*, 1998). All samples of isolated seminal plasma were stored at –70 °C until analyzed.

4.2.2. Assay of Total Protein Content of Samples

Seminal plasma samples were centrifuged at 12,000g for 15 min at 4 °C to remove cells and debris. Protein content was assayed in duplicate using the standard bicinchoninic acid (BCA) protein assay as described by Smith *et al.*(1985). For BCA assays, the supernant was diluted with PBS at an appropriate dilution rate ranging from 1:2 to 1:50. The absorbance at 562 nm was measured upon cooling to 25 °C. A standard curve was constructed for absorbance coefficients using bovine serum albumin (BSA) as the standard. The protein concentrations of the seminal plasma samples were estimated in relation to this standard curve.

4.2.3. Evaluation of PSP-I by Western Blotting

In order to precisely identify and quantify PSP-I in seminal plasma by Western blotting, different methodologies were evaluated and integrated into a standardized technique, which is described below. Pooled seminal plasma was used for this purpose.

4.2.3.1. Initial Evaluation of Western Blotting Techniques

One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins in pooled seminal plasma. Standard 180×120×1 mm separating gels were cast at a final concentration of 15% (w/v) polyacrylamide diluted from a 30% (w/v) (29 acrylamide:1 bisacrylamide) mix and overlaid with a 5% (w/v) acrylamide stacking gel (Sambrook *et al.*, 1989). Fifteen micrograms of protein were denatured in sample buffer (2% (w/v) SDS, 100 mM DTT, 60 mM Tris (pH 6.8) and 0.01% (w/v) bromphenol blue) at a concentration of 0.5 $\mu g\ ml^{-1}$ of total protein and loaded on the gel. Low MW protein markers ranging from 2,345 to 46,000 Daltons (Amersham Phamacia Biotech, Inc., Baie d'Urfé, Quebec) were used to calibrate this electrophoresis gel. Gels were run in an electrophoresis buffer (25 mM Tris, 250 mM glycine (pH 8.3) and 0.1% (w/v) SDS) for 1 to 2 h at a constant current of 16 mA per gel during the stacking gel stage and for another 3 to 4 h at 24 mA per gel in the separating gel stage. Two gels containing identical sample loadings were simultaneously run for each test.

After electrophoresis, one gel was stained for 6 h in 0.25%(w/v) Coomassie Brilliant Blue in 10%(v/v) glacial acetic acid, 45%(v/v) methanol and destained for 18 h in a solution of 10%(v/v) glacial acetic acid, 45%(v/v) methanol. The relative molecular

mass of each stained protein band was evaluated by measuring linear mobility distances of unknown electrophoretic bands and interpolating on a standard curve of mobility of the standard protein markers against their corresponding molecular weights.

The other gel was subjected to Western Blotting. The transfer of immobilized proteins from the polyacrylamide gel onto a nitrocellulose membrane was carried out by a wet electrophoretic elution, which was performed for 18 h in a transfer buffer (25mM Tris, 190 mM glycine and 20% (v/v) methanol), at a constant voltage of 50 V with a variable current, in a 4 °C cooling system. After transfer, non-specific proteins were blocked for 1 h in 5% (w/v) BSA in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6) at room temperature. After two initial brief rinses, the membrane was then washed for 25 min with 3 changes of TBS-T washing solution (20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6). The washed membrane was incubated for 1 h at room temperature with a primary rabbit antiserum raised against PSP-I and previously validated by Kwok *et al.* (1993). Non-specifically bound proteins were removed by washing as described above. The membrane was then incubated for 1 h at room temperature with a horseradish peroxidase-linked donkey anti-rabbit second antibody (Ab2) (Amersham Phamacia Biotech, Inc., Baie d'Urfe, Quebec). After the same washing steps described above, the membrane was incubated for 1 min in ECL solution (Amersham Phamacia Biotech, Inc., Baie d'Urfé, Quebec) containing luminol and chemical enhancers. The membrane was immediately exposed to Hyperfilm™-ECL™ (Amersham Phamacia Biotech, Inc., Baie d'Urfe, Quebec) for 15 seconds and the exposed film was subsequently processed in Kodak M35A X-omat Processor (Eastman Kodak Co.).

Scanning densitometry of individual immunoreactive bands on Western Blots was carried out on a Model GS-670 Imaging Densitometer (Bio-Rad Laboratories, Richmond, CA) and proteins were quantified by integrating volumes of corresponding band-enclosed rectangles using the Molecular Analyst program (Bio-Rad Laboratories, Richmond, CA). Final concentrations are expressed as the adjusted volume ($OD \times mm^2$) corrected for the loading amount and the concentration of total proteins.

Due to the observation of high background and multiple immunoreactive bands displayed on initial Western Blots, a series of dilutions were tested for both primary and secondary antibodies, but this did not substantially improve the results of our Western Blotting (Figure 4-1).

Even at high dilutions of both antibodies, only two lower relative molecular weight (M_r) bands were clearly separated from other immunoreactive bands on the blots. Based on its mobility relative to protein markers, the lowest band was putatively identified as unglycosylated PSP-I, with higher M_r's immunoreactive bands resulting from glycosylation of core PSP-I to varying degrees (Kwok *et al.*, 1993, Calvete *et al.*, 1993a and b). Since these were overlapping in their M_r, it was difficult to discriminate and quantify each of them. In addition, the existence of high background between individual species made the task of densitometric quantification very difficult. Relevant literature (Rutherfurd *et al.*, 1992; Calvete *et al.*, 1994a and 1995) suggested that enzymatic deglycosylation offered the possibility of clearer, more efficient quantification of PSP-I.

4.2.3.2. N-Glycanase Deglycosylation of Samples

Recombinant N-Glycanase™, or peptide-N-glycosidase F (Enzyme Immunobiologicals, Cambridge, MA) cloned from *F. meniingsepticum* and expressed in *E. coli* was used to remove intact N-linked oligosaccharides from PSP-I glycoproteins in order to obtain a single PSP-I band. Lyophilized seminal plasma was reconstituted at 5 mg ml^{-1} and denatured by heating for 2 min at 100 ^{0}C in a phosphate buffer (20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.02%(w/v) sodium azide) supplemented with 0.5% (w/v) SDS and 5% (v/v) β-mercaptoethanol. Both 5% (v/v) Nonidet P-40 (final concentration of 2.5% (v/v)) and phosphate buffer (1:1) were added to this denatured solution prior to digestion for 18 h at 37 ^{0}C with N-Glycanase™ (0.5 U μl^{-1} in 20 mM Tris HCl, 1 mM EDTA, 50 mM NaCl, 0.02%(w/v) sodium azide, pH 7.5) at a dose of 20 μg of total protein per unit. The resulting deglycosylated protein solution was dialyzed for 24 hr in 1000 ml of deionized distilled water (DDW) to remove excess salt and EDTA and then lyophilized. To confirm that resulting immunoreactive bands other than PSP-I were not due to insufficient N-Glycanase digestion, incubations were also carried out using twice as much enzyme as recommended by the manufacturer.

Enzymatic digestion significantly altered the protein profiles (Figure 4-2). Multiple bands between 12 to 20 kDa seen previously were reduced to a major doublet on the Coomassie Blue stained gel (Panel A) and on the basis of their relative mobility, these two proteins were evaluated as 13.8 and 12.1 kDa in molecular mass and designated as P13 and P12, respectively. Three major immunoreactive bands were now observed on the Western Blots with molecular masses of 27.5, 12.0 and 6.9 kDa and designed as P27, PSP-I and P7, respectively (Panel B). Although there were minor species observed

between 14.3 and 21.5 kDa, these were believed to be residual, undigested, resistant or partially deglycosylated forms which persisted even after doubling the concentration of N-Glycanase used (Lane 3, Panel B of Figure 4-2).

Enzymatic digestion results suggested that these three positive bands, including what is considered to be authentic PSP-I, could be effectively quantified using Western blots, in addition to the P12 and P13 proteins on the Coomassie-stained gels.

4.2.3.3. Separation of Heparin-bound Proteins and Heparin-unbound Proteins

An earlier study showed that PSP-I is present in both heparin-bound and heparin-unbound fractions of seminal plasma (Sanz *et al.*, 1993). Both isolates of the protein have an identical protein core, but possess different modifications (post-translational or co-translational), which affect their biological function, including their ability to dimerize with PSP-II (Dostálová *et al.*, 1994; Calvete *et al.*, 1995). This property of PSP-I led us to use affinity chromatography on Heparin Sepharose CL-6B to examine heparin-bound and heparin-unbound species of PSP-I, prior to further quantification by Western blotting.

One gram of freeze-dried Heparin Sepharose CL-6B (Amersham Phamacia Biotech, Inc., Baie d'Urfé, Quebec) was reconstituted to approximately 4 ml of swollen gel material. After swelling for 15 min in 20 ml DDW, Heparin Sepharose was transferred into a sintered glass filter for washing with 200 ml DDW. After resuspension in 20 ml PBS (20 mM phosphate, 150 mM NaCl, pH 7.3), gel was loaded into a 10×150mm chromatographic column and washed with 20 ml PBS. Prepared columns were stored at 4 °C until use.

After further washing the gel with 2 gel volumes of PBS, 8 mg of total protein in 2 ml of PBS-diluted seminal plasma (4 mg ml^{-1}) was loaded onto the Heparin Sepharose column. Once the seminal plasma had been loaded, 1 ml fractions of the flow-through (non heparin-bound protein) were collected into numbered 1.5-ml centrifuge tubes on a Bio-Rad fraction collector attached to a peristaltic pump at a rate of 6 ml h^{-1} by elution with 20 ml of 0.15 M NaCl in 0.02 M PBS. Absorbance of individual fractions was monitored at 280 nm in a GeneQuant RNA/DNA Calculator. When the absorbance values had reached a baseline level (usually below 0.020), 25 ml of a sodium chloride gradient (0.15 to 3.0M NaCl) was applied at a rate of 0.0114 M min^{-1} to the column to elute Heparin-bound protein. Absorbance was again monitored until it fell to baseline levels.

Before further processing, the major flow-through fraction from tubes 19 to 31 was pooled as the heparin-unbound fraction and half of the eluate collected in tubes 61 to 70 were pooled as the heparin-bound fraction for use in Western blotting (Figure 4-4). Consecutive tubes from the heparin-bound fraction were pooled for use in Coomassie-stained gels (Figure 4-3). After pooling, eluates were dialyzed for 24 h in cellulose membrane tubing (Fisher, Nepean, ON) against 1000 ml DDW with one change, prior to the protein being quantified by BCA assays. Eluates, balanced for protein, were then lyophilized.

A representative profile of a Heparin affinity chromatographic run and further SDS-PAGE separation of the resulting fractions is illustrated in Figure 4-3. BCA protein assays indicated that the ratio of bound proteins to unbound proteins was 2:3. The

heparin-bound fraction was collected into tubes 61-70 at concentrations of 1.18 to 2.20 M NaCl, with three distinct peaks of eluted proteins within this fraction (Figure 4-3A).

Following digestion of both pooled unbound and bound fractions with N-Glycanase, further separation by SDS-PAGE and subsequent quantification by Western blotting showed that PSP-I was more abundant in the unbound fraction than in the bound fraction, although some of the protein was possibly present as PSP-I/PSP-II heterodimers in the unbound fraction (Figure 4-4B). According to the integration of band volume, the deglycosylated PSP-I was distributed in the ratio of 1.0:0.9:2.5:11.1 in the untreated total seminal plasma, enzyme-treated bound fraction, untreated unbound fraction and enzyme-treated unbound fraction, respectively, whereas it was undetectable in the untreated bound fraction. In particular, glycosylated PSP-I in the bound fraction appeared to be more resistant to the enzyme, resulting in much higher background on the Western blots of the bound fraction than the unbound fraction. It was concluded that the heparin-bound fraction of PSP-I could not be completely deglycosylated, and therefore, accurately quantified. These results led us to quantify PSP-I in total seminal plasma rather than in the heparin-bound or unbound fraction, following N-Glycanase deglycosylation.

