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University of Alberta

ASSESSMENT OF IN VITRO MATURATION AND FERTILIZATION TECHNIQUES FOR EVALUATION OF OOCYTE MATURATION AND SPERM QUALITY IN PIGS

by

XIAOJI XU 

A thesis submitted to the Faculty of Graduate Studies and Research in Partial Fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Animal Science

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

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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 ASSESSMENT OF IN VITRO MATURATION AND FERTILIZATION TECHNIQUES FOR EVALUATION OF OOCYTE MATURATION AND SPERM QUALITY IN PIGS submitted by XIAOJI XU in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL SCIENCE.

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ABSTRACT

Two studies evaluated the use of IVM-IVF systems for assessment of boar sperm fertilizing ability and pig oocyte maturation. In the first set of experiments, the fertilizing ability of sperm from different boars and different fractions within an ejaculate was assessed using standardized in vitro matured pig oocytes. In Experiment 1, use of three ejaculate fractions representing the first sperm-rich fraction (F1), the seminal-rich fraction (F2) and the second sperm-rich (F3) demonstrated significant fraction effects on IVF parameters. F1 sperm showed superiority in fertilizability and less variability. Also the criteria used allowed discrimination between boars in apparent semen quality. The results of Experiment 2 showed that penetration, polyspermy and MPN rates were significantly affected by boars, sperm:oocyte ratios, and their interactions. The MPN formation rate was found to be an effective discriminator for in vitro comparisons of semen quality among boars at specific sperm:oocyte ratios. Experiment 3 examined the relationship between estimates of semen quality using IVM-IVF techniques and fertility in vivo when semen from the same boars was used for artificial insemination of sows in a nucleus herd. No single in vitro characteristic was consistently correlated to fertility in vivo. However potential embryo production rate appeared to be a promising predictor of semen quality and boar fertility. Overall, the results support the suggestion that the sperm population tested in vitro may be different from that responsible for fertilization in vivo. In the second set of experiments, optimized IVM-IVF systems were applied to test the hypothesis that follicles recovered from prolific Meishan pigs provide a more favorable

environment for oocyte maturation in vitro than those from Large-White hybrid pigs. A higher percentage of oocytes cultured in Meishan follicle conditioned medium (CM) underwent sperm penetration and male pronuclear formation than oocytes cultured in Large-White hybrid CM, which supported the hypothesis. In conclusion, the IVM-IVF system adopted proved to be effective for sperm quality and oocyte maturation evaluation in vitro.

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1. INTRODUCTION

To achieve successful fertilization and normal embryo development both in vivo and in vitro, both gametes must be mature and of a good quality. Either an immature oocyte or a poor quality sperm will cause problems and the oocyte will fail to fertilize or develop normally. Therefore it is important to develop reliable methods for evaluating oocyte maturation status and sperm quality.

From the practical point of view in the AI industry, although routine semen examination gives some information about semen quality, currently used measurements are often not correlated to field data (Barth, 1992; Linford et al., 1976; Saacke et al., 1991). Also in normal commercial practice, a high number of sperm (for example 3 billion) per AI dose is commonly used for breeding to maximize the farrowing rate and litter size. However, the use of 3 billion sperm per dose could be very conservative and it is likely that in many boars lower doses of sperm could be used for AI if a better assessment of semen quality was available. An increase in the number of insemination doses per ejaculate would greatly increase the efficiency of boar AI studs and increase the genetic impact of superior boars in their working lifetime. Therefore, when using high sperm numbers for AI it is hard to identify boars with medium fertility from others with high fertility, and almost impossible to compare the difference in semen quality among boars with generally high fertility, since these high sperm numbers exceed the semen doses that would yield a 'linear' fertility response (see Saacke, 1982; Saacke et al., 1994). To enhance the use of artificial insemination, the AI industry needs reliable in vitro techniques to assess semen quality and to compare boar fertility. Among existing in vitro tests, in vitro fertilization of oocytes with sperm of the same species is the most informative method used so far, since it is more suitable for assessing overall sperm fertilizing function (Harrison et al., 1995).

To provide background knowledge for the studies presented in this thesis, a comprehensive review of oocyte maturation and acquirement of developmental competence, sperm maturation and capacitation, and the current status of IVF in the pig is presented in Chapter 2.

Based in the literature reviewed, porcine in vitro maturation and fertilization (IVM-IVF) systems have been established and proved to be effective in our laboratory (Ding and Foxcroft, 1994a,b). Although these systems may not be ideal in terms of the number of normally developing embryos obtained, they still can be effectively used to evaluate sperm quality and oocyte maturation status. The objectives of the two sets of studies presented in this thesis therefore were to use our IVM-IVF systems for evaluation of sperm fertilizing ability and oocyte maturation in pigs. In the first set of studies, attempts were made to optimize the IVM-IVF systems to provide a better discrimination among boars in semen quality assessments and then to compare the IVF results with field fertility data.

Previous experiments in our and other laboratories showed that when used in standard IVM-IVF systems there were large variations in semen quality among boars and even variations between ejaculates from the same boar (Ding et al., unpublished observations; Sirard et al., 1993). Variability in semen quality is therefore one of the major problems in establishing reliable IVM-IVF procedures in the pig. Einarsson (1971) had previously demonstrated that the characteristics of semen within an ejaculate varied from fraction to fraction, suggesting that sperm fertilizing ability in each fraction might be different. Therefore the objectives of the experiments presented in Chapter 3 were to determine the effect of variability of different fractions of a single ejaculate on boar semen used for IVF and to standardize IVM-IVF systems for evaluating boar semen quality. The results showed that use of a standardized sample of the sperm-rich fraction of an ejaculate produced the best IVF results and less variability. Also, the in vitro criteria used in this study allowed for discrimination between the three boars in terms of apparent semen quality. However, use of a high concentration of sperm for IVF results in both high oocyte penetration and polyspermy rates, which did not allow the difference in sperm quality between the two boars with high fertility to be demonstrated. In Chapter 4, therefore, experiments were designed to examine the effect of using decreasing sperm concentrations of standardized ejaculate fractions from three boars on different IVF parameters. As Rath (1992) suggested that using sperm:oocyte ratios in stead of using sperm concentration would give a better comparison of IVF outcomes from different laboratories, five sperm:oocyte ratios used represented five serially diluted sperm concentrations. Among these parameters used to evaluate IVM-IVF results, the characterization of male pronuclear formation rate was found to be an effective discriminator for comparisons of semen quality in vitro among boars at specific sperm:oocyte ratios. The results support our assumption that quality of spermatozoa, as well as the maturational state of the oocyte, contribute to successful male pronuclear formation. It is suggested that serial dilution of semen used in IVM-IVF systems could therefore provide better discrimination of sperm quality among boars. The optimization of the sperm concentration used in the IVM-IVF systems would also improve in vitro fertilization and pig embryo production.

Results from experiments in both Chapter 3 and 4 showed that the IVM-IVF systems can be adopted for comparing boar semen quality in vitro. However, it was still necessary to determine how well our IVM-IVF results would correlate to the in vivo fertility data and whether IVF parameters can be used to predict boar fertility. In the pig, sperm penetration tests using immature pig oocytes and matured zona-intact pig oocytes have been reported to be useful for providing information about fertilizing ability of boar sperm (Matínez et al., 1993; Ivanova and Mollova, 1993), but these in vitro results were not compared with in vivo data. A series of experiments presented in Chapter 5 were therefore designed to validate the semen serial dilution method for discrimination of in vitro sperm quality among boars, and to correlate field data with both IVF results and routine laboratory semen characteristics to predict sperm fertilizing ability. Both our preliminary experiment (using 8 boars) and main experiment (using 6 boars) confirmed that the semen serial dilution used in IVM-IVF procedures provided sperm quality information useful for discrimination among boars, and was also crucial for the effective

analysis of correlations between in vivo and in vitro data. A number of IVF parameters such as oocyte penetration rate, polyspermy rate, average number of sperm per penetrated oocyte and average number of sperm attached per oocyte were parameters useful for identification of sub-fertile boars. Furthermore, potential embryo production rate, an integral term incorporating penetration, monospermy and MPN formation rates, was positively correlated to in vivo fertility and might be used as a useful indicator of boars fertility in vivo.

In the second set of studies, the IVM-IVF systems were applied to evaluate oocyte maturation in order to test the hypothesis that Meishan preovulatory follicles provide a 'better' environment for oocyte maturation than those of Large-White hybrid pigs. The Chinese Meishan pig is characterized by its prolificacy, producing an average of 3-4 more piglets per litter than European breeds (Bolet et al., 1986; Haley and Lee, 1993). Substantial differences in the characteristics of follicle maturation between Meishan and Large-White have been observed (Biggs et al., 1993; Hunter et al., 1994). Also results from this and other laboratories provided evidence that follicular cells or follicular fluid play a crucial role in the regulation of oocyte maturation (Ding and Foxcroft, 1992; Niwa, 1993; Sirard et al., 1993). Based on this knowledge, the objective of the experiments described in Chapter 6 was to use IVM-IVF techniques to test the hypothesis that Meishan follicles provide a more favorable environment for oocyte maturation than Large-White hybrid follicles. The results of these IVM and IVF experiments supported the above hypothesis and showed that the IVM-IVF systems could be adopted to evaluate oocyte maturation in vitro.

In Chapter 7, a general discussion is presented which integrates all the experimental results of the studies presented in this thesis and provides further insight into the application of the IVM-IVF techniques to an evaluation of sperm quality and oocyte maturation in pigs.

The material in Chapters 3 and 4 has been published in amended form in Theriogenology (X. Xu, J. Ding, P.C Seth, D.S. Harbson and G.R. Foxcroft, 1996a; X. Xu, P.C Seth, D.S. Harbson, A.P. Cheung and G.R. Foxcroft, 1996b). The material in Chapter 6 has been resubmitted for publication in Molecular Reproduction and Development (X. Xu, L.S. Faillace, R.T. Hardin, G.R Foxcroft and M.G. Hunter). Aspects of the work described in this thesis were also presented in a paper to the Third International Conference on Boar Semen Preservation in Mariensee, Germany in 1995 (X. Xu and G.R. Foxcroft, Reproduction in Domestic Animals 31:31-36), and to the Alberta Agriculture Research Institute, Farming for the Future Conference, in Red Deer, Alberta, Canada, in 1994 (G.R. Foxcroft, X. Xu, S.D. Harbison and P.C. Seth). During the completion of this doctoral research program, the IVM-IVF system developed was also used in a collaborative study to evaluate the impact of previous metabolic state on oocyte maturation in the sow: this study was originally described in the PhD thesis of Louisa Zak (University of Alberta, 1997) and has been published in modified form in the Journal of Reproduction and Fertility (L. Zak, X. Xu, R.T Hardin and G.R. Foxcroft, 1997). (Literature cited in this chapter is listed in Chapters 2, 5 and 6).

2. LITERATURE REVIEW

Mammalian embryos result from a complex series of events taking place in a rapidly changing environment and with the participation of numerous tissues. The understanding of mechanisms of fertilization has been greatly facilitated in recent years by manipulation of spermatozoa and oocytes throughout the entire fertilization process in vitro (Roger 1978; Parrish, 1990; Niwa, 1993; Sirard et al., 1993, 1996; Nagai, 1996). There are at least three types of maturation process involved in fertilization: follicular maturation, oocyte maturation and sperm maturation (see review by Sirard et al., 1993). As the formation of a successfully fertilized oocyte which has the potential to develop into a healthy embryo requires the contribution of both matured gametes, effective maturation of the spermatozoon is of the same importance as maturation of the oocyte in both in vivo and in vitro fertilization. In this chapter, relevant literature about oocyte maturation, sperm maturation and gamete interactions, especially in pigs, will be reviewed to provide the background for the studies presented in the following chapters.

2.1 PREPARATION OF OOCYTES FOR FERTILIZATION

The mammalian oocyte is matured inside a particular ovarian structure, the follicle. The particular cellular relationship between the oocyte and somatic follicular cells is fundamental to oocyte maturation. In addition to providing nutrients to the oocyte, the follicular cells play a crucial role in regulating oocyte meiotic arrest and the resumption of meiosis. An oocyte must reach full nuclear and cytoplasmic maturity before it is capable of fertilization and completion of embryonic development (Thibault et al., 1987). Nuclear maturation comprises the completion of the first meiosis and cytoplasmic maturation includes the reprogramming of synthetic activity, re-organization and utilization of products sequestered during oocyte growth (Moor et al., 1990). Cytoplasmic differentiation events are not dependent upon nuclear regulation but instead control both the progressions of the meiotic cycle and the other intracellular events that confer developmental competence on the fully-grown oocytes (Moor et al., 1990; Parrish and First, 1993).

2.1.1 Maturational competence and developmental capacity

Based on the ultrastructure and transcriptional activity of the bovine oocyte and relating these features to the developmental competence of the oocyte, Hyttel et al. (1997) categorized follicular oocyte development into three phases: 1) oocyte growth; 2) "oocyte capacitation", and 3) final oocyte maturation.