4.2.4. Identification and Quantification of PSP-I and Other Proteins in Experimental Samples

After N-Glycanase deglycosylation, each of three collections of twelve seminal plasma samples (6 boars × 2 fractions), harvested as described previously, was run on duplicate gels. One gel was used for analysis of protein profiles and quantification of low MW proteins with Coomassie staining and the other gel was for quantification of PSP-I,

P7 and P27 with Western Blotting. The first lane in the Coomassie-stained gel was loaded with protein markers. The first lane in the other gel used for transfer for Western blotting was loaded with a pooled seminal plasma sample as an internal control for the quantification of proteins.

4.2.5. Statistical Analysis of Data

Data were analyzed with the GLM procedure in version 7.0 of Statistical Analysis System (SAS, 1999). As no effect of time of collection of seminal plasma samples (three occasions within the study period) was found using repeated measures analysis, a split-plot model was used with boar as the main plot and fraction as the sub-plot. The three collections were considered as replicates with 12 combinations (6 boars × 2 fractions) each. In addition to effects of boars and fraction, interaction between boar and fraction was also tested against replicate as error term. Where applicable, Duncan's multiple comparisons of means were made among six boars. Simple regression analysis was used to evaluate correlations between any two parameters. All of the differences were evaluated at the level of $P<0.05$.

4.3. Results

4.3.1. Concentrations of Total Proteins

Total protein concentrations were higher ($P<0.01$) in F2 than F1 seminal plasma and differed among boars ($P<0.01$). There was no interaction between boar and fraction for total protein concentrations (Figure 4-5).

4.3.2. Characterization of PSP-I and Other Low M_r Proteins

Complete profiles of proteins in F1 and F2 seminal plasma were obtained on Coomassie-stained gels after SDS-PAGE electrophoresis (Figure 4-6). Computerized analyses of data from the two fractions indicated different protein distributions (Figure 4-7). More high MW proteins (≥20 kDa) were present in F1 than in F2 seminal plasma. In contrast, F2 contained more low MW proteins than F1. The high M_r proteins indicated by Peaks 1, 2 and 3 in Figure 4-7 were not evaluated further in the present study. Besides P12 and P13, three distinct bands were detected in F1. They were estimated to be 38.4, 20.4 and 15.4 kDa in molecular mass and designated as P38, P20 and P15, respectively (Figure 4-6). Peak 4 was dominant in F2 but was not quantified. Due to the effect of dilution caused by pooling of two fractions, these proteins were not apparent in pooled seminal plasma (Figure 4-2 and 4-4). Peaks representing P12 and P13 were common in both fractions. The observation that F2 possessed higher protein peaks (Figure 4-7), and more intensely staining bands (Figure 4-6), indicated potential quantitative differences in individual proteins between the two fractions.

4.3.3. Quantification of PSP-I and Other Low M_r Proteins

PSP-I, P7 and P27 were quantified by integrating the volumes of the respective bands on Western Blots (Figure 4-8, Table 4-1). P12 and P13 were quantified in both fractions of seminal plasma on Coomassie-stained gels using the same method (Figure 4-6, Table 4-1), whereas P15, P20 and P38 were only quantified in F1 (Figure 4-6, Table 4-2). F2 had consistently more PSP-I ($P<0.01$) and P7 ($P<0.0001$) than F1 seminal plasma and concentrations of both PSP-I ($P<0.05$) and P7 ($P<0.0001$) were different among

boars. There was no difference in other proteins either between fractions, or among boars and no interaction between boar and fraction for concentrations of any of the seminal plasma proteins. A significant difference in P20 was found among boars ($P<0.05$).

4.4. Discussion

Our results showed that the majority of boar seminal plasma proteins were under 21.5 kDa and that the most predominant proteins by Coomassie-staining were P12 and P13 (Figure 4-2 and 4-6). The most intense immunoreactive band found by Western Blotting was estimated to be 12.0 kDa in molecular weight. This molecular mass is consistent with the calculated molecular weight 11.98 kDa of PSP-I (Rutherfurd *et al.*, 1992). On Coomassie-stained gels, proteins P12 and P13 were estimated to have M_r's of 12.1 and 13.8 kDa, respectively. On the basis of its molecular weight and relative abundance, P12 was considered to be PSP-I, while P13 was considered to be PSP-II. It is possible, however, that the P12 band contained other proteins with a molecular weight similar to PSP-I, although estimated concentrations of PSP-I and P12 were highly correlated ($R^2=0.42$, $P<0.01$). According to Rutherfurd *et al.*(1992), estimates of the M_r of PSP-II varied from 13.6 to 14.1 kDa depending on which method or chromatographic fraction was used. These authors further demonstrated that digestion with N-Glycanase did not alter the molecular weight of PSP-II. It was clear from our results that PSP-I and PSP-II are two of the most predominant proteins in boar seminal plasma.

In addition to PSP-I, P7 and P27 were predominant immunoreactive species on the ECL films (Figure 4-2 and 4-8) and their molecular weights were estimated to be 6.9

and 27.5 kDa, respectively. The latter is consistent with the M_r of PSP-I/PSP-II heterodimers and were visible in the enzyme-treated unbound fraction, but not in the enzyme-treated bound fraction (Figure 4-4). The disappearance of PSP-I/PSP-II heterodimers in the heparin-bound fraction seemed to be consistent with an abrogation of heparin binding ability resulting from their dimerization (Calvete *et al.*, 1995). Protein bands at P7 and P27 appeared either in the enzyme-treated total seminal plasma (Figure 4-8) or in the enzyme-treated unbound fraction (Figure 4-4), but not in the enzyme-untreated bound fraction (Figure 4-4), nor in the untreated total seminal plasma (Figure 4-4). The lack of PSP-I/PSP-II heterodimers in the enzyme-untreated total seminal plasma was due to dissociation of non covalent bonds linking between PSP-I and PSP-II. It is indicated theroretically and in the present result that the P27 band were derived from N-Glycanase digestion of heparin-unbound proteins rather than PSP-I/PSP-II heterodimers.

In addition to the above non-specific bands, strong background immunoreactivity was seen in the enzyme-treated total seminal plasma. Western blotting of column-separated fractions of proteins demonstrated that the background reactivity was derived from the bound fraction, not from the unbound fraction (Figure 4-4). These findings suggest that heparin-bound glycosylated PSP-I was less sensitive to N-Glycanase than non-bound PSP-I since high background immunoreactivity and little evidence of specific bands were seen in the enzyme-treated heparin-bound fraction. In the unbound fraction, multiple immunoreactive bands existed along with a single low molecular weight band of deglycosylated PSP-I (Figure 4-4B, Lane EH-). The deglycosylation of this fraction of proteins with N-Glycanase reduced them to a single far more intense band of PSP-I with little apparent background. These findings indicate that PSP-I existed in both

glycosylated and non-/or partially deglycosylated forms in seminal plasma. No visible band corresponding in molecular weight to PSP-I/PSP-II dimers were seen on the Coomassie gel profiles either of total seminal plasma or of enzyme-untreated unbound fraction. This may be due to post-denaturation breaking of non-covalent bonds between PSP-I and PSP-II. Although PSP-I theoretically possesses heparin-binding ability (Sanz *et al.*, 1993 and even though an excess of heparin-binding sites were available in our columns, a considerable amount of glycosylated PSP-I was detected in the heparin-unbound fraction. It appears, therefore, that this component of PSP-I was derived from disassociation of PSP-I/PSP-II heterodimers during the SDS-PAGE separation. Since the unbound fraction contained 11.5 times higher levels of enzyme-deglycosylated PSP-I than the bound fraction, PSP-I appeared to be present mostly as PSP-I/PSP-II heterodimers in seminal plasma, with low levels of glycosylated monomers. Since deglycosylated PSP-I forms were found in the unbound fraction, but not in the bound fraction, we conclude that deglycosylated PSP-I had limited ability to bind heparin, and that it was the oligosaccharide moiety, rather than the peptide core, that conferred PSP-I heparin-binding ability.

PSP-I is reported to be a unique protein, since a sequence homology search of existing protein databases did not reveal any significant homology with other proteins reported to date (Rutherfurd *et al.*, 1992). It has been previously demonstrated that PSP-I binds to a number of proteins such as α-casein, IgG, IgA, soybean trypsin inhibitor and endo-β-galactosidase-digested ZP3, but not to native ZP3 (Kwok *et al.*, 1993). After capacitation, heparin-bound, glycosylated, PSP-I was present on the sperm acrosomal surface (Calvete *et al.*, 1995), while PSP-I/PSP-II heterodimers were found to bind only

loosely to the sperm surface, and were absent from *in vitro* capacitated spermatozoa (Dostálová *et al.*, 1994). Although heparin-bound, glycosylated, PSP-I might play a more direct role in sperm capacitation and/or fertilization, low abundance and incomplete deglycosylation of heparin-bound glycosylated PSP-I in our study made it difficult to accurately evaluate this species of PSP-I. Although PSP-I might not be involved in the initial stage of sperm-egg recognition, its subsequent involvement in sperm-egg binding cannot be ruled out. An earlier study indicated that different varieties of ligands found on spermatozoa might function at different stages of fertilization (Jones, 1991) Another possibility is that interactions among several members of the spermadhesin family might play an important role in sperm-egg interactions. Like AQN-1 and AWN, the ability of PSP-I to bind soybean trypsin inhibitor allows it to bind to acrosin inhibitor and thereby play a role in preventing a premature acrosome reaction. It was also postulated that PSP-I might function as an immunosuppressive factor, protecting the spermatozoa from immunological attack during their transport in the female genital tract (Kwok *et al.*, 1993). Although these proposed functions remain to be confirmed, in our study we established that significant differences exist in PSP-I abundance between the two seminal plasma fractions and among boars. This suggests that PSP-I is a potentially useful protein marker for the assessment of semen quality in pigs in boars.

It was shown in our study that F2 seminal plasma possessed higher concentrations of total protein than F1. Individual boars were also different from each other with respect to total protein. Our previous IVF results, presented in Chapter 3, indicated F1 sperm co-incubated with their original F1 seminal plasma had higher *in vitro* penetration rates than F1 sperm co-incubated with F2 seminal plasma. Different rates of *in vitro* penetration

were also found among boars. These findings suggested that seminal plasma proteins, on the whole, may have more negative than positive effects on fertilization. Certainly, it is possible that some varieties of proteins inhibit and the others promote sperm maturation and fertilization. In boars, some seminal plasma proteins are derived from the epididymis, but mostly are produced from the seminal vesicles, and secreted into semen sequentially first from epididymis and then from seminal vesicles (White, 1980; Shivaji *et al.*, 1990). Most of the low M_r proteins (10-20 kDa) are of vesicular origin (Leonardi *et al.*, 1983; Balerna *et al.*, 1984). Specific glandular origins and successive patterns of secretion may result in an unequal distribution of individual proteins in different ejaculate fractions. This would explain the observation of more concentrated low MW proteins (<20 kDa) in F2 seminal plasma than in F1, and more higher MW proteins in F1 than in F2.

As a major member of the spermadhesin family in boar seminal plasma, PSP-I was also more abundant in F2 than in F1. Whether or not the negative effect of F2 seminal plasma is associated with its higher concentration of PSP-I remains to be determined. Although PSP-I is thought to be involved in sperm capacitation and gamete recognition and binding (Sanz *et al.*, 1993; Töpfer-Petersen *et al.*, 1994 and 1998), it has not been conclusively shown whether its effect is positive or negative with respect to fertilization. The significant difference in levels of PSP-I among boars makes it a potentially useful parameter for assessment of semen quality provided that differences are ultimately correlated with fertility. PSP-I was significantly correlated with P12 in our study, but the latter did not exhibit any difference among boars. It is, therefore, inappropriate for the P12 band, as a substitute for PSP-I, to be quantified on Coomassie gels.

4.5. Bibliography

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Table 4-1. Relative Densitometric Values of PSP-I, P7, P27, P12 and P13 in Seminal Plasma (OD* × mm^2 ml^{-1})

Protein	Fraction	A	B	C	D	E	F	Fraction Mean	Pooled SEM
PSP-I	F1	47181	65496	38208	67246	32857	100673	58610^{x}	
	F2	59805	190063	107673	102894	65918	167450	115634^{y}	11974
	Boar Mean	53493^{b}	127780^{a}	72941^{ab}	85070^{ab}	49387^{b}	134062^{a}		
	Pooled SEM			20739					
P7	F1	5088	9004	2618	6714	2015	17114	7092^{x}	
	F2	8519	26881	18248	15320	7280	34776	18504^{y}	1599
	Boar Mean	6804^{c}	17942^{ab}	10433^{bc}	11017^{bc}	4648^{c}	25945^{a}		
	Pooled SEM			2770					
P27	F1	2197	10083	1023	2855	1470	11770	4900	
	F2	8274	35986	22758	25823	12034	38436	23885	4004
	Boar Mean	5236	23035	11891	14339	6752	25103		
	Pooled SEM			6936					
P12	F1	7884	4846	1824	2301	789	5758	3900	
	F2	2951	11314	7654	7003	2881	11185	7165	1187
	Boar Mean	5418	8080	4739	4652	1835	8472		
	Pooled SEM			2056					
P13	F1	1363	4717	1975	2108	784	5042	2665	
	F2	2234	9704	6608	5848	2165	8104	5777	822
	Boar Mean	1799	7211	4292	3978	1475	6573		
	Pooled SEM			1424					

*OD is abbreviated from 'optical density'. A, B, C, D, E and F represent six boars, respectively. F1 and F2 represent the first sperm-rich fraction and subsequent sperm-depleted fraction of seminal plasma. Means in the table were calculated from three replicates. Pooled SEM: Standard error of the mean for six boars and two fractions collected on three occasions. For each protein, means labeled with the same superscript within the same column or row are not significantly different at the level of $P<0.05$.

Table 4-2. Relative Densitometric Values of P15, P20 and P38 in F1 Seminal Plasma (OD*×mm^2 ml^{-1})

Protein	A	B	C	D	E	F	Mean	SEM
P15	454	993	197	323	421	500	481	261
P20	168^b	478^a	215^b	503^a	225^b	227^b	303	77
P38	72	107	34	65	62	62	67	19

*OD is abbreviated from 'optical density'. A, B, C, D, E and F represent six boars, respectively. Means in the table were calculated from three replicates. SEM: Standard error of the mean from three replicates. Means labeled with the same superscript within the same row are not significantly different at the level of $P<0.05$.

Figure 4-1. Western blot of pooled seminal plasma proteins showing positive staining to a primary rabbit antiserum raised against PSP-I (Ab1) and a horseradish peroxidase-linked donkey antibody (Ab2). Serial dilutions of Ab2 from 1:2,000 to 1:16,000 were used with the same dilution of Ab1 (1:4,000) in this test.

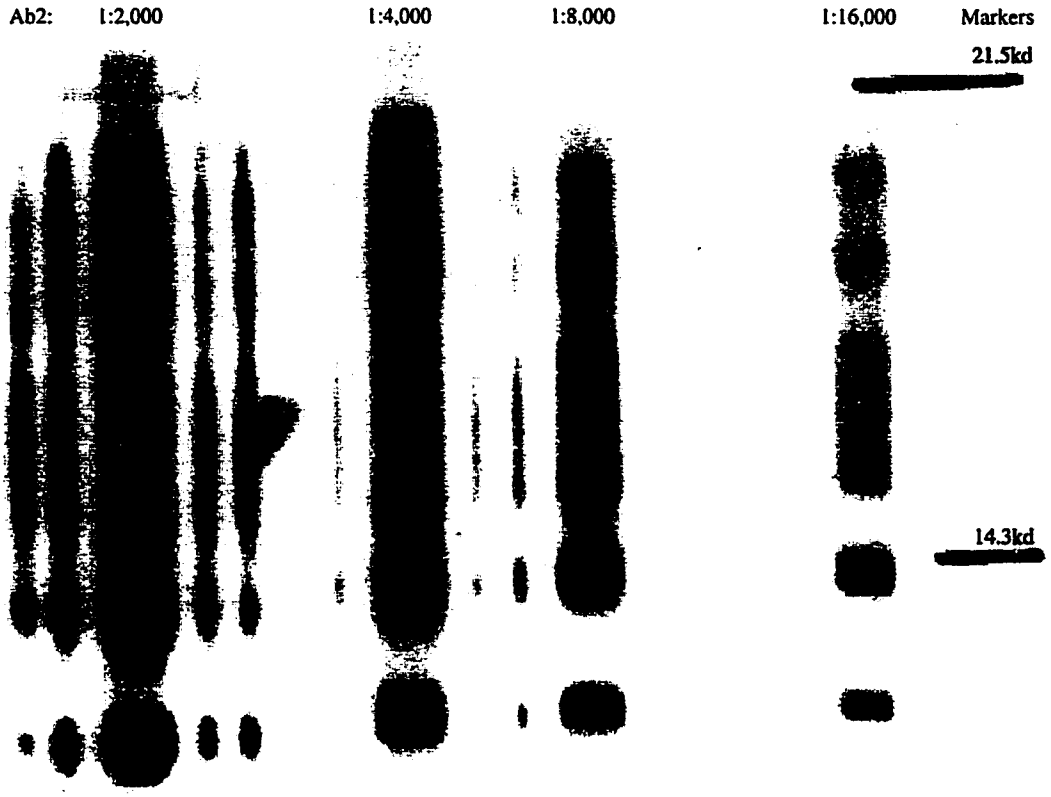

Ab2:
1:2,000
1:4,000
1:8,000
1:16,000
Markers
21.5kd
14.3kd

Figure 4-2. N--Glycanase deglycosylation of pooled seminal plasma. Panel A: Coomassie-stained gel of pooled seminal plasma proteins deglycosylated with N-Glycanase; Panel B: Western blot of pooled seminal plasma proteins immunoblotted with a primary rabbit antiserum raised against PSP-I (Ab1) and a horseradish peroxidase-linked donkey antibody (Ab2) following N-Glycanase deglycosylation. Lane 1: Untreated control; Lane 2: Enzyme treatment; Lane 3: Double-enzyme treatment; Lane 4: Protein markers. See text for identification of specific proteins (P12, P13, PSP-I, P7 and P27).

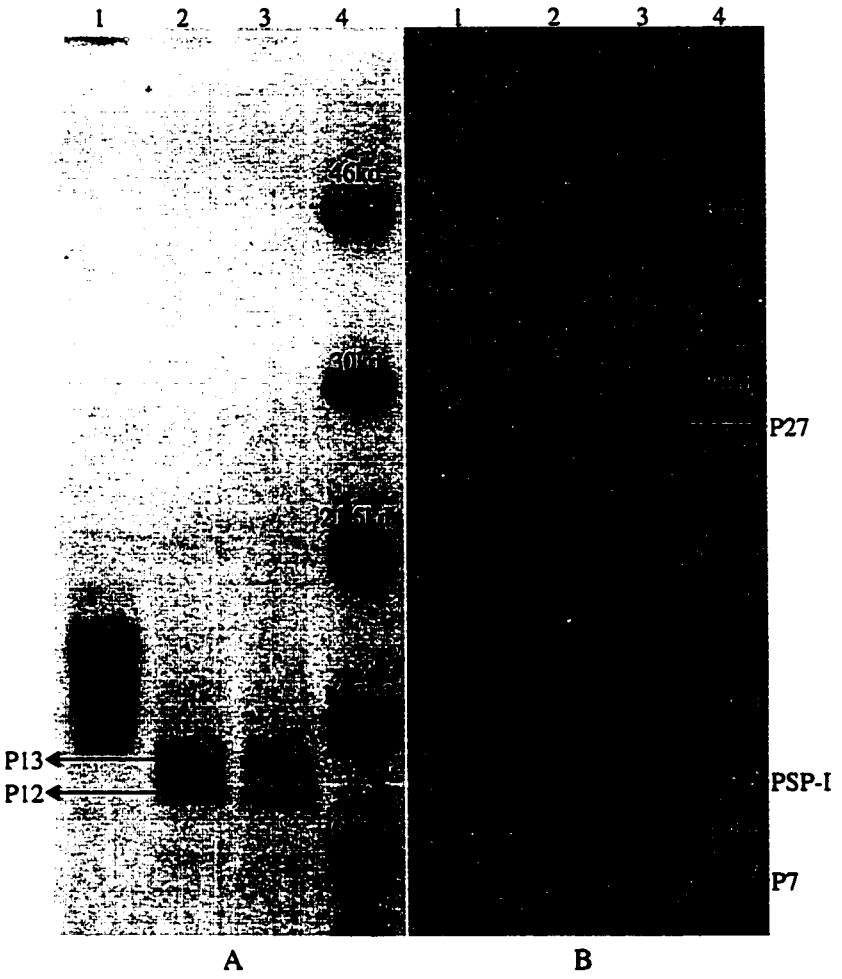
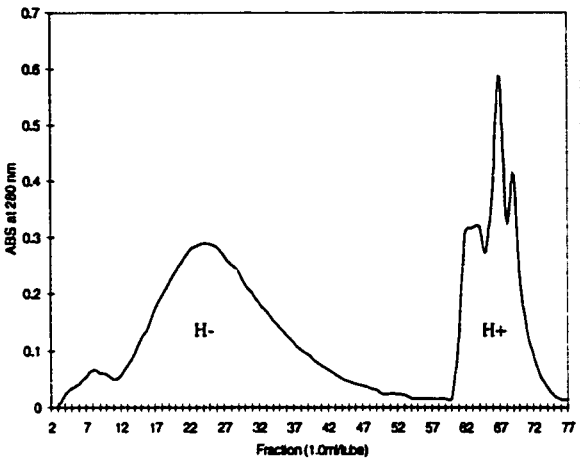
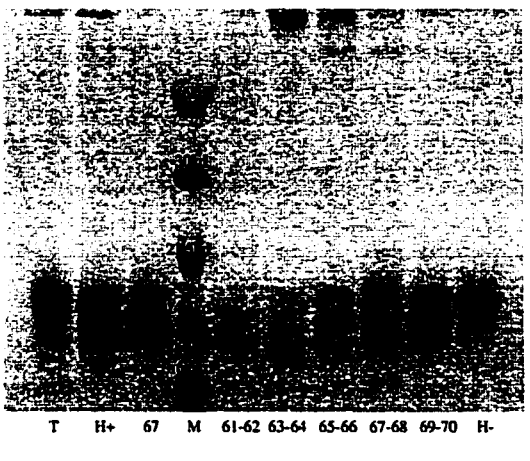

1
2
3
4
1
2
3
4
46kd
30kd
P27
P13
P12
PSP-I
P7
A
B

Figure 4-3. A): Results of flow-through of heparin-unbound and elution of heparin-bound proteins from pooled seminal plasma by affinity chromatography on Heparin Sepharose CL-6B; B): SDS-PAGE of collected fractions. In panel B, T: Total (pooled) seminal plasma; H+: Heparin-bound fraction pooled from Tubes 61-70 (0.4 ml of each). Consecutive tubes (61-62, 63-64, 65-66, 67-68 and 69-70) from the bound fraction (0.4 ml each) were also pooled for detailed examination; Tube 67, as the most concentrated tube, was individually loaded. M: Protein markers. H-: Heparin-unbound fraction pooled from Tubes 19-31.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABS at 280 nm
H-
H+
2 7 12 17 22 27 32 37 42 47 52 57 62 67 72 77
Fraction (1.0ml/Tube)

A


T H+ 67 M 61-62 63-64 65-66 67-68 69-70 H-

B

Figure 4-4. Coomassie-stained gel (Panel A) and Western blot (Panel B) of Heparin-bound (H+) and heparin-unbound (H-) proteins immunoblotted with a primary rabbit antiserum raised against PSP-I (Ab1) and a horseradish peroxidase-linked donkey antibody (Ab2) following N-Glycanase deglycosylation. T: Total (pooled) seminal plasma; H+: Heparin-bound fraction; H-: Heparin-unbound fraction; E: Enzymatic treatment; B: Buffer used as control for enzymatic treatment. See text for description of identified proteins P27, PSP-I and P7.