2.1.1.1 Oocyte growth stage

During the oocyte growth phase, the inside zona diameter of the gamete increases from less than 30 µm in the primordial follicle to more than 120 µm in the tertiary follicle. In the primordial and primary follicle the communication between the oocyte and the granulosa cells is mediated through an endocytotic pathway as signaled by the abundant coated pits and vesicles present in the oocyte. This communication between the oocyte and the granulosa cells is switched to gap junctions in the secondary and succeeding follicle stages. At the same time in the ooplasm, a number of organelles and inclusions such as Golgi complexes, smooth endoplasmic reticulum, lipid droplets and membrane bounded vesicles are gradually built up and undergo a peripheral dislocation. Oocyte- specific structures such as the cortical granules and the zona pellucida appear in the secondary follicle (Hyttel et al., 1997). During oocyte growth, changes of transcription activities in the oocyte nucleus have also been observed (Crozet et al., 1986; Fair et al., 1996). It is believed that transcripts are required for the synthesis of proteins for use within the oocyte or for export out the cell, while the storage of certain other transcripts and proteins are required for building up the meiotic and developmental competence of the gamete. The transcriptional activity is normally reflected in the ultrastructure of the nucleolus, which is the site of rRNA transcription and subsequent synthesis of the ribosomal subunits. The actively transcribing oocyte nucleolus shows well defined fibrillar centers and granular components but lacks the dense fibrillar component; the inactive non-transcribing oocyte nucleolus consists of a sphere of densely packed fibrils (Fair et al., 1996). The oocyte nucleolus is gradually activated in the primary and secondary follicle and is inactivated again at the end of the growth phase (at an oocyte diameter of about 100 µm). However, transcription is not completely inactivated in the fully-grown oocyte (Hyttel et al., 1997).

Increasing meiotic competence during follicular growth has been demonstrated in the pig (Motlik and Fulka, 1986; Motlik et al., 1984), cow (Fuhrer et al., 1989; Blondin and Sirard, 1995) and goat (Crozet et al., 1995) in which species a substantial improvement in the meiotic competence of the oocyte occurs with increasing follicle size. In all species, full meiotic competence appears to be acquired at a follicle size of about 3 mm, which corresponds well to an oocyte diameter of about 110 µm (Fair et al., 1996). In vitro studies of bovine oocytes with different diameters showed that the competence for resumption of meiosis is acquired at an oocyte diameter of about 100 µm; whereas the competence for reaching MII and the full competence for sustaining embryonic development is acquired at an oocyte diameter of about 110 µm. Similar results have been reported in the pig by Hirao et al. (1994). Meiotic resumption was observed in porcine oocytes with a diameter greater than 90 µm. Oocytes with a diameter of 100 µm could not progress beyond metaphase I, whereas those with a diameter of 115 µm appear to complete meiotic maturation.

2.1.1.2 Oocyte 'capacitation' stage

Sequential ultrastructural changes have been observed during oocyte development in ruminant species from the attainment of follicular dominance up to ovulation. The oocyte in the dominant follicle continues the expansion of its lipid compartment whereas the Golgi complexes are reduced in size. The envelope of the nucleus becomes undulating and the nucleolus transforms into a ring-like structure including the fibrillar center with a central and several secondary vacuoles. Nucleolus vacuolization is observed in both cow and ewe oocytes from dominant follicles before the end of the luteal phase and immediately before the occurrence of the LH surge (see Hyttel et al., 1997).

Several studies have demonstrated that the developmental capacity of in vivo matured oocytes is superior to that of in vitro matured oocytes. This difference is assumed to reflect capacitating actions of the dominant follicle rather than suboptimal conditions during maturation in vitro (Sirard and Blondin, 1996). In vitro maturation and fertilization of bovine oocytes have provided evidence to support the hypothesis that oocyte competence is acquired before the beginning of nuclear condensation, which starts a few hours after the aspiration from the follicle or the LH surge (Sirard and Coenen, 1994; Blondin et al., 1995). Based on these observations, a new hypothesis is postulated by Sirard and Blondin (1996) that the growing follicle must reach a minimum of 3-4 mm in size to acquire the capacity to respond to a developmental signal. This capacity will increase as the follicle reaches a plateau or a reduction of its growth rate, either by dominance or early atresia. The triggering signal is provided by the follicular conditions around the time of the LH surge in vivo or can be mimicked by post-mortem conditions in vitro. Since the period of follicular dominance is probably bypassed during the standard IVM-IVF procedure, warm incubation (about 30°C) of the bovine ovary for 4 hours before collection of the oocytes for IVM-IVF was reported to mimic this effect and to produce significantly more blastocysts after 7 days of in vitro development (Blondin et al., 1997).

Since the oocyte undergoes significant modifications in the dominant follicle that play a key role in the acquisition of full developmental competence, it is only during this phase of development that the oocyte reaches its full capacity and the term 'oocyte capacitation' is suggested (Hyttel et al., 1997).

2.1.1.3 Final oocyte maturation

Final oocyte maturation occurs in the ovulatory follicle upon stimulation by the LH surge. Rather than being the activation of a quiescent gamete, final oocyte maturation is the culmination of a long series of preparatory processes. The objective of final oocyte maturation is to produce a haploid secondary oocyte that is equipped with the biological machinery necessary for successful fertilization and initial embryonic development (Hyttel et al., 1997). Oocytes undergo nucleus breakdown (or Germinal Vesicle Break Down, GVBD) in the pre-ovulatory follicle after the LH peak and the perivitelline space develops further. In the oocyte, the mitochondria tend to arrange around the lipid droplets,

the nuclear envelope is dissolved into tubules of smooth endoplasmic reticulum and the microtubules appear adjacent to the condensing chromosomes. When the oocyte reaches the MI stage, the number and size of the lipid droplets have increased and mitochondria have assembled around the droplets and are distributed evenly throughout the ooplasm. Numerous ribosomes appear, especially around the chromosomes. In a MII stage oocyte, Golgi complexes are practically absent. The bulk of the cortical granules are distributed individually along the oolemma, which form the structural background for the block to polyspermy. The lipid droplets and mitochondria have attained a more central location in the ooplasm leaving a rather organelle-free peripheral zone in which the most prominent features are large clusters of smooth endoplasmic reticulum. The gradual increase in the lipid compartment of the oocyte throughout capacitation and final maturation is probably of great importance for the initial phase of embryonic development as an energy pool (see Hyttel et al., 1997). Likewise, the store of ribosomes and macromolecules in the oocyte during its growth is of significance, because the embryo displays only a very limited transcriptional activity up to a species-specific cell stage (e.g. the 4-cell stage for the pig, 8-16-cell stage for cattle) at which stage of development a burst of transcription occurs concomitantly with the onset of nucleolus activation and rRNA-transcription (Kopecny et al., 1989; Prather, 1993).

2.1.2 Oocyte nuclear maturation and its regulation

Immature ovarian oocytes are characterized by having a large nucleus referred to as the germinal vesicle (GV). In the preovulatory follicle after the LH surge, the GV nuclear envelope of an immature oocyte ruptures, a process called germinal vesicle breakdown (GVBD). As described previously, GVBD is the most obvious morphological change of the oocyte nucleus and is also important as an indicator of the resumption of meiotic division. Before GVBD, the nuclear chromatin undergoes condensation. After the breakdown of the germinal envelope, the chromosomes disperse and then arrange themselves on the microtubules at the equator of the meiotic spindle at metaphase of the first meiotic division (MI). Then under the influence of the microtubules, the chromosome bivalents move to the opposite ends of the spindle, which rotates gradually through 90°, so that the axis becomes radially oriented. The set of the chromosomes at the more peripheral end, together with very little cytoplasm, is enclosed by a plasma membrane and extruded into the perivitelline space as the first polar body; the other set of chromosomes remains with almost all the cytoplasm and forms the secondary oocyte. Shortly after the completion of the first meiotic division, the oocyte enters the second meiotic division and in a very short time reaches the second metaphase stage (MII). At this point, the meiosis arrests again. The in vivo ovulated MII oocyte is considered to be a mature oocyte, since cytoplasmic maturation in these oocytes occurs in synchrony with meiotic maturation and hence the oocyte has the ability to be fertilized and subsequently to undergo embryonic development. (see Baker, 1982; Thibault et al., 1987).

2.1.2.1 Regulation of oocyte nuclear maturation

The mechanisms that regulate the maintenance of diplotene arrest in the meiotically competent oocyte, and pre-ovulatory meiotic resumption, are not precisely understood. Many factors, as well as the interactions between follicular cells and the oocyte, appear to be involved in such regulation. These factors include gonadotropins, cAMP, oocyte maturation inhibitor(s), growth factors and maturation promoting factor.

2.1.2.1.1 Hormonal regulation

The pituitary gonadotropins are the main extra-ovarian regulators. It is generally believed that the LH surge triggers the preovulatory oocyte to resume meiosis in vivo (Tsafriri, 1978; Moor et al., 1981). In rat, hamster, rabbit, cow, monkey and sheep, when isolated intact follicles are cultured in vitro, oocytes resume meiotic division with gonadotropins added to the culture medium (Tsafriri et al., 1972; Gwatkin and Andersen, 1976; Thibault et al., 1975; Thibault and Gérard, 1973; Thibault et al., 1976; Moor and Trounson, 1977). When pig oocyte-cumulus-mural granulosa complexes are co-cultured either with or without follicle shells (FS) in vitro, the addition of the gonadotropins is crucial to the resumption of meiotic division. In contrast, nuclear maturation in cumulus-enclosed oocytes cultured in the presence or absence of two follicle shells were not affected by gonadotropin supplementation (Ding, 1993). These results suggest that gonadotropins can act on mural granulosa cells, which directly connect with cumulus cells by intercellular junctions, to remove their inhibitory effect on oocytes. Naito et al. (1988) showed that gonadotropins (FSH and hCG) did not affect nuclear maturation when porcine oocytes were cultured in modified Krebs-Ringer bicarbonate solution, whereas gonadotropin greatly enhanced nuclear maturation in oocytes cultured in porcine follicular fluid. These observations demonstrate the apparent effects of gonadotropins on oocyte nuclear maturation, especially when follicular cells or follicular fluid are present.

The effect of LH on oocyte meiotic maturation is generally reported to be positive. Some studies indicate that FSH has an inhibitory effect on meiotic maturation, mainly by increasing the production of oocyte maturation inhibitor (OMI) and/or cAMP by granulosa cells (Anderson et al., 1985; Eppig et al., 1983; Moor et al., 1981). Other studies, however, show that it increases the rate of oocyte meiotic maturation (Fukui et al., 1982; Johnston et al., 1989, Naito et al., 1988; Mattioli et al., 1991a). The diversity in gonadotropin preparations together with differences in the composition of the culture medium (Fukui et al., 1982), or culture system (Nagai et al., 1993), makes it very difficult to compare the outcomes of the various studies. In particular, the presence of serum, a source with an undefined composition of a variety of hormones and growth factors may have contributed to the conflicting observations on LH/FSH supplementation seen in different laboratories (Bevers et al., 1997). The addition of gonadotropic hormones in the absence of serum enhanced meiotic maturation and fertilizing ability of IVM bovine oocytes (Saeki et al., 1991; Zuelke and Brackett, 1990).

Recently, Bevers et al. (1997) cultured bovine cumulus oocyte complexes (COCs) in serum-free medium supplemented either with hCG (a preparation devoid of FSH activity) or hFSH in vitro and fertilized these oocytes in vitro, and found a beneficial effect of FSH, but no effect of hCG on the meiotic resumption and blastocyst formation. This indicates that only FSH and not LH influences the IVM of bovine oocytes; the LH effects reported are most likely caused by FSH contamination. It seems that these in vitro results can not be simply extrapolated to the in vivo situation.

Prolactin also influences oocyte meiotic maturation; it enhances the transition of mouse oocytes from MI to MII, but does not affect GVBD (Hoshino, 1988). However, addition of prolactin to the IVM medium failed to enhance the developmental capacity of bovine oocytes (Saeki et al., 1991).

The mechanism by which the gonadotropins influence oocyte maturation is not clear. It has been proposed that the gonadotropins affect oocyte maturation by changing follicular cell metabolic pathways towards pyruvate and lactate formation indirectly (Thibault et al., 1975; Hillensjo et al., 1976), by increasing the production of progesterone (Nicosia and Mikhail, 1975), by enhancing the synthesis of glycoproteins by the Golgi apparatus (Moricard, 1968) and probably by breaking gap junctions (Thibault, 1977, Szöllösi et al., 1988).

In mammals, the role of steroids in oocyte maturation is still controversial. In vivo, the estradiol concentration of follicular fluid is high until 6 h after the LH peak; a sharp decline then occurs followed by a gradual increase in progesterone concentrations from about 20 h after the LH peak in bovine follicles (see Bevers et al., 1997). The addition of progesterone to culture medium either produces a lower rate of maturation in denuded mouse oocytes (Eppig and Koide, 1978), in corona-enclosed rabbit oocytes (Smith et al., 1978) and in cumulus-enclosed pig oocytes (Nagai et al., 1993), or enhances nuclear maturation of cumulus enclosed rabbit oocytes (Bae and Foote, 1975). Estradiol has been reported as having no effects on the meiotic maturation of porcine oocytes (Singh et al., 1993). The presence of estradiol in the culture medium of in vitro matured human oocytes had no effect on the progression of meiosis but improved the fertilization and cleavage rate, suggesting that estradiol supports cytoplasmic maturation (Tesarik and Mendoza, 1995). Maturation of bovine oocytes in the presence of high concentrations of estradiol had a negative effect on spindle formation and polar body extrusion (Kruip et al., 1988). The negative effects of androgens have been described in in vitro studies (Rice and McGaughey, 1981; Schultz et al., 1983). Anderson et al. (1985), however, demonstrated that androgen enhanced pig oocyte meiotic maturation in vitro by inhibition of oocyte maturation inhibitor(s) production by granulosa cells.

Recently, a role of growth hormone in the IVM of oocytes from different species has been reported. Acceleration of nuclear maturation by growth hormone has been observed during IVM of cow (Izadyar et al., 1996), rat (Apa et al., 1994) and pig oocytes (Hagen and Graboski, 1990). This acceleration of maturation is exerted through an effect of growth hormone on the cumulus cells (Izadyar et al., 1996).

2.1.2.1.2 Oocyte maturation inhibitor(s) (OMI)

Follicular fluid contains an inhibitory factor(s), originally termed oocyte maturation inhibitor (OMI), that maintains oocytes in meiotic arrest. OMI, initially characterized as a protein, has not been identified, but its source has been shown to be the granulosa cells (Downs, 1993). Use of pig follicular fluid in culture of pig, rat and hamster oocytes inhibits spontaneous meiotic maturation (Tsafriri and Channing, 1975; Gwatkin and Andersen, 1976). These observations led to the search for OMI from follicular fluid. Channing et al. (1982) have concluded that there are probably two and possibly three OMI polypeptides present in pig follicular fluid which are not species specific. OMI may exert its inhibitory effect via cumulus cells, since it prevents maturation of cumulus-enclosed oocytes, but not oocytes denuded of cumulus cells (Hillensjo et al., 1979; Tsafriri and Bar-Ami, 1982). It is suggested that the OMI might be small enough (2,000Da) to pass through the gap junction between the oocyte and cumulus. Recently, two specific types of molecules, cyclic nucleotides and purines, have been shown to be important in controlling meiotic maturation (Downs, 1993).

Cyclic adenosine monophosphate (cAMP): cAMP is considered to be one of the possible inhibitors of oocyte meiotic resumption in mammals. The block of the spontaneous meiotic maturation of oocytes in culture has been repeatedly demonstrated in rodents. Culture of murine oocytes in the presence of cAMP derivatives such as dibutyryl cAMP (dbcAMP) and 8-bromo-cAMP, or activators of adenylate cyclase such as forskolin, or inhibitors of phosphodiesterase (PDE) such as isobutyl methyl xanthine (IBMX), inhibit the resumption of meiotic division (Cho et al., 1974; Wassaman et al., 1976; Dekel and Beers, 1978; Magnusson and Hillensjo, 1977; Schultz et al., 1983a; Bomslaeger and Schultz, 1985; Dekel et al.,1984; Olsiewski and Beers, 1983; Racowsky, 1985a, b; Sato and Koide, 1984; Urner et al., 1983). If oocytes are maintained in the germinal vesicle stage with cAMP or IBMX, and are microinjected with an inhibitor of cAMP-dependent protein kinase (PK-A), resumption of meiosis occurs (Bornslaeger et al., 1986). In addition, the cAMP antagonist, Rp-cAMP, reverses the suppressive effect of exogenous inhibitors of meiotic maturation (Downs, 1993). These data suggest that cAMP-dependent pathways play an important role in the mechanisms controlling meiotic maturation in mammalian oocytes. This mechanism would involve activation of protein kinase-A and resultant phosphorylation of specific proteins that either directly or indirectly bring about meiotic arrest (Downs, 1993). However, in bovine, treatments that elevate cAMP levels are not particularly effective in preventing spontaneous maturation in vitro (Sirard and First, 1988; Sirard, 1990).

Purines: Purines such as hypoxanthine and adenosine have been found at millimolar concentrations in preparations of follicular fluid that maintain oocytes in meiotic arrest in vitro. The spontaneous maturation of oocytes from a wide variety of species is suppressed when hypoxanthine is added to culture medium (Downs, 1990), though a limited inhibitory effect has been reported in bovine oocytes (Sirard, 1990). Microinjection of nanomolar amounts of the purine base prevents GVBD in mouse oocytes (Shim et al., 1992). Hypoxanthine and adenosine have shown to act

synergistically to maintain meiotic arrest and this is directly related to inhibitory actions on cAMP PDE activity and elevation of meiosis-arresting levels of cAMP. The inhibitory effects of hypoxanthine and adenosine are reversible and non-toxic (Downs, 1993).

2.1.2.1.3 Maturation promoting factor (MPF)

Exept in the mouse mammalians oocytes have an absolute requirement for protein synthesis in the period immediately before GVBD for the normal progression of the meiotic cycle. Inhibition of de novo protein synthesis during maturation blocks GVBD in cow (Hunter and Moor, 1987; Kastrop et al., 1991), sheep (Moor and Crosby, 1986), pig (Fulka et al., 1986b; Kubelka et al., 1988) and rat (Ekholm and Magnusson, 1979) oocytes. The protein factor(s) which induces oocyte maturation is named maturation-promoting factor (MPF) or M-phase promoting factor. MPF promotes the G2 to M phase transition in the cell cycle.

Meiotic maturation involves two key aspects. The first is MPF and the role MPF plays in the control of the G2/M cell cycle transitions. The second involves the modulation of centrosomes and microtubule (MT) assembly in meiotic and mitotic cells. Mammalian oocytes exhibit a series of cell cycle transitions that coordinate the events of meiosis with the onset of embryogenesis at fertilization. The execution of these cell cycle transitions, at G2/M of meiosis-I, and metaphase/anaphase of meiosis-I and II, involve both biosynthetic and post-translational modifications that directly modulate centrosome and microtubule behavior (see Albertini, 1992).

MPF has been identified as a complex comprised of two proteins, cyclin B and p34*cdc2* kinase (p34), that exists in an inactive form (pre-MPF) in immature oocytes (Murray et al., 1989). Crucial MPF-like activity reached a threshold level at 8 -16 h after the induction of maturation and peaked at the MI and MII stages, but was low at anaphase I-telophase I stages in pig oocytes (Mattioli et al., 1991b). It is proposed that the appearance of MPF activity in oocytes requires protein synthesis (Mattioli et al., 1991b; Ding et al., 1992). The accumulation of an initiator protein to a critical level plays a key role in determing the cell-cycle timing of p34 kinase activation. Lévesque and Sirard (1996) identified the initiator protein that controls meiosis in the cow; they indicated that cyclin B is synthesized only in maturing bovine oocytes and the quantity of cyclin in the oocytes is critical to meiotic resumption.

Meiotic inhibitors have a profound effect on MPF activation, and elevation of cAMP in frog oocytes has been shown to antagonize the activation of MPF; PK-A is also a negative controlling factor (Rime et al., 1992). This could explain why purines and other cAMP-elevating agents maintain mammalian oocytes in meiotic arrest in culture. It is known that IBMX prevention of MPF activation in mouse oocytes is associated with maintenance of p34*cdc2* phosphorylation (Choi et al., 1991).

As Downs (1993) pointed out, control of MPF activation is a complicated process dependent upon a cascade of kinase and phosphatase activities. It is evident that many

substrates exist for these enzymes, and their impact on the G2/M transition may be fine-tuned by their phophorylation state. An important challenge will be to determine how the meiosis-inducing hormonal signal is transduced within the follicle to produce active MPF and what phosphorylations and dephosphorylations mediate this process. One hypothesis is that, upon hormonal stimulation, follicle cells release a factor(s) that travels to the oocyte to stimulate cAMP phosphodiesterase; the resultant drop in cAMP levels and subsequently, cAMP-dependent protein kinase activity, would then permit MPF activation.

2.1.2.1.4 Cumulus oocyte uncoupling

As discussed previously, cAMP and OMI seem to exert their actions on oocyte meiotic maturation via follicular cells. Therefore, the spontaneous resumption of meiosis by removal of the follicular cells from oocytes may be due to the oocytes escaping from an inhibitory follicular environment (Pincus and Enzmann, 1935). Culture of oocytes with follicular cells results in a decrease of meiotic resumption of oocytes, which is reversed by the addition of gonadotropins to the culture media (Ding, 1993). Thus the positive hormonal effects on oocyte maturation may also be due to the removal of inhibitory factors from the follicular compartment.

Dramatic changes in the cumulus oophorus take place after the gonadotropin surge in vivo or during in vitro culture when gonadotropin is present. The cumulus expands and the cellular processes of cumulus cells lose their intimate contacts with the oocyte plasma membrane (Dekel and Kraicer, 1978; Moor et al., 1980). It is suggested that uncoupling of cumulus-oocyte gap junctions is essential for meiotic resumption; this is consistent with the observation of spontaneous resumption in artificially denuded oocytes. However, cumulus-oocyte uncoupling does not occur before GVBD (Moor et al., 1980; Moor and Gandolfi, 1987; Mattioli et al., 1988b). Szöllösi and his colleagues (1988) demonstrated that at the time of meiotic resumption the heterocellular gap junctions between oocyte and cumulus cells underwent substantial changes, fully or partly losing contacts with the oocyte plasma membrane, although a cellular relationship was still present. Thus full or partial loss of contact between cumulus cell processes and oocyte plasma membrane may reduce the quantity of maturation inhibitors entering the oocyte. Experiments with COCs cultured in vitro have suggested a relationship between the resumption of meiosis and disruption of gap junctions (Racowsky, 1984; Racowsky and Satterlie, 1985; Schultz et al., 1983b). Using quantitative freeze-fracture analysis, Wert and Larsen (1989) revealed that the loss of cumulus-to-cumulus cell gap junctions occurred prior to the completion of meiotic resumption. In contrast, Isobe et al. (1996) found that the removal of cumulus cells from porcine COCs after 20 h or 24 h of cultivation accelerated the induction of GVBD and subsequent meiotic progression, suggesting that cumulus cells, per se, may produce the suppressive factor in meiotic progression. It is proposed that the gonadotropins are responsible for the induction of cumulus expansion and uncoupling of the cumulus from the oocyte (Moor et al., 1980). The changes in cumulus cell morphology induced by gonadotropins appear to be induced by a cAMP-mediated,

energy-dependent process in which microfilaments play an important role (Lawrence et al., 1979).

Different degrees of cumulus expansion from in vitro cultured porcine COCs are shown in Figure 2-A, -B, -C and -D.

2.1.2.1.5 Growth factors

At the level of the follicle the action of growth factors is modulated by locally produced paracrine and autocrine acting growth factors. Production and secretion of these factors, that exert their biological action by binding to membrane-bound receptors of either theca cells and/or granulosa cells, seem strongly related to the quality and size of the follicle (see Bevers et al., 1997).

The presence of several growth factors and their receptors in developing follicles, and their effects on granulosa and theca cell proliferation and differentiation contributes evidence for a putative involvement of growth factors in the regulation of oocyte maturation. It was shown that follicular fluid contains epidermal growth factor (EGF) (Hsu et al., 1987), insulin-like growth factors (IGF-I and II) (Mondschein et al., 1988), and transforming growth factor-alpha (TGF-a) (Lobb et al., 1989). There are also binding sites for EGF within the ovary that flux in relation to the physiological state of the follicle (St-Arnaud et al., 1983; Feng et al., 1987). Studies from several groups indicate that some growth factors affect oocyte meiotic resumption.

EGF has been shown to induce cumulus expansion and promote nuclear maturation in several species including pig (Coskun and Lin, 1994a; Ding and Foxcroft 1994b; Wang and Niwa, 1995) and cow (Coskun et al., 1991; Harper and Brackett, 1993; Lorenzo et al., 1994). Nuclear maturation was not affected when denuded oocytes were cultured with EGF, indicating mediation by cumulus cells (Lorenzo et al., 1994; Wang and Niwa, 1995). It is proposed that the stimulating activity of EGF is independent of the cyclic AMP pathway and probably transduced by the PKC pathway (Coskun and Lin, 1994b).

TGFα acts similarly to EGF, since it is structurally and functionally related to EGF and binds to the EGF receptor (Kobayashi et al., 1994). In the pig, TGFα only promotes oocyte maturation of cumulus-enclosed oocytes (Coskun and Lin, 1994a). A beneficial effect of IGF-1 on IVM of porcine oocytes was reported by Xia et al. (1994), who reported an increase in the number of embryos developed beyond the 8-cell stage. IVM of bovine oocytes in the presence of IGF-1 increases the number of MII oocytes but did not increase cumulus expansion. The maturation of cumulus-free oocytes was not affected by IGF-1 (Lorenzo et al., 1994). TGFβ and activin, both members of the TGFβ superfamily, inhibit IVM of porcine, but do not affect bovine oocytes (Coskun and Lin, 1994a; Van Tol et al., 1994). Insulin, structurally related to IGF-1, improves fertilization and cleavage rate of in vitro matured pig oocytes (Zhang et al., 1991a), induces cumulus expansion and has a positive effect on blastocyst cell number in the bovine (Zhang et al., 1991b).

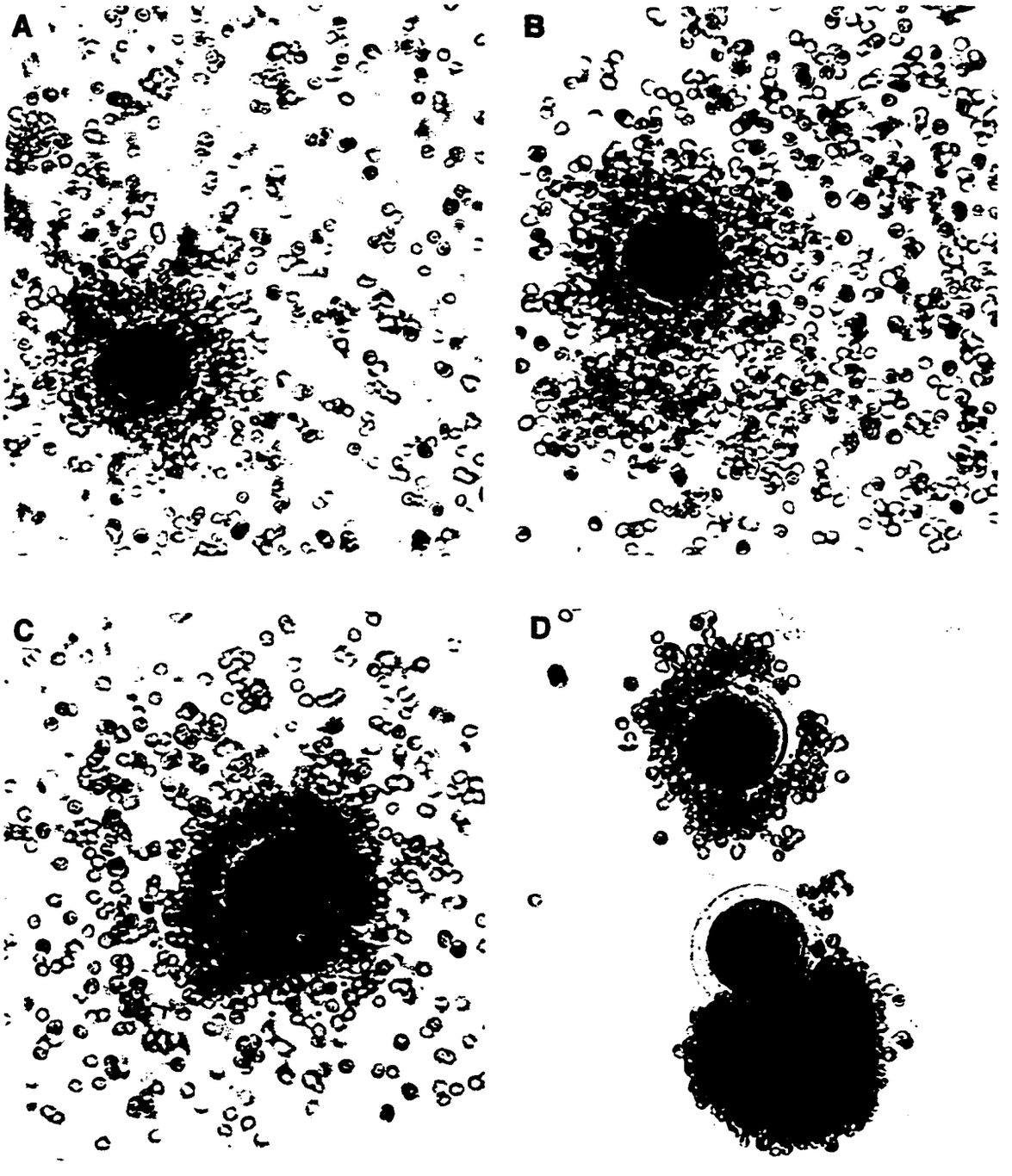
Figure 2-1 A-D Photomicrographs of live cumulus-oocyte complexes with different degrees of cumulus expansion. Figure A, B, C and D represent four classes of COCs after 44 h in vitro maturation culture.