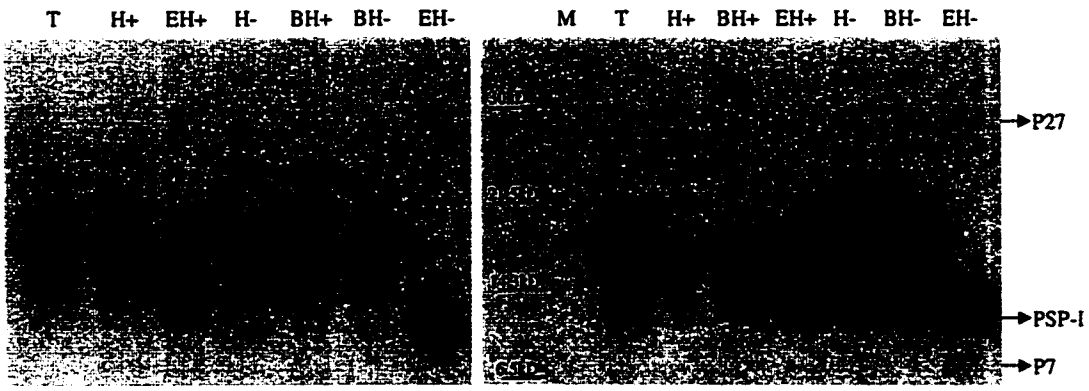

T
H+
EH+
H-
BH+
BH-
EH-
M
T
H+
BH+
EH+
H-
BH-
EH-
P27
PSP-I
P7
A
B

Figure 4-5. Concentrations of total proteins in two fractions (F1, the first sperm-rich fraction; F2: the sperm-depleted fraction) of seminal plasma from six boars (A, B, C, D, E and F). Means were calculated from three replicates. Pooled SEMs were 1.64 and 2.83 for effects of fraction and boar, respectively. a, b, c, in histogram bars representing mean concentrations of total proteins from two fractions collected on three occasions, indicate differences among boars ($P<0.01$). x, y, in the two histogram bars representing average concentrations of total proteins in the two fractions (F1 and F2) of seminal plasma from six boars collected on three occasions indicate a difference between the two fractions of seminal plasma ($P<0.01$).

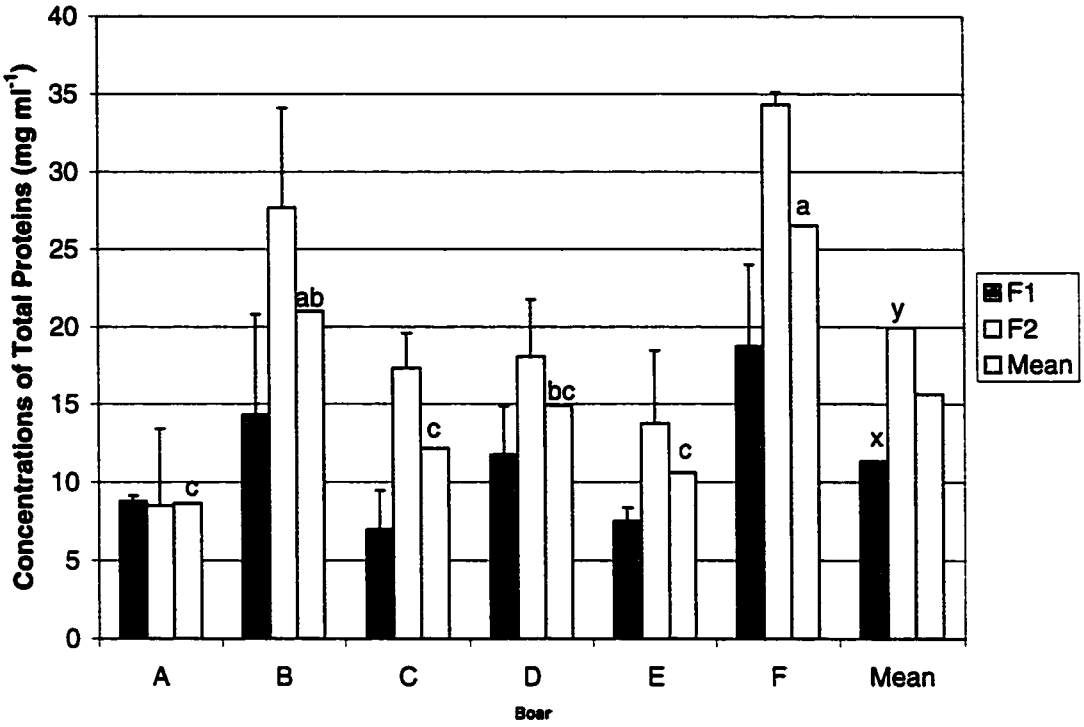

Concentrations of Total Proteins (mg ml-1)
40
35
30
25
20
15
10
5
0
A
B
C
D
E
F
Mean
Boar
F1
F2
Mean
c
ab
c
bc
c
a
x
y

Figure 4-6. Representative Coomassie-stained gel of proteins in two fractions (F1: The first sperm-rich fraction; F2: The sperm-depleted fraction) of seminal plasma from six boars (A, B, C, D, E and F). M: Protein markers. See text for description of identified proteins P38, P20 and P15.

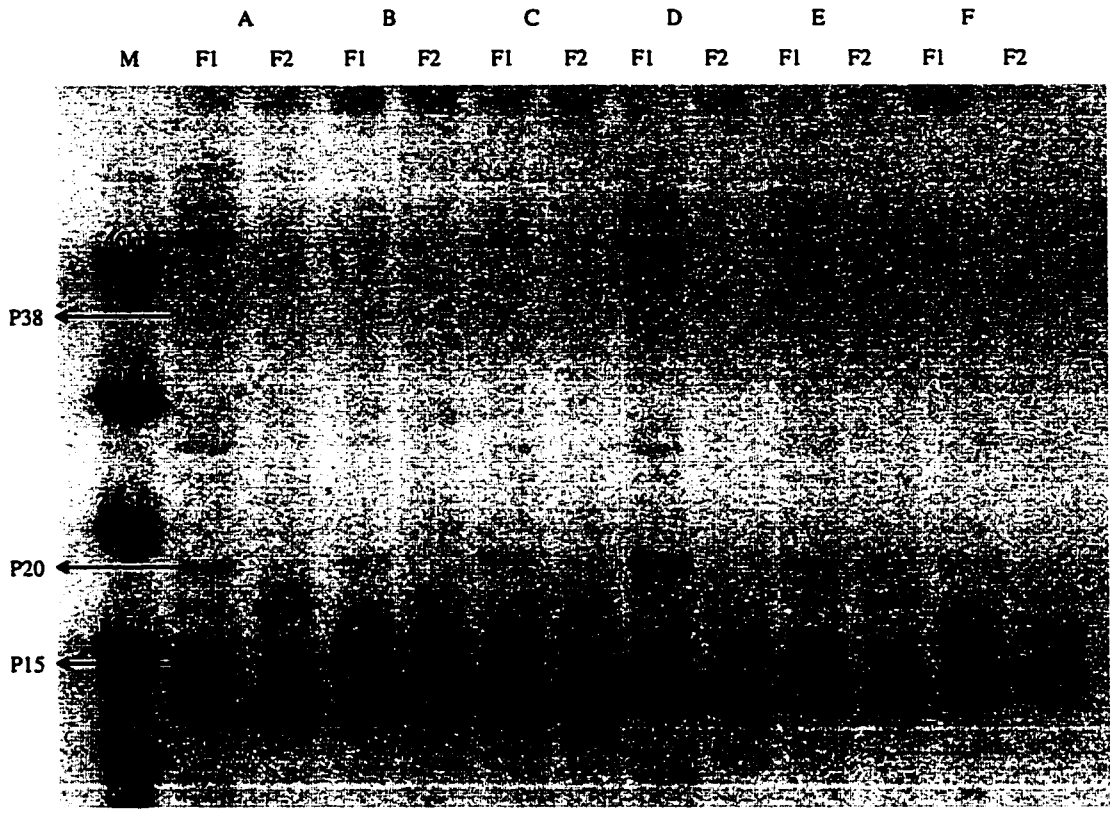

A
B
C
D
E
F
M
F1
F2
F1
F2
F1
F2
F1
F2
F1
F2
F1
F2
P38
P20
P15

Figure 4-7. Coomassie-stained gel profiles of proteins from the two fractions (F1: the first sperm-rich fraction; F2: the sperm-depleted fraction) of seminal plasma from six boars (A, B, C, D, E and F) after N-Glycanase digestion. F1-Grey line; F2-Black line.

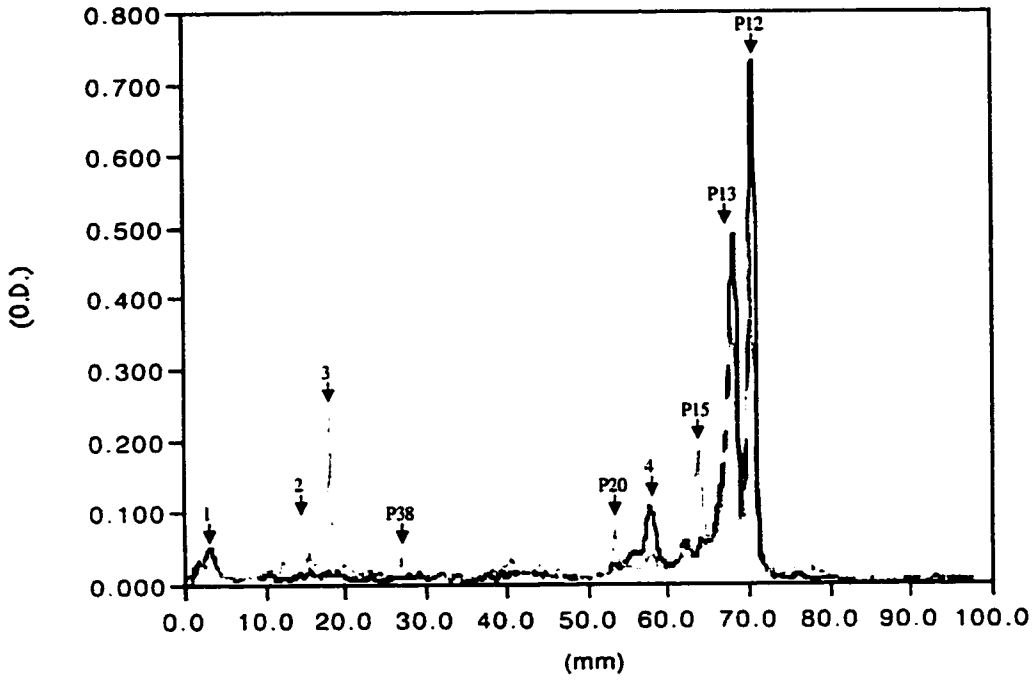
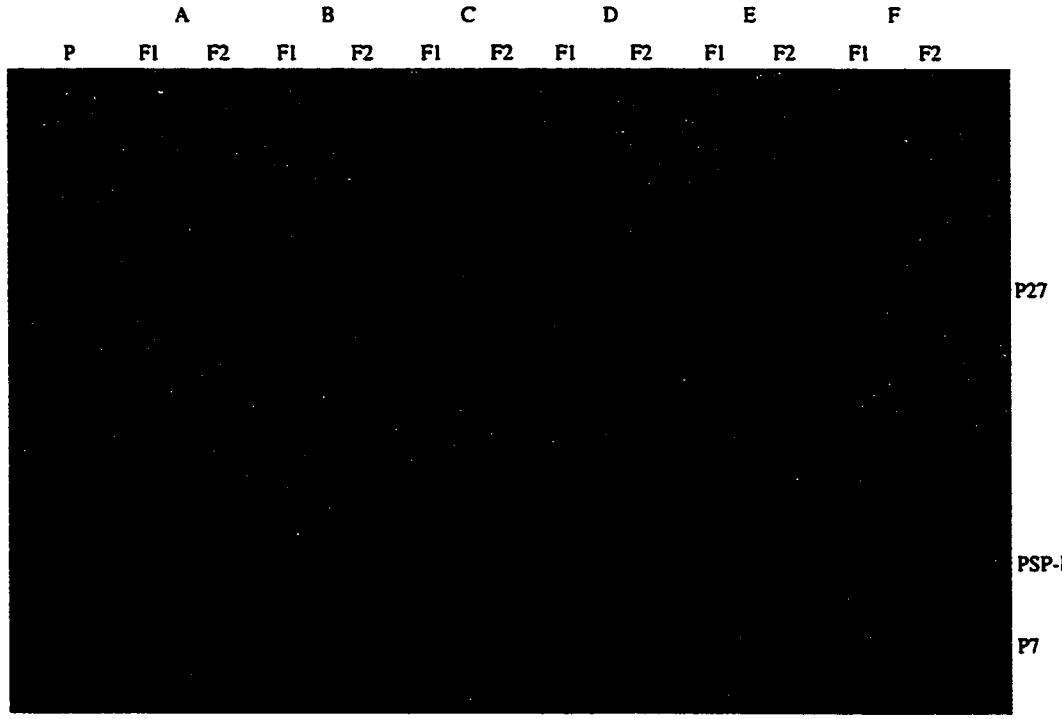

0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
(O.D.)
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
100.0
(mm)
1
2
3
4
P38
P20
P15
P13
P12

Figure 4-8. Representative Western blot of proteins in two fractions (F1, the first sperm-rich fraction; F2: the sperm-depleted fraction) of seminal plasma from six boars (A, B, C, D, E and F), immunoblotted with a primary rabbit antiserum raised against PSP-I (Ab1) and a horseradish peroxidase-linked donkey antibody (Ab2). Means were calculated from three replicates. P: Pooled seminal plasma used as an internal control for the quantification of proteins. See text for description of identified proteins P27, PSP-I and P7.