A: COC with excellent cumulus expansion. Cumulus cells including corona radiata cells are evenly dispersed around the oocyte. Cumulus is very sticky and resistant to manipulation because of mucification of cumulus cells. Oocyte has a uniform, dark, healthy looking cytoplasm. This type of COC is classified as degree 4 or excellent, and will be selected for IVF.

B: COC with good cumulus expansion. Most cumulus cells excepting corona radiata cells are evenly dispersed around the healthy looking oocyte. Cumulus is very sticky. This type of COC is classified as degree 3 or good and will also be used in IVF experiment.

C: COC with only outer layers of cumulus cell expanded. Several layers of cumulus cells close to the oocyte are still compact which gives the appearance of a dark area around the oocyte. Cumulus is slightly sticky and easy to transfer. This type of COC is classified as degree 2 or medium expansion, and normally will not be used for IVF.

D: COC with cumulus not expanded at all (upper) or denuded but still associated with an unexpanded cumulus mass (lower). Usually these oocytes have an uneven colored cytoplasm which is a sign of degeneration. This type of COC is classified as degree 1 or poor expansion, and will be definitely excluded from the IVF experiments.


A
B
C
D

In conclusion, many factors have been reported to be involved in mammalian oocyte nuclear maturation although their mechanisms of action are not yet clear. Gonadotropins have apparent effects on nuclear maturation by removing cumulus cell inhibitory effects on oocytes through changes in granulosa cells and cumulus cells communication. The role of prolactin and steroids is still controversial. Growth hormone and growth factors have promoted nuclear maturation in several species and this effect is mediated by cumulus cells.

Examples of mature porcine oocytes with MII chromosomes and the first polar body are shown in Figure 2-E, -F, -G and –H.

2.1.3 Cytoplasmic maturation and its regulation

In preparation for fertilization, not only must meiotic maturation occur, but also the cytoplasm of the oocyte must undergo some critical changes in order to achieve competency to support sperm chromatin decondensation and subsequent male pronuclear (MPN) formation (Thibault et al., 1987). As mentioned previously, in in vivo matured oocytes, cytoplasmic maturation usually runs parallel with meiotic maturation, and ovulated MII oocytes thus usually possess the ability to be fertilized and to undergo subsequent embryonic development. However, in vitro, the nuclear maturation of oocytes is frequently dissociated from cytoplasmic maturation, resulting in a low male pronuclear formation rate and low frequency of embryonic development (Motlik and Fulka, 1974a, b; Thibault 1977; Moor and Trounson, 1977). It has been proposed that mature ooplasm contains components that promote the formation of the male pronucleus and this has been referred to as male pronuclear growth factor (MPGF) (Thibault, 1977). The nature of MPGF is still not known, although glutathione may be one of the candidates, as reviewed by Niwa (1993).

Cytoplasmic changes during final oocyte maturation have been described previously, including the relocation of mitochondria and cortical granules, and an increase of lipid droplets. This cytological remodeling is accompanied by a major reprogramming of protein synthesis, involving the utilization of approximately 30% of the polyadenylated RNA stored during oocyte growth (Bachvarova et al., 1985). The pattern of de novo protein synthesis undergoes dramatic changes during oocyte maturation, especially during GVBD, in mouse (Schultz and Wassarman, 1977; Wassarman et al., 1978), rabbit (Van Blerkom and McGaughey, 1978), sheep (Moor and Gandolfi, 1987), goat (Le Gal et al., 1992), pig (McGaughey and Van Blerkom 1977) and human (Schultz et al., 1988). The changes in protein synthetic pattern during oocyte maturation have significant physiological importance for nuclear maturation and subsequent fertilization and embryonic development. It is suggested that proteins synthesized during maturation may be utilized during fertilization and early embryonic development (Ding et al., 1992b; Prather, 1993).

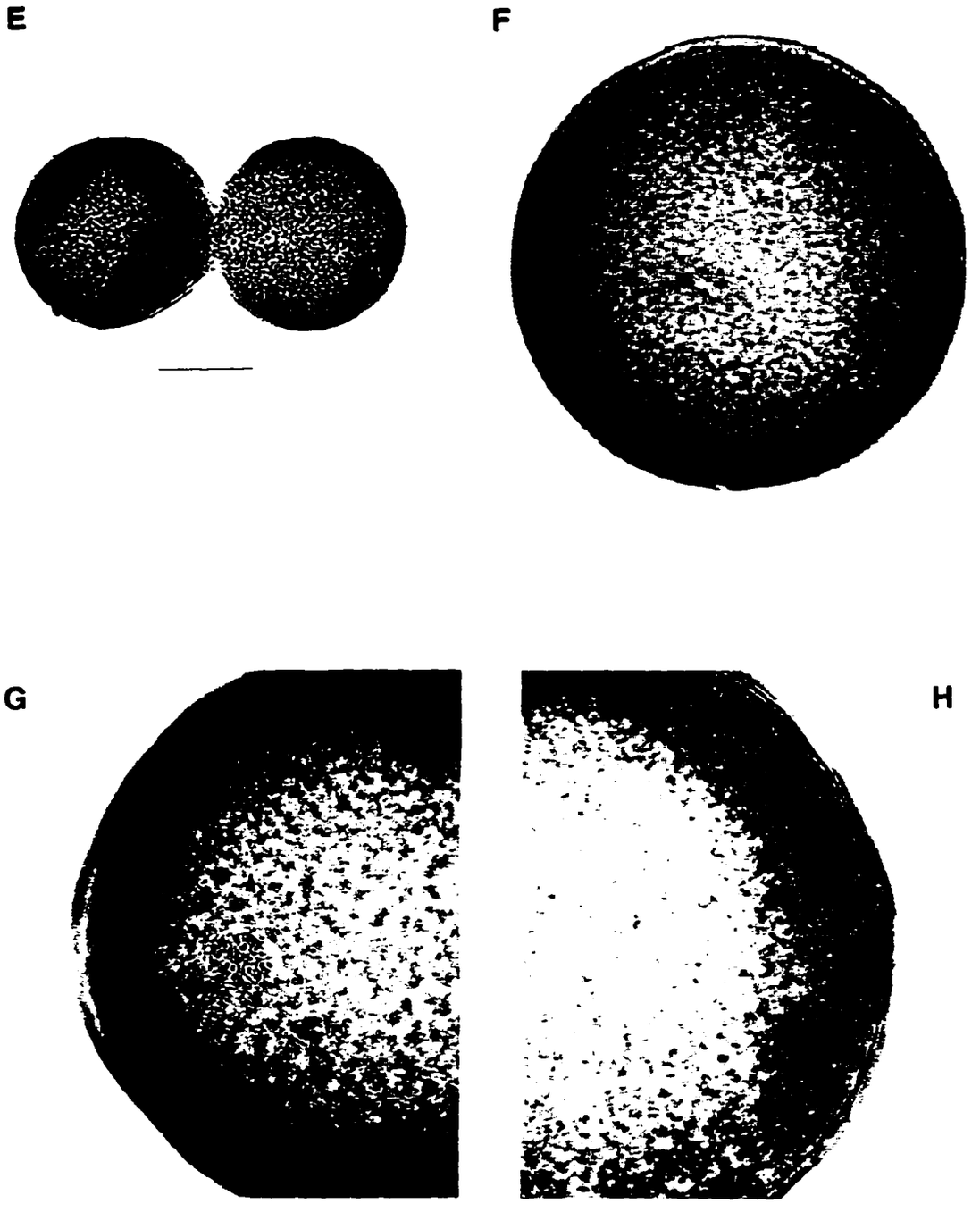
Figure 2-2 E-H Photomicrographs showing metaphase II porcine oocytes after 44 h IVM. Oocytes have been whole mounted, fixed in ethanol-acetic acid (3:1) for 48 h and stained with 1% Lacmoid in 45% acetic acid solution.

E: Two MII porcine oocytes examined under a phase contrast microscope at a magnification of 100×. Two sets of dark red colored chromosomes can been seen in the oocytes.

F: A MII porcine oocyte examined under a phase contrast microscope at a magnification of 250×. The metaphase II chromosomes and the first polar body are clear.

G: Part of a MII porcine oocyte examined under a phase contrast microscope at a magnification of 400×. Metaphase II chromosomes dispersed at a metaphase plate and the first polar body are clearly seen.

H: Part of a MII porcine oocyte examined under a phase contrast microscope at a magnification of 400×. The metaphase spindle and the first polar body are clearly seen.


E
F
G
H

2.1.3.1 Regulation of cytoplasmic maturation

In the pig, the most common criterion for measuring cytoplasmic maturation of oocytes is male pronuclear formation (Mattioli et al., 1988a). Incomplete or inadequate conditions in vitro result in partial and/or delayed sperm decondensation (Moor et al., 1990). Also male pronuclear development could be a measure of MPGF. In the normal sequence of pig fertilization, detachment of the sperm tail and early decondensation occur at about 4 h after insemination in vitro and are synchronized with the beginning of the second meiotic division (anaphase II) in the oocyte. At 6 h after insemination, incipient nuclear membranes appear around the decondensed sperm nucleus and around the female chromosomes; by 7 h well formed male and female pronuclei could be observed and by 10 h most of oocytes contain both male and female pronuclei (see Ding et al., 1992a).

2.1.3.1.1 Follicular cell effects

Follicular cells not only control nuclear maturation, but also facilitate cytoplasmic maturation in mammalian oocytes (Thibault et al., 1987; Buccione et al., 1990; Moor et al., 1990). Cumulus-enclosed oocytes cultured alone in vitro failed to form male pronuclei or had low developmental ability after sperm penetration in pigs (Motlik and Fulka, 1974a; Mattioli et al., 1988a), cattle (Thibault et al., 1975, 1976; Trounson et al., 1977; Leibfried-Rutledge et al., 1986) and sheep (Moor and Trounson, 1977), suggesting that the cumulus alone can not support full oocyte cytoplasmic maturation in vitro in these species. Furthermore, culturing cumulus-enclosed oocytes in these species together with granulosa cells or follicle shells greatly improved cytoplasmic maturation and developmental potential (Crozet et al., 1987; Critser et al., 1986; Lu et al., 1987, 1989; Lutterbach et al., 1987; Fukui and Ono, 1988; Mattioli et al., 1988a, b; 1989; Zheng and Sirard, 1992).

The development of successful co-culture systems resulted in the birth of live young after fertilization of oocytes matured in vitro (sheep, Staigmiller and Moor, 1984; Crozet et al., 1987; cattle, Critser et al., 1986; Lu et al., 1987, 1989; pig, Mattioli et al., 1989). However, recent reports showed that cumulus-enclosed oocytes matured in vitro without additional follicular cells also possess full developmental potential to term (bovine, Fukuda et al., 1990; porcine, Jiang et al., 1991; Yoshida et al., 1993b). These results suggest that additional follicular cells are not absolutely required for cumulus-enclosed oocytes to achieve full cytoplasmic maturation in vitro. The discrepancy probably results from the use of different culture systems in different laboratories.

Regardless of whether induction of cytoplasmic maturation is dependent on the presence of granulosa cells, cytoplasmic maturation is enhanced in all species by association with adherent cumulus cells (Vanderhyden and Armstrong, 1989). When the cumulus cells are removed before maturation culture, the percentage of such cumulus-free oocytes that support normal male pronuclear formation is substantially reduced (Fukui and Sakuma, 1980; Sirard et al., 1988; Vanderhyden and Armstrong, 1989). Recently,

Niwa (1993) obtained a high male pronuclear formation rate of 73% using cumulus-enclosed oocytes matured and fertilized in vitro without removal of the expanded cumulus, compared to cumulus enclosed oocytes matured in vitro and fertilized with their expanded cumulus removed. Kikuchi et al. (1993) also reported that the expanded cumulus cells surrounding oocytes play an important role in increasing male pronuclear formation during fertilization in vitro and that supplement denuded oocytes with cumulus cells during fertilization did not improve male pronuclear formation. The underlying mechanism is not clear. Two possibilities have been suggested: one possibility is that IVM oocytes may be damaged by the treatment used (hyaluronidase and/or mechanically pipetting) for removing cumulus cells, resulting in a reduced male pronuclear formation rate (Ball et al., 1983; Kikuchi et al., 1993); the other possibility is that the expanded cumulus has a beneficial effect on sperm selection and function in IVF.