A
B
C
D
E
F
P
F1
F2
F1
F2
F1
F2
F1
F2
F1
F2
F1
F2
P27
PSP-I
P7

Chapter 5

Seminal Plasma Proteins and Boar Fertility, Part 2: Associations among Seminal Plasma Protein and Steroid Concentrations, Semen Quality Measured *in vitro*, and Boar Fertility

5.1. Introduction

In the pork industry, boar fertility is a key factor that directly affects productivity. Special attention is paid to semen quality in artificial insemination. Sperm density, motility and normality, and ejaculate volume, become routine measures in a typical procedure for artificial insemination. These observations are simple and practical, but they are subjective and of limited use in precise analysis of boar fertility. Although *in vitro* maturation and *in vitro* fertilization (IVM/IVF) techniques have been developed to evaluate semen quality, they are expensive and time-consuming. Therefore, it would be valuable to find a more objective technique for the assessment of semen quality.

Seminal plasma, the liquid fraction of ejaculated semen, comprises secretions of the epididymis, vas deferens and other accessory sex glands. In boars, it is mainly derived from the seminal vesicle, prostate and Cowper's gland. As with the spermatozoa component, the seminal plasma component of ejaculate secreted from these glands varies among various fractions of the ejaculate. Diverse proteins constitute the predominant

components in seminal plasma. As a major spermadhesin, PSP-I is a glycoprotein with 109 amino acid residues and accounts for one quarter of total protein content (Rutherfurd *et al.*, 1992; Calvete *et al.*, 1993). It was reported that this protein might be involved in sperm capacitation, gamete recognition, and binding (Sanz *et al.*, 1993; Töpfer-Petersen *et al.*, 1994 and 1998).

Hormones are another category of macromolecules in seminal plasma. In the boar, most of them are derived solely from testicular Leydig cells (Claus *et al.*, 1983; Reeves, 1989). Testosterone (T), androstenedione (A), prostaglandins (PGE_2 and $PGF_{2\alpha}$) and progesterone (P4) have been identified in boar seminal plasma (Claus *et al.*, 1983; Anderson, 1993). Interestingly, a large amount of estrogens including estrone (E1), estradiol-17β (E2) and estrone-sulphate (E1-S) was found to exist in boar semen, with considerable differences in mean concentration and total amount among individual ejaculates (Claus *et al.*, 1983, 1985 and 1987; Schopper *et al.*, 1984). The addition of a physiological amount of estrogens to semen extender used for artificial insemination was reported to increase both conception rate and litter size born (Claus *et al.*, 1989). It was shown in a subsequent study that this positive effect of seminal estrogens resulted from stimulation of ovulation and sperm transport by inducing secretion of $PGF_{2\alpha}$ from the endometrium (Claus *et al.*, 1990). Another study indicated that an appropriate ratio of E2 to androgen is imperative for proper maturation and post-fertilization development of oocytes (Anderson, 1993). Testosterone might be indirectly involved in sperm capacitation and fertilization. In the bull, heparin-binding proteins derived mainly from seminal vesicles bind to cauda epididymal spermatozoa in a testosterone, dose-dependent, manner (Nass *et al.*, 1990). As in the boar, bull seminal plasma heparin-bound proteins,

derived from seminal vesicles, may play a role in fertilization by attaching to sperm surfaces, allowing heparin-like glycosaminoglycans in the female reproductive tract to be involved in sperm capacitation.

Concentrations of both testosterone and estradiol-17β in boar seminal plasma samples analyzed for protein content in the preceding chapter, were analyzed and are presented in the first part of this chapter. Along with these steroid data, data on total and individual seminal plasma proteins reported in the preceding chapter were then used to evaluate their associations with fresh semen characteristics, IVF estimates and *in vivo* insemination data reported previously (Xu *et al.*, 1998), in order to find one or more biochemical markers for the assessment of boar fertility.

5.2. Materials and Methods

5.2.1. Collection and Isolation of Semen

The collection and storage of seminal plasma samples used for steroid analysis was described previously (Xu *et al.*, 1998; Chapter 4 of this thesis). Briefly, semen was collected at a frequency of three times over a two-week period from six fertile boars at Pig Improvement (Canada) Ltd. (PIC) Boar Stud in Acme, Alberta. A standardized method was applied to harvest semen (Xu *et al.*, 1998). Semen was used routinely for artificial insemination during a 20-wk breeding period. Routine physiological parameters of ejaculates were recorded in that period. The three batches of samples used for *in vitro* assessment, and characterization and quantification of seminal plasma proteins and steroids, were harvested before, during and after completion of the 20-week insemination

period. Immediately following ejaculation, semen characteristics were evaluated (Appendix Table 1). Seminal plasma was subsequently isolated and shipped to the University. It was kept in a –70 ^{0}C freezer in our laboratory until analyzed.

5.2.2. Radioimmunoassays of Testosterone (T) and Estradiol-17β (E2)

Testosterone was extracted from seminal plasma in duplicate, using Waters Oasis HLB 3 extraction cartridges (Waters Corporation, Milford, Massachusetts) with a Supelco Visiprep Solid phase Extraction Vacuum Manifold (Sigma-Aldrich Canada Ltd., Mississauga, Ontario). Extraction consisted of four steps, all carried out at a maximum flow rate of 1 ml per minute; 1) preparation: Flushing of cartridge with 2 ml of 90 % ethanol (Fisher Scientific HPLC grade Reagent Alcohol, Nepean, Ontario), followed by elution with 2 ml of deionized and distilled water 2) loading: Application of 0.5 ml seminal plasma 3) wash: Flushing of cartridge with 4 ml of 4.5 % ethanol and 4) elution: Elution of cartridge with 2 ml of 90 % ethanol and collection into a 12×75 mm glass tube. The ethanol fraction was dried in a rotary evaporator and reconstituted with 0.15 ml of 0.1% (v/w) PBS gelatin (PBS gel) buffer (Sigma Aldrich Canada Ltd., Oakville, ON). Samples were extracted and reconstituted over several days, stored at 4° C, and assayed in a single assay. Reconstituted samples were assayed using Diagnostics Product Corporation Coat–A–Count Total Testosterone kit (Diagnostic Products Corporation, Los Angeles, California) with the following modifications: two additional standards were made by diluting the lowest standard provided with PBS gel buffer, having established no difference in binding when using zero calibrator and PBS gel buffer. The range of curve standards was, therefore, 0.0025, 0.005, 0.01, 0.05, 0.2, 0.4, and 0.8 ng per 50 µl. The

volume of reconstituted sample assayed was 50 µl. Parallelism was established by demonstrating no significant deviation between the slopes of the standard curve and a binding curve established by extracting 500, 250, 125, and 62.5 µl of a pool of seminal plasma. Recovery of 0.2 ng testosterone from seminal plasma, measured on two occasions, was 123.3±0.3 %. Assay sensitivity, calculated as 2 standard deviations below maximum bound (94.4 %) was 0.00309 ng per tube, equivalent to 0.0256 ng ml^{-1} of seminal plasma. The intra-assay CV was 11.4 %. Hot recoveries using seminal plasma spiked with I^{125}-labeled testosterone averaged 88.5±1.1 %. Concentrations were not corrected for recovery.

Estradiol was extracted from seminal plasma using the same procedure as used for testosterone, with two exceptions: 1) the load step used 0.125 ml seminal plasma plus 0.375 ml PBS buffer and 2) dried samples were reconstituted with 0.2 ml PBS gel buffer. Samples were again extracted and reconstituted over several days, stored at 4° C, and assayed in a single assay. Due to a solvent effect on the estradiol assay, glass tubes containing 2 ml of 90 % ethanol were dried in a rotary evaporator and used for the standard curve, non-specific bound, and maximal bound tubes. Samples were assayed using Estradiol Double Antibody kit (Diagnostic Products Corporation, Los Angeles, CA), with the following modifications: use of PBS gel buffer, use of in-house standards (estradiol-17β, E2, Sigma Aldrich Canada Ltd., Oakville, ON) prepared in PBS gel buffer at 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 pg per 0.1 ml. The volume of standards tubes was adjusted to 0.2 ml by adding 0.1 ml PBS gel buffer. In order to improve assay sensitivity, reconstituted kit estradiol antiserum was further diluted three-fold with distilled and deionized water. Sample non-specific bound was significantly higher than

buffer non-specific bound (6.8 versus 2.64 %). Since this had a significant effect on sample binding, the difference was subtracted from the raw cpm and data were reprocessed to give the corrected binding. Parallelism was shown by demonstrating no significant deviation between the slopes of the standard curve and a curve derived from extraction of 125 µl, 62.5 µl, 31.25 µl and 15.625 µl of a pool of seminal plasma. Recovery of 12.5 pg of estradiol from seminal plasma, measured on one occasion, was 81.06 %. Assay sensitivity at the linear cut-off (84 %) was 0.39 ng per tube, equivalent to 3.13 pg per ml of seminal plasma. The intra-assay CV was 8.87 %. Hot recoveries using seminal plasma spiked with I^{125}-labeled estradiol averaged 93.45±1.43 %. No correction of concentrations was made for recovery.

5.2.3. Characterization and quantification of Seminal Plasma Proteins

After deglycosylation with N-Glycanase, total seminal plasma was separated by SDS-PAGE. PSP-I, P7 and P27 were quantified by Western blotting. P12, P13, P15, P20 and P38 were quantified by scanning Coomassie-stained gels. The detailed technologies for identification and quantification of proteins were described in the preceding chapter. The protein data used for regression analyses are presented in Appendix Table 2.

5.2.4. *In vitro* Penetration Assays of Spermatozoa

Complete IVF procedures and methods were reported previously (Xu *et al.*, 1998). The penetration rate, monospermy rate, number of sperm inside each penetrated oocyte, number of sperm attaching on each oocyte and potential embryo productivity, represented as the percentage of penetrated monospermic oocytes with both a female and a single male pronucleus, were evaluated and presented after arcsine transformation. The

parameters were definited in detail in the same reference (Xu *et al.*, 1998). The IVF data used for regression analyses are shown in Appendix Table 3.

5.2.5. *In vivo* Insemination

After routine microscopic observation, fresh semen was immediately diluted into two doses of 3×10^9 and 2×10^9 sperm from each ejaculate, with AndroHEP extender (Minitube, Canada), packed in 100-ml plastic AI bottles and delivered to the breeding farm as soon as possible for AI use. A total of 345 sows were inseminated in the 20-week breeding period. Pairs of sows exhibiting similar weaning-to-estrus intervals were inseminated with the two different doses, with three inseminations within two days of standing estrus. Conception rate and litter size at birth were recorded and the breeding data used for regression analyses are presented in Appendix Table 4.