2.1.3.1.2 Follicular secretion effects

Follicular fluid and follicular cell conditioned medium have been shown to have a supportive effect on oocyte cytoplasmic maturation (Niwa, 1993). Naito et al. (1988) found that porcine cumulus-enclosed oocytes cultured in medium containing porcine follicular fluid without gonadotropin supplementation severely inhibited nuclear maturation; however, this inhibitory effect was removed by gonadotropin supplementation. Evidence has been provided by different research groups that the addition of follicular fluid in the maturation medium with gonadotropins greatly improves male pronuclear formation rate in pig oocytes (Naito et al., 1992; Yoshida et al., 1990, 1992b, 1993b; Funahashi and Day, 1993c). The effective factors in the follicular fluid supporting cytoplasmic maturation is heat (56°C)-treatment stable (Naito et al., 1990; Funahashi and Day, 1993c). Follicular cell conditioned medium is also capable of supporting cytoplasmic maturation of cumulus-enclosed, but not cumulus-free oocytes (Mattioli et al., 1988a, b; Ding et al., 1994a). Collectively, it can be suggested that follicular cell secretions contain factors to support cytoplasmic maturation through cumulus cells and the coupling between follicular cells and the oocyte is essential.

2.1.3.1.3 Hormonal effects

Hormonal action on oocyte cytoplasmic maturation is also likely to be mediated via follicular cells. Using either follicular cell co-culture (Ding et al., 1994a) or follicular fluid (Naito et al., 1988), both nuclear and cytoplasmic maturation can be properly achieved only in the presence of gonadotropins. Mattioli and his colleagues (1991a) examined LH and FSH effects on cytoplasmic maturation separately or in combination and showed that LH but not FSH is effective in stimulating cytoplasmic maturation of pig oocytes co-cultured with everted follicle shells. However, Ding and Foxcroft (1994a) demonstrated that factors secreted by follicular cells stimulated by FSH alone provide better support for full oocyte maturation than by combined FSH, LH and prolactin treatment. Funahashi and Day (1993c) reported that culturing pig cumulus-enclosed oocytes in medium supplemented with eCG, hCG and estradiol for the first 20 h, then in gonadotropin-free medium for another 20 h, enhanced cytoplasmic maturation (67%

MPN formation) when compared with those cumulus-enclosed oocytes continuously cultured in the presence of gonadotropins for 40 h (36% MPN formation). They suggest that hormonal effects on cytoplasmic maturation are achieved in the first 20 h of culture and that over exposure of oocytes to gonadotropins, and probably also to steroid hormones produced by cumulus cells stimulated by gonadotropins, has adverse effects on cytoplasmic maturation (Funahashi and Day, 1993c).

2.1.3.1.4 Growth factors

EGF has been suggested to improve oocyte cytoplasmic maturation in some domestic species. Coskun et al. (1991) and Harper and Brackett (1993) reported that EGF supplementation enhanced the developmental potential of bovine oocytes matured in vitro. Wang and Niwa (1995) reported a positive effect of EGF on male pronucleus formation in the pig which was synergistically enhanced when EGF and gonadotropins were added. In contrast, Ding and Foxcroft (1994b) reported a positive effect of EGF only when it was used in conjunction with gonadotropins. Procházka et al. (1997) found that EGF only stimulated expansion of porcine COCs originating from large antral follicles. IGF-1 has also been reported to improve porcine oocyte cytoplasmic maturation (Xia et al., 1994).

Effects of growth factors via follicular cells clearly exist, since there are binding sites for growth factors on follicular cells. EGF, for example, binds to its receptors and induces the activation of tyrosine-specific kinases, an essential primary event in the EGF pathway. In turn, tyrosine kinase activation initiates phosphorylation of several cellular proteins as well as phosphorylation of the receptor itself (Harper and Brackett, 1993). It is suggested that the interactions between EGF and gonadotropins in the regulation of receptors may play an essential role during oocyte maturation (Harper and Brackett, 1993).

2.1.3.1.5 Glutathione

Glutathione, the major intracellular free thiol, has been implicated as playing a key role in protamine disulphide bond reduction, which in turn is a prerequisite for sperm chromatin decondensation, and thus for male pronuclear formation (see Niwa, 1993). When glutathione synthesis is blocked during the early stages of oocyte maturation in vitro, the cytoplasm of chromosomally normal MII oocytes is incapable of supporting sperm nuclear decondensation (Perrault et al., 1988). Recent studies in pig oocytes also confirm the effect of glutathione on sperm decondensation and pronuclear formation (see Niwa, 1993). More details on glutathione and its effect on oocyte IVM-IVF will be given in a later section of this literature review.

2.1.3.1.6 Other factors

The size of a follicle from which an oocyte originates has effects on its subsequent ability to be fertilized and to undergo early embryonic development in vitro. Porcine

oocytes from larger antral follicles have a larger oocyte diameter and a greater ability to complete meiosis (Tsafriri and Channing, 1975; Motlik et al., 1984; Motlik and Fulka, 1986). Similarly, the developmental competence of goat oocytes is acquired progressively during follicular growth and only those isolated from large antral follicles have the capacity to progress to the blastocyst stage following in vitro maturation and fertilization (Crozet et al., 1995). In cattle, many studies demonstrated that the size of follicles from which oocytes are derived affects their developmental competence (Tan and Lu, 1990; Galli and Moor, 1991; Pavlok et al., 1992; Longergan et al., 1994; Arlotto et al., 1996). Blondin and Sirard (1995) described the fate of individual oocytes according to exact follicular size and confirmed an increased competence with size. However, not all of the oocytes from large follicles develop to embryos, and some oocytes from medium sized follicles already have this developmental capacity (Sirard and Blondin, 1996).

Studies in the pig (Tsafriri and Channing, 1975; Anderson and Hillensjo, 1982; Motlik et al., 1984; Motlik and Fulka, 1986; Homa et al., 1988), sheep (Moor and Trounson, 1977) and human (Gougeon and Testart, 1986) have shown that large oocytes, contained in large follicles, mature at higher rates. Large oocytes extrude the first polar body slightly faster than smaller oocytes, suggesting that there may be differences in the speed of maturation between small and large oocytes (Arlotto et al., 1996). In bovine, the development to the morula and blastocyst stages increases as the diameter of the oocyte increases; this difference in developmental potential may indicate cytoplasmic differences between oocytes that are able to undergo nuclear maturation (Arlotto et al., 1996). Sirard and Blondin (1996) also suggest that a thick cumulus may be a characteristic associated with oocyte in vitro developmental competence. Also, as reviewed by Sirard and Blondin (1996) the oocyte handling procedure before the beginning of in vitro maturation is very important for full developmental competence.

In conclusion, as important as controlling nuclear maturation, follicular cells facilitate cytoplasmic maturation in mammalian oocytes. Also, follicular cell secretions have supportive factors that support cytoplasmic maturation through cumulus cells. The addition of follicular fluid in the culture medium or use of follicular cell conditioned medium would improve male pronuclear formation rate in pig oocytes. Using either follicular cell co-culture or follicular fluid, oocyte maturation can be properly achieved only in the presence of gonadotropins.

2.2 PREPARATION OF SPERMATOZOA FOR FERTILIZATION

Mammalian spermatozoa, although highly differentiated by the time they leave the testis, do not yet have the ability to move progressively and to penetrate the zona pellucida, a glycoprotein matrix that protects the oocyte and the early embryo from physical damage. After leaving the testis, spermatozoa undergo a series of changes in the epididymis where they are exposed to epididymal secretions, at ejaculation when they are exposed to the secretions of the various male accessory glands, and in the female genital

tract where the final stages of sperm preparation for fertilization involve capacitation and the acrosome reaction. Capacitated spermatozoa may recognize and bind to the zona pellucida. The exocytotic event of the acrosome reaction then leads to the release of enzymes facilitating the passage of a motile hyperactivated spermatozoon through the zona pellucida and enables the sperm to fuse with the oocyte (reviewed by Yanagimachi, 1994). Studies related to the acquisition of fertilizing ability by boar spermatozoa and fertilization in pig have been reviewed by Hancock (1962), Baker and Polge (1976), Einarsson (1980), Hunter (1980, 1990, 1991, 1995) and Sirard et al. (1993).

2.2.1 Epididymal maturation

One of the major events in the life of a spermatozoon that regulates its ability to fertilize a mature oocyte is the completion of maturation within the epididymis (Parrish and First, 1993). When the spermatozoa leave the testis, they are immotile and incapable of fertilizing eggs (Orgebin-Crist and Olson, 1984). Both these limitations are overcome during the period of time the sperm spend in the epididymis, 12 days in the boar, 7 days in the bull (Swierstra, 1968) and 16 days in the ram (Amann, 1981). The process by which spermatozoa gain the ability to fertilize eggs while slowly passing through the male epididymal duct is called sperm *epididymal maturation* (Yanagimachi, 1994). The site where the spermatozoa begin to acquire their fertilizing ability varies from species to species. It becomes recognizable in the distal corpus epididymis in rabbit, boar and ram and proximal cauda in mouse, rat, hamster and man (Reviewed by Orgebin-Crist, 1987a). Sperm motility and fertility increase from 50% to 80% in boar and 12% to 80% in ram when spermatozoa move from mid corpus to posterior cauda (see Parrish and First 1993). The sequential changes in the epididymal luminal fluid probably play an essential part in sperm maturation, since simply retaining the sperm for an equivalent time in one segment of the epididymis does not allow them to reach maturity (Bedford, 1975). It is also unlikely that all the spermatozoa gain their fertilizing capacity simultaneously. Some spermatozoa apparently become fertile much faster (or in a more proximal region of the epididymis) than others. However, it is not until they enter the cauda epididymis that the great majority of spermatozoa attain their full fertilizing potential (Yanagimachi, 1994).

The development of fertilizing ability, as summarized by Bedford (1975) and Suzuki (1990), is associated with changes in several aspects of the functional integrity of the spermatozoa: (a) development of the potential for sustained active, progressive motility upon exposure to physiological salt solutions; (b) alteration of the metabolic patterns and the structural state of specific tail organelles (the dense fibers, sheath and mitochondrial shell in the tail) engendered by oxidation of protein-bound -SH to -S-S-; (c) stablization of nuclear chromatin by steady disulfide bond formation; (d) movement and loss of the protoplasmic droplet; (e) modification, at least in some species, of the form of the acrosome; (f) disappearance of flocculent material from the space between the plasma and the outer acrosomal membrane, and (g) changes in the surface of the plasma membrane.

The epididymis has a very high fluid-absorbing and secreting activity and the osmolality and chemical composition of the fluid secreted by the epididymis vary from one segment to another (Yanagimachi, 1994). Therefore, it would be expected that the sperm plasma membrane would be altered step by step as spermatozoa pass through the different regions of the epididymis. There is no doubt that the sperm plasma membrane is one of the most prominent sites of change during epididymal maturation (Olson and Orgebin-Crist, 1982).

2.2.1.1 Membrane changes during epididymal maturation

Numerous studies have demonstrated that the mammalian sperm surface proteins undergo extensive alteration during maturation. Only recently has it been possible with monoclonal antibodies and polyacrylamide gel electrophoresis to identify and partly characterize some of the membrane components involved. First, new surface components become expressed on the sperm surface during epididymal transit, often associated with the development of sperm-fertilizing capacity, or more precisely, the acquisition of binding receptors for the zona pellucida. Second, the localization of antigens by monoclonal antibodies within the lateral plane of the sperm plasmalemma is modified during maturation. This suggests that membrane moieties may become more mobile and the membrane fluidity of spermatozoa is altered as sperm pass through the proximal epididymis. Finally, the inhibition of fertilization in vivo and in vitro with monoclonal antibodies and specific antisera suggest that some of these antigens may be involved in gamete interactions (Moore et al., 1987).

2.2.1.1.1 Changes in lipid content

Singer and Nicholson (1972) proposed that membranes are a fluid mosaic, with the lipids and proteins diffusing laterally in the plane of the membrane. The phospholipid/protein ratio is approximately 0.68 on a weight basis in plasma membranes isolated from boar spermatozoa, suggesting that the amounts of total lipid and protein in the sperm plasma membrane are about the same. However, the amount and type of lipids and the lipid/protein ratios are probably different in various domains (Eddy and O'Brien, 1994) and therefore affect the basic fluidity of these domains (Buhr and Pettitt, 1995). Lipids are largely in a fluid phase at body temperature, although localized domains of gel phase lipids are detectable. Proteins affect the overall membrane fluidity and tend to restrict the mobility of the so-called annular lipids which surround the proteins. In boar spermatozoa, phospholipids make up about 70% of the total plasma membrane lipid, sterols are the next abundant, glycolipids are less abundant and free fatty acid is a relatively small amount of the membrane lipid. The lipid content of whole sperm has been reported to decrease and the composition of lipid changes during epididymal maturation in many species including the boar. The loss of sperm lipids during maturation indicates that these lipids may serve as an endogenous source of metabolic fuel for spermatozoa in the epididymis (Scott and Dawson, 1968). The lipid content and compositional changes may explain the increase in membrane fluidity (Wolf and Voglmayr, 1984) and the greater sensitivity to cold shock in ejaculated sperm compared

to testicular spermatozoa (Hammerstedt et al., 1979). Lipid composition has also been suggested to be at least partially responsible for the species-specific differences in cold-shock sensitivity (Parks and Lynch, 1992). The composition of sterols in mammalian spermatozoa has been reported (Awano et al., 1993). In boar spermatozoa, an appearance of desmosterol, an intermediate of cholesterol synthesis, in the plasma membrane was observed during sperm maturation (Nikolopoulou et al., 1985). The presence of desmosterol and its conversion to desmosterol sulfate would cause a change in surface charge of the spermatozoa, which may contribute, at least in part, to the increase in net negative charge at the plasma membrane during epididymal maturation (Yanagimachi et al., 1972). The removal of cholesterol from the plasma membrane was found to be associated with capacitation and the acrosome reaction, and the time required for capacitation was considered to correlate with the sperm cholesterol/ phospholipid molar ratio of the spermatozoa (Davis, 1980).