5.2.6. Statistical Analysis of Data

Using the same model as used for analyses of protein data in the preceding chapter, steroid data were analyzed using the GLM procedure of SAS 7.0 (SAS Institute Inc., 1999). Using the stepwise procedure (SAS Institute Inc., 1999), fresh semen characteristics (Appendix Table 1), IVF estimates (Appendix Table 3) and breeding results (Appendix Table 4) (dependent variables) were regressed on observations of seminal plasma steroids (Appendix Table 5) and total and individual proteins (Appendix Table 2) (independent variables), reported in Chapter 4. Only significant general models at the $P<0.05$ level were subject to the stepwise procedure. The correlation between

dependent variables and independent variables is expressed as model correlation. All variables left in the model were significant at the 0.15 level.

5.3. Results

5.3.1. Concentrations of Steroids

Data on steroid concentrations are listed in Appendix Table 5. Concentrations of both T and E2 differed among boars ($P<0.0001$ for both) and E2 was more concentrated in F1 than in F2 seminal plasma ($P<0.0001$). Interactions between boar and fraction existed for estradiol ($P<0.01$), but not for testosterone ($P>0.5$) (Figures 5-1). A positive linear correlation was observed between the two steroids ($R^2=0.61$, $P<0.0001$).

5.3.2. Correlation between IVF Estimates and Protein Concentrations in Seminal Plasma

All IVF parameters, for each of the three dilutions used (D1, D2 and D3), were regressed on total and individual proteins in both fractions of seminal plasma analyzed.

5.3.2.1. IVF Parameters and F1 Proteins

IVF observations using a dilution of 50,000 sperm to 1 oocyte (D1), were regressed on total and individual proteins in F1 seminal plasma. Significant correlations existed between number of sperm (penetrating) inside each oocyte (SpermN), number of sperm (attaching) on each oocyte (AttachN), potential embryo productivity (Embryo) and concentrations of specific proteins.

SpermN (D1) = 18.95 - 0.023 P20 (F1) - 0.00069 P12 (F1).....Model 1

$R^2 = 0.36$, $P = 0.04$.

AttachN (D1) = 38.01 - 0.00027 PSP-I (F1).....Model 2

$R^2 = 0.23$, $P = 0.04$.

Embryo (D1) = 2.36 + 0.0010 P27 (F1) - 0.0020 P7 (F1).....Model 3

$R^2 = 0.67$, $P = 0.0002$.

IVF observations using a dilution of 12,500 sperm to 1 oocyte (D2) were regressed on total and individual proteins in F1 seminal plasma. Significant correlations existed between number of sperm (penetrating) inside each oocyte (SpermN) and protein P13.

SpermN (D2) = 8.65 - 0.00093 P13 (F1).....Model 4

$R^2 = 0.24$, $P = 0.04$.

When sperm were diluted at a ratio of 3,125 sperm to 1 oocyte, no correlation existed between any variable of IVF estimates and any species of proteins.

5.3.2.2. IVF parameters and F2 Proteins

Multiple regression analysis of IVF estimates using the dilution of 3,125 sperm to 1 oocyte on the individual proteins in F2 seminal plasma indicated a significant correlation

between potential embryo productivity (Embryo) and three proteins, P7, P27 and P13, for D1 IVF results.

Embryo (D1) = -0.83 - 0.00039 P7 (F2) + 0.00012 P27 (F2) + 0.0014 P13 (F2)...Model 5

$R^2 = 0.67$, $P = 0.001$.

5.3.3. Seminal Plasma Proteins and Other Measured parameters

No correlations existed between seminal plasma proteins, and laboratory assessments of semen characteristics and litter size at birth. When sperm:oocyte ratio decreased to 12,500:1 or 3,125:1, no variable of IVF estimates was correlated with any species of proteins.

5.3.4. Seminal Plasma Steroids and Other Parameters

No correlation was found between steroid concentrations and seminal plasma proteins, IVF parameters, semen characteristics and litter size at birth.

5.4. Discussion

The above results indicate that IVF estimates are more strongly correlated with F1 than with F2 protein concentrations in seminal plasma. This finding is likely due to the fact that the sperm estimated *in vitro* were derived from the same first sperm-rich fraction of the ejaculates that provided the F1 seminal plasma in which individual proteins were quantified. As F1 sperm underwent co-incubation with their F1 original seminal plasma

for approximately 20 h prior to IVF, they would have been affected directly by F1 seminal plasma proteins.

When a high dose of sperm (D1) was used for IVF, number of sperm penetrating each oocyte, number of sperm attaching to each oocyte and potential embryo productivity were correlated with different proteins. With a decrease in the ratio of sperm to oocyte to the medium level (D2), only the number of sperm penetrating per oocyte was correlated with Protein P13. At the highest level of dilution (D3) used for IVF, no correlation existed between IVF parameters and seminal plasma protein concentrations. The results indicate that as the ratio of sperm to oocyte decreased, fewer and fewer IVF parameters correlated with seminal plasma proteins concentrations. These data suggest that the effects of proteins on IVF depend on their density in seminal plasma. With a decrease in the ratio of sperm to oocyte in the given volume of fertilization medium, seminal plasma-derived proteins, either on sperm acrosomes or in the medium became sparse in proportion to the level of dilution. More importantly, some of the initially sperm-bound proteins might dissociate from the sperm heads into the medium, resulting in a partial loss of protein functions. Similar dynamic transformations have been observed in ram sperm incubated in uterine, oviduct and cauda epididymal fluids *in vitro* (Voglmayr and Sawyer, 1986).

At different levels of dilution, a few IVF parameters exhibited association with different proteins. Even the same dependent variables were correlated with different proteins in different fractions of seminal plasma, e.g. Model 3 vs. 5, or at different levels of dilution, e.g. Model 1 vs. 4. This may again be attributed to effects of protein concentrations. For example, when the first dilution (D1) was used, potential embryo

productivity was related to Proteins P7 and P27 in F1 seminal plasma (Model 3) and also to Proteins P7, P27 and P13 (Model 5) in F2 seminal plasma. The discrepancy might be due to P13 not being at sufficient concentrations in F1 seminal plasma to exert a biological effect. At the same (D1) level of dilution, the number of sperm penetrating each oocyte was associated with proteins P20 and P12 (Model 1), but with P13 (Model 4), rather than P20 and P12, at the medium level of dilution (D2).

As indicated in Model 2, the number of sperm attaching to each oocyte was correlated with PSP-I at the level of D1. PSP-I is one of the two most predominant spermadhesins in seminal plasma (Rutherfurd *et al.*, 1992) and it was proposed that this glycoprotein is involved in sperm capacitation and gamete recognition and binding (Sanz *et al.*, 1993; Calvete *et al.*, 1994b; Topfer-Petersen *et al.*, 1994 and 1998). It has also been shown that heterodimerization of specific glycoforms of PSP-I with PSP-II exerts a profound effect on the ligand-binding characteristics and compartmentalization of these boar spermadhesins (Calvete *et al.*, 1995a). The interaction between lectins of the sperm surface and oligosaccharides of the oocyte's *zona pellucida* glycoproteins (Yanagimachi, 1994) mediates sperm capacitation, acrosomal reaction and gamete recognition (Calvete *et al.*, 1992). These findings were verified in Model 2. As ligands, free PSP-I in seminal plasma is likely to compete for binding to some of glycoprotein receptors (ZP2 and ZP3) on *zona pellucida* (Wassarman, 1992; Wassarman and Litscher, 1995) with the spermatozoa-associated PSP-I and other spermadhesins such as AQN-3 and AWN (Topfer-Petersen *et al.*, 1998). It is, therefore, suggested that a high density of free PSP-I gave rise to the decrease in the number of sperm attaching onto oocytes. As a competitive result, oocytes get less chance to be penetrated by capacitated sperm coated with PSP-I

monomers. A higher concentration of PSP-I in F2 than in F1 seminal plasma ($P<0.01$), as described in the preceding chapter, might, in part, contribute to poorer quality of this fraction of seminal plasma, which adversely influences *in vitro* penetration ability of sperm co-incubated with it (see Chapter 3). That would explain the significant negative correlation between the number of sperm attaching onto each oocyte and free PSP-I.

Other proteins such as P12, P13 and P20 may affect IVF outcome through different mechanisms. As shown in Model 1, the number of sperm penetrating inside each oocyte was negatively correlated with P20 and P12 at the first level of dilution (D1). When *in vitro* insemination was carried out at a ratio of 12,500 sperm to 1 oocyte, the penetration ability became negatively associated with P13. P20 might, therefore, be another inhibitor of sperm penetration. In both Models 3 and 5, potential embryo productivity was consistently and positively correlated with P27 and negatively correlated with P7. This indicated that P27 might have a beneficial effect, while P7 might have an adverse effect on *in vitro* embryo development. P13 may also promote embryo development. Overall, however, more protein species appeared to be detrimental than beneficial to *in vitro* fertilization.

Of eight varieties of proteins identified in the present study, PSP-I, P7, P27, P12, P13 and P20, quantified in both fractions of seminal plasma, were found to be associated with different IVF characteristics. According to their molecular weights and relative abundance, P12 and P13 are considered to be PSP-I and PSP-II, respectively (see preceding chapter), although both bands could contain others proteins similar, in molecular mass, to PSP-I and PSP-II. As has also been demonstrated previously, P7 and P27 are believed to be two products resulting from N-Glycanase deglycosylation of

heparin-unbound proteins rather than from PSP-I/PSP-II heterodimers. Although the true origins of these two bands remains to be identified, these two proteins were still included in the regression analysis. However, the two other proteins, P15 and P38, were not included in the final models. Although the latter two proteins were more abundant in F1 than F2 seminal plasma, their functions were not discovered in our present study. Whether they contributed to the beneficial effects of F1 seminal plasma on *in vitro* fertilization remains to be investigated. As an independent variable, total protein was unable to enter any of the five models. Thus, this variable is of little value in indicating semen quality and boar fertility.

No correlation existed between any of the individual proteins and the dependent variables of fresh semen characteristics and litter size born. There are several possible reasons for this lack of functional relationships among measured variables. One is that little correlation exists between sperm motility or morphology and fertilization ability, provided that motility and morphological normality are not extraordinarily low. The lack of protein effects on the average number of piglets per litter born, may be due to the lack of variance in litter size, even when using the lower dose of 2 billion sperm per insemination.

Although boars were different from each other, neither testosterone nor estradiol-17β concentrations in seminal plasma were found to associate with any IVF estimates, other dependent variables of fresh semen characteristics, or litter size born. Therefore, concentrations of the two seminal plasma steroids appear to have less direct effects on *in vitro* fertilization compared with seminal plasma proteins.

It is worthy of note that available data on fresh semen characteristics and litter records (Xu *et al.*, 1998) were averaged over the whole insemination period used, thus preventing more extensive regression analysis using data associated with each of the three estimates of seminal plasma proteins and steroids generated in the present study. This may, in part, contribute to the lack of effects of either seminal plasma proteins or steroids on semen quality characteristics and insemination outcomes. Additionally, the whole ejaculate, including the second sperm-rich fraction (F3) and possibly the steroid-rich gel fraction, was used for *in vivo* insemination, whereas only F1 or F2 seminal plasma was evaluated for protein and steroid concentrations. This suggests that the macromolecules evaluated in either F1 or F2 seminal plasma may not sufficiently indicate semen quality of the whole ejaculate associated with differences in litter size born.

A positive correlation between testosterone and estradiol concentrations probably relates to the fact that in the biochemical pathway of steroid synthesis, estrogen is derived from testosterone. As a major active form of androgens, testosterone is mainly produced by the interestitial cells (Leydig cells) of the testes (Claus *et al.*, 1983; Reeves, 1989). It stimulates the later stage of spermatogenesis, prolongs the life span of epididymal sperm and promotes growth, development and secretory activity of the male accessory sex glands such as the prostate, seminal vesicles, bulbourethral gland, vas deferens and the external genitalia (penis and scrotum) (Reeves, 1989). Seminal plasma proteins, especially low M_r spermadhesins including PSP-I, are synthesized and secreted by these male accessory sex glands, and especially by the seminal vesicles. In particular, low M_r spermadhesins including PSP-I are predominantly derived from seminal vesicle epithelial

cells (Sinowatz *et al.*, 1995). In spite of this linkage, no correlation was observed between steroids and proteins. Therefore, neither of the seminal plasma steroids appeared to be as useful as seminal plasma proteins in judging semen quality and sperm performance.