2.2.1.1.2 Changes in protein compositions

Most proteins found in the epididymal fluid are synthesized and secreted by the epithelium. In the boar, a total of 146 epididymal proteins were found to be secreted by the epididymis and some major proteins were identified, such as clusterin, glutathione peroxidase, retinol-binding protein, lactoferrin, EP4, β-N-acetyl-hexosaminidase, α-mannosidase, and procathepsin L (Syntin et al., 1996). Some of the proteins interact with spermatozoa and may alter the sperm membrane properties in various ways. They may be adsorbed onto the sperm surface (Okamura et al., 1990), or may modify preexisting compounds through the action of proteases, glucosidases, glucosaminidases, or glycosyltransferases (Syntin et al., 1996). Biochemical approaches have been used to identify protein changes during maturation in spermatozoa from rat (Cameo and Blaquier, 1976; Brooks 1981, 1983; Usselman and Cone, 1983), rabbit (Moore, 1980), hamster (Moore, 1980), mouse (Flickinger, 1983), ram (Alumot et al., 1971), boar (Russell et al., 1984), bull (Amann et al., 1973) and human (Tezon et al., 1985).

Epididymis-Specific Proteins: In 1976 Cameo and Blaquier showed the presence of at least four epididymis specific proteins (ESPs) in the rat and designated them as proteins B, C, D, and E, according to their increasing electrophoretic mobility under nondenaturing conditions. Subsequent studies have confirmed the presence of ESPs B-E in the rat. They are all glycoproteins and their synthesis is under hormonal control (Brooks 1981, 1983; Shabanowitz and Killian, 1987). Regional distribution of ESPs in rat epididymis and binding of these proteins to spermatozoa have been determined (Brooks 1983). Scanning electron microscopic studies indicated that antibodies adhered to ESPs on the plasma membrane in the acrosomal and postacrosomal region of cauda spermatozoa (Cameo et al., 1986). The amount of ESPs bound to spermatozoa increases progressively from the caput to the cauda epididymis spermatozoa. Incubation of cauda epididymal spermatozoa in utero, or in a capacitating medium for 6 h, reduces the amount of bound protein to the level observed in caput sperm, suggesting that some of the ESPs may act as antagonists of capacitation and thus prevent the occurrence of a premature acrosome reaction in the male genital tract (Kohane et al., 1980). It was also found that

ESPs associate with the swollen acrosome, with the vesicles formed during the acrosome reaction, and with the postacrosomal region (Cameo et al., 1986). Incubation of cauda epididymal spermatozoa with rat ESP protein D/E antibodies prior to use for AI resulted in blocked fertilization, suggesting that the epididymal protein D/E might be a component of a sperm structure involved in the process of fertilization (Cuasnicu et al., 1984). Similar ESPs have been found in rabbits (Moore, 1980) and ram (Voglmayr et al., 1982). The proteins first appear to bind to spermatozoa in the distal caput and proximal corpus region, the same regions where rabbit sperm acquire their fertilizing ability (Bedford, 1967). The exact function of these glycoproteins is unknown. They may influence the maturation of spermatozoa or act as a decapacitation factor (Eng and Oliphant, 1978).

The Antagglutinin Protein: Spermatozoa obtained from the caput or cauda epididymal regions generally undergo head-to-head agglutination on dilution. This process is time dependent and was not influenced by Ca^{2+}, EDTA, milk, egg yolk, BSA, or testicular fluid (Dacheux et al., 1983). However, the dilution-induced agglutination can be prevented or reversed by the addition of cauda epididymal fluid or ejaculated plasma prior to or after the agglutination, suggesting that the epididymal fluid contains a substance that prevents agglutination of the spermatozoa. In ram, boar and bull, agglutination involves the membrane overlying the acrosome (Senger and Saacke, 1976). The antagglutinin is present in the semen of several mammals, including human, and does not appear to exhibit any species specificity (Shivaji et al., 1990). β-N-acetyl-hexosaminidase has been purified and identified as an antagglutinin in the boar (Dacheux et al., 1983). The physiological role of epididymal fluid antagglutinin is unknown.

The Forward-Motility Protein: Mammalian spermatozoa acquire forward motility as they transverse the epididymis, which suggests that factors contributed by the epididymis are responsible for the acquisition of this ability. To understand the mechanism of the initiation of motility in sperm cells, Hoskins et al. (1975) tried to induce motility in nonmotile caput bull spermatozoa by addition of phosphodiesterase inhibitors, such as caffeine and theophylline, which can stimulate motility of mature bull spermatozoa. It was observed that only very high concentrations of such inhibitors could initiate flagellar activity in caput sperm but did not cause forward motion. However, forward motility could be induced in the caput spermatozoa if both phosphodiesterase inhibitors and seminal plasma were added. The seminal plasma factor responsible for the induction of forward motility in bovine caput sperm was subsequently purified and named the "forward-motility protein" (FMP) (Acott and Hoskins, 1981). Molecular studies showed the existence of multiple forms of FMP in aggregate equilibrium and the molecular weight of this monomeric protein was 37,500 Da. FMP is heat-stable but is completely inactivated on digestion with trypsin. FPM is of epididymal origin and is confined exclusively to the reproductive tract fluids (Brandt et al., 1978). FMP is not species-specific, as bovine seminal plasma can induce forward motility in the caput sperm of a variety of mammals (bull, rabbit, boar, monkey and guinea pig) and seminal plasma from different mammals also induces forward motility in bovine caput sperm (Acott et al., 1979). The mechanism by which FMP converts the whiplash movements of immature caput sperm into coordinated progressive motility is not clear. Since the forward motility

of cauda sperm is independent of the presence of FMP, it has been suggested that FMP acts like a switch and converts the FMP-dependent caput sperm into FMP-independent sperm. It can be concluded that FMP binds to sperm membranes during sperm transit through the epididymis and acts in concert with the increase in the intracellular level of cAMP during maturation to induce forward motility in spermatozoa.

Calcium-Binding Proteins: Calcium has been suggested to be involved in sperm motility and the acrosome reaction (Yanagimachi, 1994) and is known to play a major role in gamete interaction. Both the sperm plasma membranes and outer acrosomal membranes are the preferential sites for Ca^{2+} sequestration (Perterson et al., 1989). In mammalian spermatozoa, calmodulin is a well-known major Ca^{2+}-binding protein associated strongly with the plasma membrane during epididymal transit (Jones et al., 1980). In pig, six major Ca^{2+}-binding proteins (CBPs) were identified (Perterson et al., 1989), which have molecular weights of 30, 35, 38, 42, 52 and 66 kDa, respectively, and develop an association with the plasma membrane during epididymal maturation. These calmodulin-like proteins interact with hydrophobic matrices and appear to play a role in the regulation of calcium function.

Ap_z - A Sperm Protein Involved in Zona Binding: A boar sperm integral plasma membrane protein was identified as being involved in the adhesion of capacitated and uncapacitated sperm to porcine oocytes (Perterson and Hunt, 1989). This zona adhesion protein (AP_z), with a molecular weight near 55 kDa, is localized in the plasma membrane overlying the anterior and posterior regions of the sperm head and becomes active during sperm maturation in the epididymis (Peterson et al., 1991). Antibodies to a plasma membrane protein fraction containing this protein lost their ability to block sperm-egg binding when absorbed against the purified protein. It was found that these antibodies were not absorbed by caput sperm plasma membranes but were absorbed by membranes from the corpus and cauda epididymis, which coincides with the observation that boar sperm gain the ability to bind to porcine oocytes as they pass into the proximal corpus (Perterson et al., 1985). Peterson et al (1991) described a rapid method for isolating AP_z by preparative polyacrylamide gel electrophoresis (PAGE). They showed that antibodies raised against AP_z, isolated directly from PAGE gels, inhibit the adhesion of ejaculated sperm to the zona pellucida in vitro. In the zona pellucida (ZP) glycoprotein families, it is the alpha group of ZP_3, not the beta form of ZP_3 that binds to AP_z in the intact sperm cell (Peterson et al., 1991). The structures of AP_z in the bovine and porcine plasma membrane are similar (Peterson et al., 1991). It is possible that other proteins participate in sperm zona adhesion and that AP_z is a necessary but not sufficient for this interaction. In pig, AP_z is probably a main participant in the initial steps of sperm-zona adhesion.

2.2.1.1.3 Changes in sperm surface carbohydrates

Another way that sperm surface components are altered in the epididymis is by glycosylation. Studies on rat epididymis showed that the epididymis contains androgen-dependent glucosyl and mannosyl transferases, and the corpus has the highest β-N-glucosaminidase, β-N-acetyl-galactosaminidase and β-N-galactosidase activities. Also N-

acetylneuraminyl transferase activity is higher in caput than cauda epididymis, although some sialoglycoproteins in the epididymal fluids are produced only in cauda (Eddy and O'Brien, 1994). There is evidence that the increase in negative surface charge by ram and bull spermatozoa in the epididymis is due to the addition of sialic acid groups to the sperm surface ((Holt, 1984). Other studies have suggested that lactosaminoglycans present on the surface of testicular sperm are fucosylated by an epididymal fucosyltransferase. Galactosyltransferase activity is also involved in sperm surface modifications during maturation by galactosylation of exposed N-acetylglucosamine residues (Eddy and O'Brien, 1994). It has been hypothesized that the sperm-surface galactosyltransferase binds to N-acetylglucosamine on the zona pellucida as part of the fertilization process. Evidence for this is that (a) purified galactosyltranserase produced a dose-dependent inhibition of sperm binding to the zona pellucida and caused sperm bound to the zona to be released and (b) monoclonal antiserum to the enzyme produced a dose-dependent inhibition of sperm binding to the zona pellucida and concomitantly blocked sperm galactosyltransferase activity (Eddy and O'Brien, 1994).

2.2.1.1.4 Changes in the nature of calcium transport systems

The functional changes in the sperm plasma membrane are directly or indirectly responsible for epididymal sperm maturation. For example, the activity of adenylylcyclase on the sperm plasma membrane increases during epididymal maturation, resulting in the increase in intracellular cyclic AMP (cAMP), which is known as an intracellular messenger of sperm maturation (Okamura and Sugita, 1983). In addition to cAMP, calcium is also well known as an important intracellular regulator of various sperm functions including epididymal maturation. It has been proposed that various kinds of transport systems are involved in the regulation of Ca^{2+} concentration in mammalian sperm, such as the ATP-dependent, Ca^{2+} pump, the Na^{+} /Ca^{2+} exchanger, the voltage-dependent Ca^{2+} channel, the calmodulin-dependent, energy-requiring Ca^{2+} transporter and the receptor-operated Ca^{2+} channel (see Okamura et al., 1992). Changes in the activities and properties of calcium transport across the plasma membrane during epididymal maturation of porcine sperm were studied by Okamura et al. (1992). The observation that the rate of Ca^{2+} uptake in boar sperm is similar to that of ram but lower than bovine suggests that the Ca^{2+} uptake activity of sperm varies among species and experimental conditions. Their results indicate that the Ca^{2+} uptake by porcine sperm is highly dependent on energy levels (glucose or pyruvate) and mitochondrial functions; the net activities of Ca^{2+} uptake increase while sperm are transported from caput to corpus epididymis; the accumulation of Ca^{2+} by sperm becomes more feasible and more dependent on the concentration of extracellular Na^{+} during epididymal transit (Okamura et al., 1992). Although the mechanisms for the changes in the nature of Ca^{2+} transport remain to be elucidated, such changes must influence the sperm activities controlled by Ca^{2+}. The Na^{+}-dependency of Ca^{2+} efflux may be used as a good indicator for porcine sperm maturation in the epididymis.

2.2.1.2 Factors involved in motility regulation

2.2.1.2.1 Intracellular pH

Intracellular pH controls the activity of several enzymes of metabolism, adenylate cyclase, and Ca^{2+}-binding protein. Increases in internal pH (pH_i) stimulate the motility and metabolism of ejaculated bull sperm (Vijarayagavhan et al., 1985). It has been proposed that during epididymal transit, the motility of sperm of several mammalian species is controlled by internal pH (Carr et al., 1985). In mammalian sperm, pH_i is strongly dependent on the external pH (pH_e); immediately after sperm dilution, the pH_i equilibrates toward the value of pH_e. In rat, the pH_i of sperm from the cauda is controlled by a sodium-proton exchange system; however, in boar and ram, pH_i is unaffected by the external concentration of sodium or potassium. It has been demonstrated that a membrane anion (HCO_3^- and $SO4^-$) transporter exists in the boar (Dacheux et al., 1990). Motility of epididymal sperm depends strongly on the pH of the dilution medium and less on its ionic composition. For boar sperm, an acidic pH strongly inhibits motility, which is observed only at neutral or alkaline pH (Dacheux et al., 1990). Motility can be affected by an internal pH shift either by a direct effect on dynein ATPase, which is fully active at pH 7.8-8.0, or on adenylate kinase present in the sperm flagella (Schoff et al., 1989). Interestingly, a significant population of ram sperm can still be motile when pH_e reaches extreme values such as pH 6 or 9 in the presence of sodium and potassium, which indicates that the pH control of motility is only a part of a more complex regulation system (Dacheux et al., 1990).