In conclusion, differences in seminal plasma protein concentrations are associated with IVF outcomes. Of the eight proteins identified in this study of seminal plasma, most exhibit negative associations with IVF results. PSP-I has an inhibitory effect on sperm attachment to the oocyte. Both P20 and P12 (mainly PSP-I) inhibit sperm penetration. As two new products revealed by N-Glycanase deglycosylation, P7 is detrimental to, and P27 is of benefit to, embryo development. High concentrations of free PSP-I and most of the other spermadhesins in seminal plasma might inhibit sperm-egg attachment and subsequent binding by competition, and eventually result in a decrease in sperm penetration and fertilized oocyte development. It is suggested by the current study that these proteins, especially PSP-I, may be useful for the assessment of sperm fertilizing ability.

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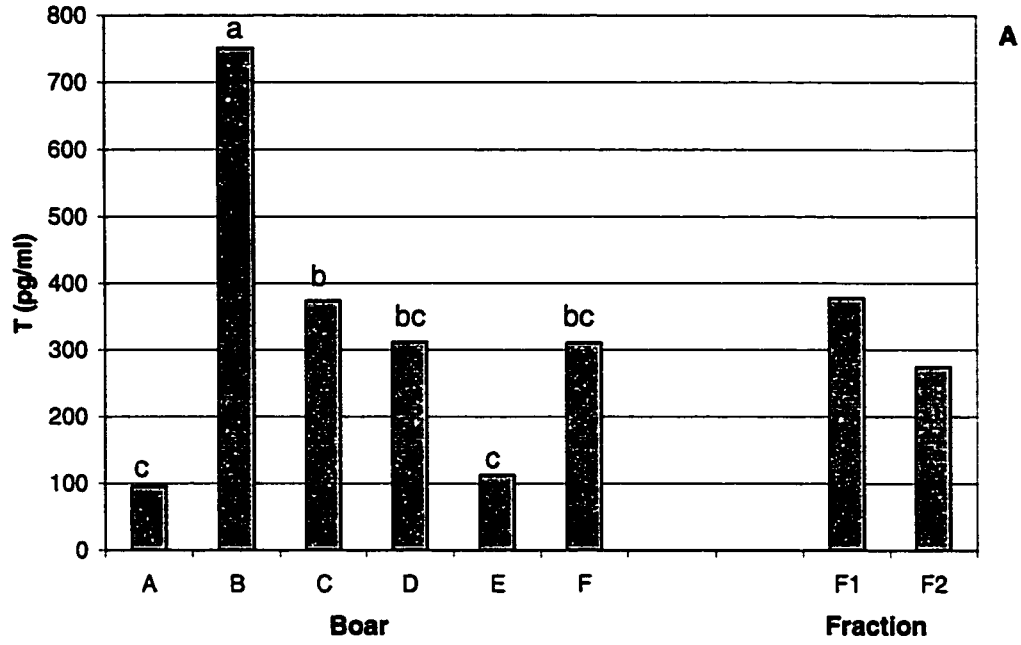
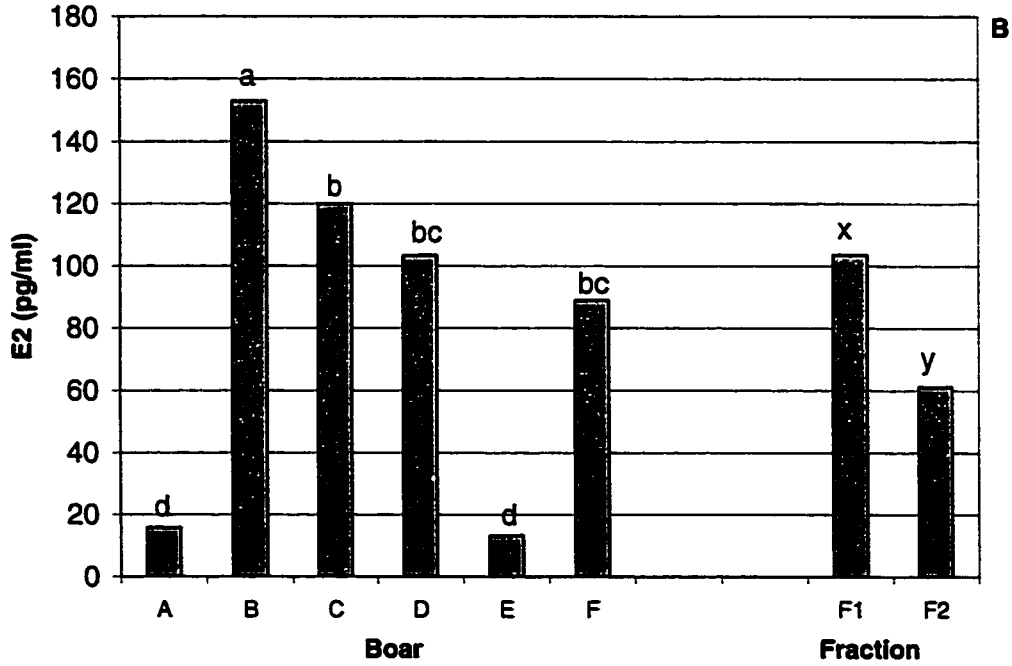
Figure 5-1. Overall boar and fraction effects on steroids in seminal plasma resulting from analysis of data in Appendix Table 5. A, B, C, D, E and F stand for six boars, respectively. F1 and F2 represent the first sperm-rich fraction and the subsequent sperm-depleted fraction of seminal plasma, respectively.

A). Concentrations of testosterone (T) in seminal plasma (pg ml^{-1}). Pooled SEMs were 69 and 40 for effects of boar and fraction, respectively. Histogram bars labeled with same letters (a, b and c) indicated no differences among boars ($P>0.05$).

B). Concentrations of estradiol-17β (E2) in seminal plasma (pg ml^{-1}). SEMs were 5.5 (n=18) and 5.7 (n=17) for F1 and F2, and 9.6 (n=6), 10.7 (n=5), 9.6 (n=6), 9.6 (n=6), 9.6 (n=6) and 9.6 (n=6) for the six boars, respectively. a, b, c, d; histogram bars labeled with the same letters indicate no differences among boars ($P>0.05$). x, y indicate an overall difference between F1 and F2 seminal plasma across the six boars in mean concentrations of E2 ($P<0.0001$).


A
T (pg/ml)
800
700
600
500
400
300
200
100
0
a
b
bc
bc
c
c
A
B
C
D
E
F
F1
F2
Boar
Fraction


B
E2 (pg/ml)
180
160
140
120
100
80
60
40
20
0
a
b
bc
bc
d
d
x
y
A
B
C
D
E
F
F1
F2
Boar
Fraction

Chapter 6

General Discussion and Conclusion

6.1. General Discussion

Based on the previous findings in our laboratory, that F1 sperm have superior IVF performances than either F2 or F3 sperm (Xu *et al.*, 1996), the first trial of the study demonstrated that IVF differences among the three fractions are, at least partially, derived from seminal plasma. Although the same F1 ejaculate fraction of sperm was used, co-incubation with different fractions of seminal plasma resulted in different IVF outcomes. F1 sperm co-incubated with their original F1 seminal plasma for 14-16 h had a higher penetration ability than F1 sperm co-incubated with F2 seminal plasma. The results implied that some seminal plasma components might play an important role in sperm capacitation and fertilization.

The spermadhesins, a predominant family of proteins in boar seminal plasma (Calvete *et al.*, 1997), are sperm-associated, low-molecular mass (12-20 kDa) lectins, proposed to have major roles at different steps of fertilization (Jones, 1991; Sanz *et al.*, 1993; Calvete *et al.*, 1994b;Töpfer-Peterson *et al.*, 1994 and 1998). PSP-I is a major member of the family (Rutherfurd *et al.*, 1992) and its glycosylated monomers are still bound to sperm heads after *in vitro* capacitation (Calvete *et al.*, 1995a), although most spermadhesin is lost by then (Töpfer-Peterson *et al.*, 1998). These implications led us to

assess PSP-I and other low M_r proteins in both F1 and F2 of seminal plasma from six boars in the second trial.

Quantitative analyses by Western blotting techniques indicated that total proteins, PSP-I and N-Glycanase deglycosylation-derived Protein P7 were less concentrated in F1 than F2 seminal plasma. Boars differed in these proteins and in Protein P20 as well. Specific glandular origins (Leonardi *et al.*, 1983; Balerna *et al.*, 1984) and successive patterns of secretion (White, 1980; Shivaji *et al.*, 1990) may result in an unequal distribution of individual proteins in different ejaculate fractions. These low M_r seminal plasma proteins are suggested to have negative effects on sperm fertilization. Many previous reports implicated roles for PSP-I and other spermadhesins in the fertilization process, but none clarified whether they were beneficial or detrimental to sperm capacitation and fertilization. For this reason, multiple regression analysiswas used to determine associations between IVF estimates, fresh semen characteristics, litter size born and the amounts of quantified proteins.

Aa a result of a series of multiple regression analyses run using the stepwise procedure, five models were presented in Chapter 5. PSP-I and most of the low M_r seminal plasma proteins were negatively associated with sperm attachment and penetration, and embryo development, whereas P13 and N-Glycanase deglycosylation-derived protein P27 were positively associated with *in vitro* embryo production.

The interaction between lectins of the sperm surface and oligosaccharides of the oocyte's *zona pellucida* glycoproteins mediates sperm capacitation (Yanagimachi, 1994), the acrosomal reaction and gamete recognition and binding (Calvete *et al.*, 1992, Wassarman 1992 and 1999). As ligands, free PSP-I and other spermadhesins, or other

low M_r glycoproteins in seminal plasma, are likely to compete for binding to some of the glycoprotein receptors (ZP1, ZP2 and ZP3) on the *zona pellucida* (Wassarman, 1992; Wassarman and Litscher, 1995) with the spermatozoa-associated PSP-I, and other spermadhesins such as AQN-3 and AWN (Topfer-Petersen *et al.*, 1998). This may reduce the chance of sperm attachment and even penetration by capacitated sperm coated with the spermadhesins. As a result of such competition, F2 seminal plasma that contains more concentrated PSP-I and other low M_r proteins than F1 seminal plasma, may exert negative effects on the fertilization potential of co-incubated sperm. Protein P13 is one of five proteins identified and quantified on Coomassie-stained gels and although this band possibly contains more than one protein, it was tentatively identified as PSP-II based on its molecular mass and abundance in seminal plasma. This may be why P13 exhibited a dual role of inhibiting sperm penetration, yet promoting embryo development, in the *in vitro* fertilization system. P27 was consistently and positively associated with embryo development when IVF was conducted at the two lower sperm dilutions, but its origin remains to be identified.

No correlation was found between total proteins and IVF estimates. This may be due to negative effects of PSP-I and most of the low M_r proteins being partially antagonized by positive effects of a small group of proteins, including P13 and P27. The lack of a correlation between seminal plasma proteins and litter size born raises questions about the use of such measures to assess boar fertility. In practicai AI procedures, seminal plasma proteins are excessively diluted during extension in diluents used for artificial insemination (AI), and subsequently in the female reproductive tract. As a result, they may not exert their normal roles when AI is used. No association of either steroids that

were quantified was seen with IVF estimates and this may be attributed to less direct involvement of steroids in sperm fertilization, generally. Because the whole ejaculate, including the second sperm-rich fraction (F3), would often be diluted for *in vivo* artificial insemination, it may be inappropriate to correlate steroids or proteins from F1 or F2 seminal plasma alone with insemination outcomes. It is also worthy of note that available data on fresh semen characteristics and litter records (Xu *et al.*, 1998) were averaged over the whole insemination period used, thus preventing more extensive regression analysis using data associated with each of the three estimates of seminal proteins and steroids generated in the present study. Thus, more consistent treatments should be considered and more experimental units should be used if a further study is conducted.

6.2. Conclusion

The results presented support the concept that seminal plasma components significantly affect sperm fertilization, as determined *in vitro*. Of the molecules examined, free PSP-I and most of the other low M_r seminal plasma proteins identified are inhibitory to *in vitro* fertilization. However, both P13 and P27 appear to promote *in vitro* embryo development. These variables, especially PSP-I, can be used for evaluating sperm fertilization.

6.3. Bibliography

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Appendix

Appendix Table 1. Percentage of morphologically Normal Sperm (Normality) and Sperm with Good Mobility Measured in Fresh Semen

Characteristics	A	B	C	D	E	F
Normality* (%)	74.3	75.2	74.3	62.6	77.2	56.8
Motility* (%)	72.1	70.0	70.8	70.4	68.6	62.5

A, B, C, D, E and F stand for six tested boars, respectively. *Means of the collections made during the 20-week breeding period during which semen was used for AI.

Appendix Table 2. Concentrations of Total (mg ml^{-1}) and Individual Proteins ($OD^{*}\times mm^{2}$) in Boar Seminal Plasma Used for Regression Analysis

Boar	Fraction	Batch	Total	PSP-I	P7	P27	P12	P13	P15	P20	P38
A	F1	B1	9.10	53868	6770	4153	21331	1986	730	311	127
		B2	9.18	45051	5185	1207	1788	1506	371	81	45
		B3	8.09	42624	3310	1159	533	596	261	113	43
		M	8.79	47181	5088	2197	7884	1363	454	168	72
	F2	B1	1.80	8800	292	1133	616	511			
		B2	18.05	127177	17334	9140	6000	4272			
		B3	5.68	43437	7931	14550	2237	1920			
		M	8.51	59805	8519	8274	2951	2234			
B	F1	B1	26.95	107956	17580	28987	12539	12084	2084	578	152
		B2	10.55	65112	7688	519	1679	1635	533	447	87
		B3	5.50	23420	1743	743	319	432	361	409	82
		M	14.33	65496	9004	10083	4846	4717	993	478	107
	F2	B1	39.40	253904	27338	46112	19419	17390			
		B2	17.30	117156	17384	5365	5680	4536			
		B3	26.37	199129	35921	56482	8842	7187			
		M	27.69	190063	26881	35986	11314	9704			
C	F1	B1	11.6	69973	4533	2835	4524	4959	358	313	32
		B2	3.12	20706	1709	37	679	675	88	121	26
		B3	6.23	23946	1613	196	270	292	146	210	44
		M	6.98	38208	2618	1023	1824	1975	197	215	34
	F2	B1	21.70	120883	18755	13788	12092	11494			
		B2	14.21	87236	11465	2976	4979	4253			
		B3	16.10	114900	24523	51511	5892	4077			
		M	17.34	107673	18248	22758	7654	6608			
D	F1	B1	6.05	28364	1521	516	691	839	425	758	94
		B2	16.75	102631	10926	2917	4127	3379	224	311	80
		B3	12.50	70742	7695	5132	2084	2107	319	441	20
		M	11.77	67246	6714	2855	2301	2108	323	503	65
	F2	B1	11.55	37289	7425	6224	4658	4210			
		B2	18.45	116399	15107	46914	4703	3854			
		B3	24.25	154993	23429	24331	11648	9479			
		M	18.08	102894	15320	25823	7003	5848			

E	F1	B1	8.60	51027	3414	4000	1434	1405	569	259	62
		B2	8.12	37974	1741	272	753	735	405	242	73
		B3	5.88	9570	891	138	181	212	288	173	52
		M	7.53	32857	2015	1470	789	784	421	225	62
	F2	B1	4.84	32376	4535	3560	1048	947			
		B2	15.55	70315	8316	9093	4316	3474			
		B3	20.85	95062	8989	23450	3280	2075			
		M	13.75	65918	7280	12034	2881	2165			
F	F1	B1	25.00	169085	27808	15564	8253	7434	1031	348	96
		B2	22.95	93470	19433	15220	8123	6838	289	255	29
		B3	8.30	39465	4102	4526	897	853	180	77	60
		M	18.75	100673	17114	11770	5758	5042	500	227	62
	F2	B1	35.70	222114	28798	12076	11170	6701			
		B2	32.95	58212	33863	25661	13053	10164			
		B3	34.35	222025	41667	77570	9333	7448			
		M	34.33	167450	34776	38436	11185	8104			

*OD is abbreviated from 'optical density'. A, B, C, D, E and F stand for six tested boars, respectively. F1 and F2 stand for the first sperm-rich fraction and the subsequent sperm-deplete fraction of seminal plasma from the ejaculate, respectively. B1, B2 and B3 stand for three collections, respectively, whereas M represents means of the three batches for each boar.

Appendix Table 3. *In Vitro* Fertilization Estimates

Boar	Dilution	Batch	PR	MR	MPN	NSI	NSO	PEP
		B1	40.0	100.0	0	1.00	10.00	0
	D1	B2	100.0	0	20.0	27.93	42.20	0
		B3	100.0	0	19.6	33.20	45.00	0
		M	80.0	33.3	13.2	20.71	32.40	0
		B1	14.3	100.0	0.0	1.00	3.70	0
A	D2	B2	100.0	0	63.5	16.10	10.60	0
		B3	96.0	0	65.5	18.00	11.50	0
		M	70.1	33.3	43.0	11.70	8.60	0
		B1	0	*na*	*na*	*na*	0.90	*na*
	D3	B2	100.0	0	52.1	8.45	3.40	0
		B3	90.0	5.0	55.5	8.80	4.40	2.5
		M	63.3	2.5	53.8	8.63	2.90	1.3
		B1	41.2	100.0	58.6	1.00	9.00	2.4
	D1	B2	100.0	0	83.3	14.60	46.80	0
		B3	100.0	0	25.0	12.25	25.56	0
		M	80.4	33.3	55.6	9.28	27.12	0.8
		B1	20.0	100.0	50.0	1.00	4.00	10.0
B	D2	B2	95.0	10.0	94.1	10.80	12.80	8.9
		B3	100.0	0	80.0	8.30	7.05	0
		M	71.7	36.7	74.7	6.70	7.95	6.3
		B1	10.0	100.0	0	1.00	1.00	0
	D3	B2	90.0	11.1	72.2	4.30	3.50	7.2
		B3	95.0	15.8	73.7	3.21	2.35	11.1
		M	65.0	42.3	48.6	2.84	2.28	6.1
		B1	10.0	100.0	10.0	1.00	2.80	1.0
	D1	B2	100.0	27.3	24.5	8.20	20.15	6.7
		B3	100.0	0	40.0	10.39	34.68	0.0
		M	70.0	42.4	24.8	6.53	19.21	2.6
		B1	10.0	100.0	10.0	1.00	0.70	1.0
C	D2	B2	75.0	46.7	66.7	5.42	5.88	23.4
		B3	85.0	25.0	75.0	7.25	10.40	15.9
		M	56.7	57.2	50.6	4.56	5.66	13.4
		B1	0	*na*	na	na	0.20	0
	D3	B2	50.0	66.7	41.7	4.30	1.44	13.9
		B3	70.0	55.0	50.0	3.83	2.83	19.2
		M	40.0	60.9	45.9	4.07	1.49	11.0

		B1	22.2	100.0	25.0	1.00	3.60	5.6
	D1	B2	88.0	32.0	27.3	5.00	6.90	7.7
		B3	80.0	10.0	30.0	4.60	7.50	2.4
		M	63.4	47.3	27.4	3.53	6.00	5.2
		B1	30.0	100.0	0	1.00	1.40	0
D	D2	B2	80.0	49.5	66.7	3.81	2.14	26.4
		B3	70.0	40.0	60.0	3.50	1.90	16.8
		M	60.0	63.2	42.2	2.77	1.81	14.4
		B1	0	*na*	*na*	*na*	0.50	0
	D3	B2	70.0	55.0	43.9	1.90	0.64	16.9
		B3	50.0	60.0	40.0	1.20	0.50	12.0
		M	40.0	57.5	42.0	1.55	0.55	9.6
		B1	44.4	49.0	0	3.20	5.00	0
	D1	B2	100.0	0	67.5	19.90	66.75	0
		B3	100.0	0	30.7	22.50	64.38	0
		M	81.5	16.3	32.7	15.20	46.38	0
		B1	22.2	75.0	0	1.20	3.50	0
E	D2	B2	100.0	10.0	90.0	14.90	33.40	9.0
		B3	90.0	0	36.4	14.70	28.14	0
		M	70.7	28.3	42.1	10.27	21.68	3.0
		B1	6.7	100.0	0	1.00	0.80	0
	D3	B2	89.0	20.0	80.0	8.70	12.50	14.2
		B3	85.0	3.7	70.4	8.15	7.90	2.6
		M	60.2	41.2	50.1	5.95	7.07	5.6
		B1	20.0	100.0	0	1.00	3.40	0
	D1	B2	10.0	100.0	25.0	1.25	1.50	2.5
		B3	15.0	93.3	6.7	1.40	0.20	6.2
		M	15.0	97.8	10.6	1.22	1.70	2.9
		B1	10.0	100.0	0.0	1.00	1.30	0
F	D2	B2	5.0	100.0	40.0	1.00	0.25	2.0
		B3	7.0	100.0	28.6	1.00	1.00	2.0
		M	7.3	100.0	22.9	1.00	0.52	1.3
		B1	0	*na*	*na*	*na*	0.30	0
	D3	B2	0	*na*	0	0	0.0	0
		B3	4.5	1.0	0	0	0.0	0
		M	1.5	1.0	0	0	0.10	0

A, B, C, D, E and F stand for six tested boars, respectively. D1, D2 and D3 stand for Dilution 1(50,000 sperm:1 oocyte), Dilution 2 (12,500 sperm:1 oocyte) and Dilution 3 (3,125 sperm:1 oocyte), respectively. B1, B2 and B3 stand for Batches 1, 2 and 3, respectively, whereas M stand means from the three batches. PR, MR, MPN, NSI, NSO

and PEP stand for penetration rate (%), monospermy rate (%), male pronucleus (MPN) formation rate (%), number of sperm (penetrating) inside each oocyte, number of sperm (attaching) outside each oocyte and potential embryo productivity (%), represented as the percentage of penetrated monospermic oocytes with both a female and a single male pronucleus, respectively. *na:* data not available for analysis.

Appendix Table 4. Litter Sizes of Sows Bred with Two AI Doses of Spermatozoa*

Sperm Dose	A	B	C	D	E	F
2×10^9	9.44±0.76 (42)$^{\phi}$	10.12±0.76 (53)	9.79±0.91 (44)	9.65±0.98 (39)	10.02±0.75 (29)	9.11±1.38 (12)
3×10^9	10.70±0.63 (38)	10.21±0.60 (54)	11.45±0.73 (45)	11.53±0.73 (42)	10.48±0.63 (33)	10.40±1.03 (13)

A, B, C, D, E and F stand for six tested boars, respectively. *Means for the 20-week breeding period during which semen was collected for AI. ϕ Values in parentheses represent total number of sows inseminated. Refer to Xu *et al.*(1998) for details.

Appendix Table 5. Concentrations of Testosterone and Estradiol in Boar Seminal Plasma (pg ml^{-1}) Present by Boar and by Fraction within Boar

Steroids	Fraction	Batch	A	B	C	D	E	F
T	F1	B1	202	738	598	257	247	229
		B2	40	736	205	392	78	195
		B3	63	977	440	796	33	576
		Boar Means	100	817	414	482	119	333
	F2	B1	171	926	326	161	226	187
		B2	31	633	191	87	68	137
		B3	71	496	487	181	26	544
		Boar Means	91	685	334	143	107	289
E2	F1	B1	21	153*	153	148	9	139
		B2	14	153	153	153	26	100
		B3	24	153	153	153	8	153
		Boar Means	20	153	153	151	14	130
	F2	B1	20	153	53	32	13	44
		B2	8	153	55	34	15	25
		B3	8	*na*	153	100	8	73
		Boar Means	12	153	87	56	12	47

A, B, C, D, E and F stand for six tested boars, respectively. T and E2 stand for testosterone and estradiol-17β, respectively. F1 and F2 stand for the first sperm-rich fraction and the subsequent sperm-depleted fraction of seminal plasma from the ejaculate, respectively. B1, B2 and B3 stand for three collections, respectively. * E2 concentrations exceeding 153 pg ml^{-1} were assigned a value of 153 pg ml^{-1}. *na*-concentration not included in analysis due to a high variation between duplicate estimates.