2.2.1.2.2 Carnitine and acetylcarnitine

A relationship between the carnitine concentration in epididymal fluid and sperm motility has been suggested (Hinton and Hernandex, 1985). Carnitine is present in the epididymis at higher concentrations than in other body tissues in all species studies. In spermatozoa, carnitine is rapidly acetylated by acetyltransferase and thus can be indirectly involved in energy supply to the spermatozoa. During epididymal transit, the concentration of carnitine increases in both the fluid and the spermatozoa from corpus to the distal region (Hinton et al., 1979). Sperm membrane permeability to carnitine and the value of the acetylcarnitine/carnitine ratio do not change throughout sperm maturation (Jeulin et al., 1987). During epididymal transit, the rise in the percentage of motile spermatozoa occurs before the concentration of carnitine increases. However, the evolution of the percentage of progressive sperm is always associated with their internal carnitine and acetylcarnitine concentrations. These observations suggest that carnitine is not directly involved in the initiation of sperm motility but may be an important metabolic factor in the high energy requirement of progressive spermatozoa (Dacheux et al., 1990).

2.2.1.2.3 Platelet-activating factor

Platelet-activating factor (PAF), a biologically active phospholipid, is not only a mediator of inflammation and allergy, but has numerous physiological functions, especially those involved in reproduction. The presence of PAF in both epididymal fluid and spermatozoa has been reported in various animal species including rabbits (Kumar et al., 1988), mice (Kuzan et al., 1990), rat (Muguruma and Johnston, 1997), cattle (Parks et al., 1990) and humans (Kuzan et al., 1990; Angle et al., 1991), and PAF increases the motility of human spermatozoa, and facilitates capacitation and the acrosome reaction in the mouse (Sengoku et al., 1992), human (Angle et al., 1993; Krausz et al., 1994), rabbit (Fukuda et al., 1994) and rat (Muguruma and Johnston, 1997). Pretreatment of spermatozoa with PAF has been shown to enhance in vitro fertilization in the mouse and rabbit, as well as zona-free hamster egg penetration by human spermatozoa (see Muguruma and Johnston, 1997). PAF is synthesized via two pathways, termed the remode pathway and the de novo pathway (Snyder, 1995). The major regulatory enzymes for the synthesis of PAF via the de novo pathway have been established in spermatozoa; these include acetyltransferase and cholinephosphotransferase specific for PAF biosynthesis. PAF is converted into the biologically inactive lyso-PAF by hydrolysis of the acetyl group catalyzed by the enzyme PAF-acetylhydrolase (PAF-AH). PAF-AH exists in epididymal fluid which is secreted by epididymal cells, and also in seminal plasma (Letendre et al., 1992; Parks and Hough, 1993). It is postulated that during the storage of sperm in the epididymis, the PAF synthesized by the sperm is rapidly inactivated to lyso-PAF by the PAF-AH present in the epididymal fluid, preventing activation of the sperm. Once the spermatozoa are delivered into the female tract, they are exposed to the fluids in this environment (Muguruma and Johnston, 1997). Although a prolonged exposure of sperm to seminal plasma (which contains the PAF-AH activity) correlated well with the decline in their ability to penetrate zona-free hamster eggs and support flagellar activity (Wolf, 1986), the precise role of PAF and PAF-AH in motility, capacitation and the acrosome reaction is not clear.

In conclusion, the acquisition of fertilizing ability by the spermatozoa in the epididymis is progressive. Studies of sequential sperm modifications during epididymal maturation may provide a good correlation between biochemical modifications and fertilizing capacity.

2.2.2 Seminal plasma and sperm fertilizing ability

Twenty years ago the function of seminal plasma was controversial as is described in the article "Seminal plasma, an unnecessary evil?" by Rodger (1975). The belief that seminal plasma is of minor importance for reproduction was based on the observations that epididymal spermatozoa can be successfully used for insemination and in vitro fertilization in a number of species. During the last decades it has become evident that fertilization is not a "yes-or-no event", but the result of a large number of finely regulated processes. Today there is not doubt that seminal plasma has important regulatory functions in the processes prior to penetration of the spermatozoon into the oocyte.

Generally, the functions of seminal plasma interacting with spermatozoa include (a) providing nutrition for spermatozoa; (b) protecting sperm from damage; (c) regulating sperm motility; (d) regulating sperm capacitation, and (e) playing an important role in gamete recognition and binding. Other effects of seminal plasma indirectly affecting sperm physiology by functioning in the female genital tract include: (a) enhancement of uterine contraction; (b) relaxation of the tubal isthmus, and (c) immune modulation in the uterus (see Waberski, 1995).

Seminal plasma is a mixure of epididymal luminal fluid and the secretions of the various accessory glands (bulbourethral, prostate, seminal vesicles) in varying proportions. The proportion of the seminal plasma derived from the different glands varies widely not only between species, but also between individuals of the same species, and the same individual on different occasions (Setchell, 1993). The important constituents of seminal plasma of domesticated animals have been summarized by Setchell (1993). These seminal plasma components include proteins, fructose, sorbitol, citric acid, ascorbic acid, inositol, ergothioneine, glutamic acid, glyceryl-phosphorylcholine, sodium, potassium, calcium, magnesium, zinc, chloride, bicarbonate, α-mannosidase, and β-N-acetylglucosaminidase. The concentration of fructose from seminal vesicles has often been used as an indicator of the androgen status of the animal (Mann and Lutwak-Mann, 1976) as its secretion is so strictly governed by the level of androgens in the blood. In boar there are remarkably high concentrations of inositol, glycerophosphocholine and ergothionine in seminal plasma. Although the real function of most of these constituents is still not fully understand, there is no doubt that the complex mixture of molecules in the seminal plasma each play an important role at various levels in the fertilization capacity of spermatozoa. Some of these molecules increase the cells' motility: epinephrine and hypotaurine, for example, stimulate both the motility and the in vitro fertilization rates of bovine spermatozoa. Others, like fructose and sorbitol, serve as metabolic substrates. There are also a number of molecules involved in the protection of spermatozoa against oxidation (ergothioneine) and retroviral and bacterial infections (caltrin or seminalplasmin). Prostaglandins present in the seminal plasma of several species suppress the immune response of the female to foreign spermatozoa. Additionally, numbers of proteins are involved in the interaction between the spermatozoa and oocyte and promote the acrosome reaction. Seminal plasma, thus plays an essential role in the fertilization capacity of spermatozoa (see Leblond et al., 1993).

2.2.2.1 *Major seminal proteins and their biological function on spermatozoa*

The protein components of the seminal plasma have important functions in sperm maturation, sperm viability and fertilization in mammals. Some proteins have been isolated and identified as hormones, enzymes, growth factors and growth factor-like proteins (Bedford, 1983; Shivaji et al., 1990; Wempe et al., 1991; Einspanier et al., 1993).

2.2.2.1.1 Sperm motility inhibitor

The seminal plasma of all mammals studied so far have been shown to contain sperm motility inhibitory activity (de Lamirande et al., 1983, 1984; Agrawal and Vanhaperttula, 1987; Betsy, 1988; Gagnon et al., 1991; Iwamoto et al., 1993). This seminal plasma motility inhibitor (SPMI) is specific to the reproductive system but is not species specific, and in the bull, boar, rat, mouse, rabbit and human, SPMI was known to originate from the prostate and seminal vesicles (de Lamirande et al., 1984; Robert and Gagnon, 1996). Its concentration in the two organs differs according to the species studied. In boar, bull and human, SPMI originates exclusively from the seminal vesicles. Like other seminal vesicle proteins, SPMI is diluted 6- to 8-fold upon ejaculation (Iwamoto et al., 1993). Boar SPMI has been purified from boar seminal plasma by Iwamoto et al. (1992). The molecular mass of SPMI under native conditions has been estimated at 50 kDa by molecular sieving, but three polypeptides with molecular masses of 14 kDa, 16 kDa and 18 kDa were observed following polyacrylamide gel electrophoresis (PAGE) in denaturing conditions. Boar SPMI is stable to both a wide range of temperatures and pH. The observations that SPMI effects on motility of demembranated spermatozoa are reversed by Mg.ATP and that SPMI inhibited bull dynein ATPase (the key enzyme responsible for flagellar motility) in a concentration-dependent manner, suggest that this protein blocks the motility of demembranated spermatozoa by interfering with dynein ATPase function (Iwamoto et al., 1992). Purified human SPMI, has also been shown to block the motility of intact human spermatozoa by decreasing sperm curvilinear velocity without affecting linearity (Iwamoto and Gagnon, 1988). In human, immediately after ejaculation, semen spontaneously coagulates into a semi-solid gelatinous mass, which then liquefies within 5-20 min (Amelar, 1962). Semen coagulum appears to be mainly composed of a predominant 52 kDa protein, known as semenogelin. SPMI originates from the seminal vesicles as a very active precursor form of 52 kDa which is degraded by proteases present in prostatic secretions. These proteases transform the SPMI precursor into less active fragments in parallel with semen liquefaction. These observations suggests that these two proteins might be similar. It is interesting that semenogelin has activity on spermatozoa, temporarily immobilizing spermatozoa in coagulated semen. Proper processing of SPMI precursor/semenogelin appears to be essential in order for spermatozoa to acquire progressive motility (Robert and Gagnon, 1996).

2.2.2.1.2 Decapacitation factors

It is becoming increasingly clear that the acrosome reaction (AR) is normally restrained until oocyte ligands bind to sperm receptors. The activation of receptors on human spermatozoa leads to a number of different signal transduction events. The need for binding of appropriate ligands to receptors represents one of the methods to assure the proper timing of the AR. However, agents such as steroids or second messengers may be present in genital tract fluid that can bypass such ligand-receptor requirements and cause a premature AR. Therefore, another mechanism must be available that controls the nonspecific induction of the AR. It is likely that on or more of the decapacitation factors

represents such a mechanism (Drisdel et al., 1995). All mammalian species studied (rabbits, mice, rat, bulls, boars, and humans) so far have seminal decapacitation factors (Roberson et al., 1971; Eng and Oliphant, 1978; Reddy et al., 1978; Fraser, 1984; Amuguruma and Johnston, 1997). These factors have been shown to be glycoproteins, peptide proteinase inhibitors, sterols or lipids (Zaneveld, et al., 1975; Parrish and First, 1993).

In the rabbit, an acrosome stablizing factor (ASF) was identified as a dimeric, 160 kDa glycoprotein that inhibits the rabbit AR in vitro and also prevents in vivo fertilization. ASF is produced mainly by the principle cells of the corpus epididymidis, and first introduced to spermatozoa at a critical point during sperm maturation and may be involved in maintaining the sperm in a noncapacitated state (Renolds et al., 1989).

In human, a high molecular mass antifertility factor (AF-1) from seminal plasma has been purified as a glycoprotein composed of two subunts of 125 kDa and 72 kDa. AF-1 is capable of diminishing the number of sperm binding to intact oocytes and of preventing penetration of the oocyte investments by capacitated mouse spermatozoa. However, AF-1 does not appear to inhibit the acrosome reaction (Audhya et al., 1987). Recently an acrosome reaction-inhibiting glycoprotein from the seminal plasma ultracentrifuged pellet has been purified and partially characterized (Drisdel et al., 1995). This acrosome reaction inhibiting glycoprotein (ARIG) was found to be capable of blocking human sperm exocytosis induced by several signal transduction agonists without altering sperm motility. AGIG inhibits the calcium ionophore-induced acrosome reaction when it is added 30 min prior to ionophore, but it does not inhibit the acrosome reaction when it is added at the start of the capacitation period or after 1h of capacitation. These data suggest that ARIG is removed or inactivated during the capacitation process (Drisdel et al., 1995). Native ARIG (74 kDa) possesses oligosaccharide chains which account for approximately 12% of its molecular mass according to SDS-PAGE analysis. It is suggested that oligosaccharides of zona pellucida induce the acrosome reaction by clustering GalTase on the sperm surface (Macek et al., 1991). Seminal ARIG with N-linked oligosaccharides may bind to GalTase on the sperm surface to prevent clustering of GalTase and the acrosome reaction until they are removed or displaced during transit of the spermatozoon through the female reproductive tract or during oocyte penetration (Drisdel et al., 1995). Recently, a non-protein AR inhibitor, cholesterol in the seminal plasma has been suggested as the major inhibitor preventing sperm from becoming acrosomally responsive to progesterone, an AR inducer the sperm encounter in vivo (Cross, 1996). A proposed mechanism involves the blocking of the efflux of membrane cholesterol and of decreasing the membrane cholesterol/phospholipid ratio.

In bovine, one of the major proteins in seminal plasma, an acid seminal fluid protein (aSFP, 12.9 kDa), has been purified and its amino acid sequence obtained by cDNA cloning (Wempe et al., 1992). The aSFP shares up to 43% amino acid sequence identity with members of the recently described spermadhesin protein family. Porcine spermadhesins are thought to be involved in sperm capacitation and adhesion of sperm to the porcine zona pellucida (Sanz et al., 1993). Indirect immunofluorescence microscopy

revealed that aSFP is not present on epididymal sperm but becomes coated on ejaculated sperm to a thin region on the most apical part of the acrosomal cap. The boar spermadhesins are distributed through the whole acrosome. The average number of aSFP molecules on a single sperm is of the same range as the value reported for individual spermadhesins (12-60 $\times$ 10^6 /ejaculated boar sperm). Unlike boar spermadhesins, the population of sperm-coated aSFP decreased dramatically to (22$\pm$10) $\times$ 10^3 molecules in 1.5 h capacitated spermatozoa and was undetectable following 18 h incubation in capacitation medium. This quantitative release of aSFP from the sperm surface during capacitation indicates that aSFP could serve as a decapacitation factor in the sense defined by Florman and First (1988), that a sperm surface-associated protein whose removal during the in-utero sperm residence enables the exposure of receptors for zona pellucida or other acrosome reaction agonists.

The ultimate reason for the different sperm coating properties of bovine aSFP and boar spermadhesins remains to be established. However, since aSFP is an acidic protein (pI~4.8) (Einspanier et al., 1991) while boar spermadhesins are basic proteins (pI~8.0) (Calvete et al., 1993), their different membrane association behavior could be related to their different surface charge patterns. Alternatively, the different sperm surface binding abilities of aSFP and the boar spermadhesins might be due to structural diversity of their plasma membrane acceptor molecules (Dostalova et al., 1994). These observations suggest that homologous proteins could play different functions in the fertilization process of different mammalian species.

Other decapacitation factors in the seminal plasma have been reported. Among these, caltrin has been studied extensively in various animal species (Coronel et al., 1993). Caltrin, a calcium transport inhibitor protein, has been purified and characterized in bovine and rat (Rufo et al., 1982; Cornel et al 1993). A 54 kDa fraction (caltrin) from rat seminal vesicle content was found to react with antisera to bovine caltrin. Bull caltrin binds specifically over the sperm acrosome and on the tail but not on the posterior portion of the sperm head or on the midpiece. Caltrin of the bull changes from an inhibitor of calcium transport to an enhancer when non-covalently bound anions are removed. In guinea pig caltrins I and II, the transition form inhibitor to enhancer is accomplished by the removal of covalently bound carbohydrate residues. One of the guinea pig caltrins binds only over the acrosome and prevents calcium-induced release of hyaluronidase from the acrosome and the second caltrin binds only to the tail and prevents calcium-induced hyperactivation (Coronel et al., 1990; Agustin and Lardy, 1990). Thus, as described by Coronel et al. (1993), the caltrins appear to represent a group of small basic proteins from seminal vesicles of various species, which when bound to sperm membranes are calcium transport inhibitors. When caltrins are modified, they become enhancers of calcium uptake. This calcium influx triggers the acrosome reaction.

2.2.2.1.3 Capacitation regulation/zona binding factors

Bovine Seminal Proteins (BSPs): In bovine seminal plasma, the major proteins consist of a family of closely related acidic proteins named BSP-A1, BSP-A2, BSP-A3,

and BSP-30-kDa (collectively called BSP proteins). These proteins are the secretory products of the seminal vesicles and constitute the major protein fraction of bovine seminal plasma (20-40 mg/ml). BSP-A1, -A2, and -A3 have molecular masses of 15-16.5 kDa, whereas BSP-30-kDa has a molecular mass of 28-30 kDa. All proteins of this family are glycoproteins with the exception of BSP-A3. Since BSP-A1 and BSP-A2 have an identical amino acid sequence, with their difference residing in the degree of glycosylation, they are considered a single chemical entity named BSP-A1/-A2 (or PDC-109) (Esch et al., 1983). Interestingly, the BSP proteins bind to heparin, a glycosaminoglycan (a known capacitation factor). In bovine species, heparin and other glycosaminoglycans (GAGs) present in the female reproductive tract appear to participate in the process of capacitation (First and Parrish, 1987; Miller and Winer, 1990). The BSP proteins also bind to the surface of spermatozoa upon ejaculation (Manjunath et al., 1994). The binding sites of BSP proteins on the sperm membrane have been identified as choline phospholipids, therefore BSP proteins are also called phosphatidylcholine-binding proteins (Bleau and Manjunath, 1995). Because of their phospholipid binding properties, BSP proteins appear to participate in sperm lipid metabolism and may play an important role in capacitation. The results of Bleau and Manjunath (1995) demonstrate that the BSP proteins can potentiate capacitation of bovine epididymal spermatozoa induced by heparin. The mechanism by which these BSP proteins stimulate the heparin-induced capacitation is unknown, but it is suggested that the sperm-bound BSP proteins interact with heparin-like GAGs present in the female reproductive tract and thereby participate in the modifications of the plasma membrane that occur during capacitation. Thus they may act as regulatory factors of capacitation.

Boar spermadhesins: Porcine spermadhesins function as capacitation regulators (positive) and are involved in the adhesion of sperm to the zona pellucida (Sanz et al., 1993). In the pig, the sensitive region of the acrosomal cap is stabilized and protected by a thick protein coat built up predominantly by the spermadhesins, the major secretory products of the seminal vesicle (Sanz et al., 1993). Spermadhesins in pig (14-18 kDa) belong to a new protein family (Töpfer-Pertersen, 1995). They are called AQN-1, AQN-2, AQN-3 and AWN (Calvete et al., 1993). The other two major porcine seminal proteins, PSP-I and PSP-II were found to have 50%, 53% and 48% amino acid similarities to AWN, AQN-1 and AQN-3 respectively, therefore were thought to belong to the same spermadhesin family (Kwok et al., 1993). Spermadhesins are multifunctional proteins that combine within one molecule different ligand binding abilities to oligosaccharide/zona pellucida (AQN-1, AQN-3 and AWN), glycosaminoglycans (AQN-1, AQN-2, AQN-3, AWN, PSPI and PSPII), and serine proteinase inhibitors (AQN-1 and AWN). This indicates their role in sperm capacitation and sperm-zona interaction. A dual role for spermadhesins in fertilization has been reported by Sanz et al (1993). Interestingly, AQN-1 and AWN possess binding site(s) for both zona pellucida glycoproteins (Sanz et al., 1992a) and for an 8 kDa seminal plasma serine proteinase inhibitor (Sanz et al., 1992b). Proteinase inhibitors are known as classical decapacitation factors. Their binding to the sperm surface appears to be mediated by interaction with the spermadhesins. Those sperm surface-coated heparin-binding proteins are known to play a

pivotal role as extrinsic regulatory factors during sperm capacitation in many mammals (Florman and First, 1988).

Seminal plasma arylsulfatase : Arylsulfatases (AS-A, -B, and -C) are enzymes present in seminal plasma of several domestic mammals (Gadella et al., 1991). They remove sulfate from various glycoconjugates and steroids, molecules that are involved in the stability and permeability of the plasma membrane of spermatozoa (Farooqui and Mandel, 1977). The hydrophilic enzyme AS-A desulfates seminolipid, which is the specific sulfogalactolipid of mammalian sperm and testis. Seminolipid has been purified from spermatozoa of various mammals including boars (Ishizuka et al., 1973; Lingwood et al., 1990). Since desulfated seminolipid promotes the transition state in membrane fusion, the action of AS-A could induce the acrosome reaction. The negatively charged sulfate group of seminolipids can bind proteins via electrostatic interactions. Removal of the negative charge on seminolipid by desulfation could also play a role in capacitation (Gadella et al., 1993). The basic hydrophilic lysosomal enzyme AS-B desulfates glycosaminoglycans, and probably glycoproteins. It has been suggested that sulfated glycosaminoglycans and glycoproteins play an important role in capacitation (Domino et al., 1989; Urch and Hedrick, 1988). Desulfation of these compounds, which are present at the extracellular surface of the plasma membrane, might trigger reorganization of plasma membrane subdomains, a process that could affect the binding of sperm to the zona pellucida (Parrish et al., 1989). The third arylsulfatase, AS-C, is an integral membrane protein and desulfates steroids and sterol sulfates. It is also present in epithelia of the female genital tract and has been proposed as a promoter of sperm capacitation (see Langlais and Robert, 1985). Therefore, seminal plasma AS could function as an in vivo capacitation factor during the time before sperm swim out of the seminal plasma in the female genital tract. If AS-A and AS-B do stimulate sperm capacitation in vivo, they could be negative (deleterious) factors during the storage of boar semen, since sperm would enter the female genital tract already partially capacitated or degraded. It is suggested that washed sperm be mixed with the seminal plasma during insemination so that capacitation occurs at the proper time and place (Gadella et al., 1991).

2.2.3 Sperm reservoir in the female reproductive tract

A great deal of evidence has accumulated to indicate that there is a reservoir of sperm in the mammalian oviduct. Through carefully controlled experiments designed to determine the distribution and motility of sperm in the female tract of the rabbit, Overstreet and colleagues provided evidence for a buildup of living sperm in the caudal isthmus of the oviduct prior to ovulation, with the release of only a few sperm near the time of fertilization (Overstreet and Cooper, 1978; Overstreet et al., 1978). Through surgical ligation at various points along the oviduct, Hunter and colleagues demonstrated that the uterotubal junction (UTJ) and caudal isthmus are the sites of a reservoir of sperm capable of fertilization in pigs, sheep, and cows (Hunter, 1981; Hunter and Nichol, 1983; Hunter and Wilmut, 1984). Suarez (1987) observed high numbers of motile sperm in the caudal isthmus of transilluminated mouse oviducts, while only a few sperm were seen in more cranial regions.

2.2.3.1 Functions of the sperm reservoir

Various functions attributed to the sperm reservoir include: (a) preventing polyspermy; if the reservoir is bypassed by surgical removal of the caudal isthmus, there is an increased incidence of polyspermy in the pig (Hunter and Leglise, 1978), (b) maintaining sperm motility and fertility during estrus, since sperm incubated with oviductal epithelium in vitro remain motile and fertile longer than sperm incubated alone or with other tissues (Pollard et al., 1991; Raychoudhury and Suarez, 1991; Chian and Sirard, 1994), and (c) promoting sperm capacitation shortly before ovulation to release sperm primed for fertilization (Smith and Yanagimachi, 1991; Chian et al ., 1995).

Besides the above mentioned functions, the oviductal isthmus may also play an important role in the removal or alteration of protective proteins from the sperm surface. Einspanier et al. (1995) showed that some major protein components are liberated from the sperm surface in the oviduct. Using a new oviductal tissue culture system, they further indicated that bovine heparin-binding seminal plasma proteins BSP-A1, -A2 and A-3, which are normally bound tightly on ejaculated spermatozoa, are liberated with a higher rate after passing through the isthmic reagion (Einspanier et al., 1995). The removal or exchange of the BSP-A proteins from the sperm surface in the oviduct could be an important event leading to capacitation in the bull.

Various mechanisms have been proposed to account for the buildup of sperm in the UTJ and/or caudal isthmus (see Suarez et al., 1990; Harper, 1994). These include inhibition of sperm motility, obstruction of sperm ascent by mucus, binding of sperm to mucosal epithelium, physical obstruction created by the architecture of mucosal folds, and a narrow lumen further restricted during estrus by increased height of mucosal epithelium or by increased muscular tension.

2.2.3.2 Regulation factors involved

After in vivo insemination, some boar sperm enter the isthmus within 15 min of mating and this process is termed rapid sperm transport. It is generally agreed that rapid sperm transport does not deliver the fertilizing population of sperm to the oviduct. These sperm may however act as local messengers to the upper reproductive tract, influencing the subsequent phases of sustained sperm transport. Alternatively these sperm may sensitize the phagocytic system in the peritoneal cavity which will eventually remove excess sperm following fertilization (see Drobnis and Overstreet, 1992). Sufficient sperm to ensure subsequent fertilization are established in the isthmic reservoir within one hour of mating in nearly all animals (Hunter, 1981). In animals mated at the onset of estrus, the pre-ovulatory period of sperm arrest may last for 36-40 hours (Hunter, 1984). There are several possible regulatory factors involved in sperm retention and release from the reservoir. One such factor seems to be present in the UTJ and lower isthmus, since spermatozoa prefer to attach at these locations after entering the oviduct. Raychoudhury and Suarez (1991) showed that boar spermatozoa were bound to a greater extent in the isthmus than in the other upper parts of the oviduct and that binding was enhanced by the

estrous (pre-ovulatory) levels of steroids. They also showed that spermatozoa were entrapped in the mucous secretion that had been produced by the isthmus explants, and they concluded that this would lead to the creation of a reservoir.

Seminal estrogens, a peculiarity of the boar, improve sperm transport and are an important part of the regulating system leading to fertilization (Claus, 1990). Boar semen is rich in estrogens (up to 11.5 μg/ejaculate) and half of the estrogens are bound on the sperm membranes (Claus et al., 1985). The concentrations of conjugated and unconjugated estrogens are about 300% higher in seminal plasma compared to blood plasma (Claus et al., 1983, 1985). This high concentration of estrogens in the ejaculate increases uterine contractions by stimulating the endometrium to synthesize and release prostaglandin ($PGF_{2\alpha}$) (Claus et al., 1990). Estrogens improve prostaglandin synthesis by stimulating the activity of phospholipase A2 which is regarded as one of the rate-limiting enzymes (Kelly, 1981). Intrauterine infusion of estrogens also demonstrated that a peripheral estrogen increase may superimpose on endogenous estrodial levels and result in the positive feedback leading to the LH surge and ovulation (Claus, 1990). Therefore estrogens in the seminal plasma and on the sperm surface may play important roles in sperm transportation to the oviduct by increasing uterine contraction and in inducing ovulation.

An important factor regulating sperm redistribution in the oviduct is ovulation. Mburu et al. (1996) observed that the pattern of distribution, number and membrane integrity of spermatozoa in the UTJ and isthmus during three estrous stages were related to spontaneous ovulation in sows. This confirms that the lower isthmus is the real reservoir for spermatozoa and the middle and upper isthmus seem to facilitate transport of the spermatozoa towards the ampulla. After ovulation, the oocytes surrounded by their investing layers and follicular fluid are released into the oviductal ampulla. A study in human spermatozoa (Ralt et al., 1994) showed that follicular fluid stimulates chemotactic and chemokinetic activities of the spermatozoa as well as hyperactivation-like motility. It is possible that an 'attractant' (chemotactic active fraction of the follicular fluid) released at ovulation and may play a role in sperm redistribution in both human and in the pig (Ralt et al., 1994; Mburu et al., 1996). In the cow, two small proteins appearing during rupture of the mature follicle at ovulation have been purified (Einspanier et al., 1995). In vitro studies demonstrated that these two 14 kDa hemoglobins are absorbed on sperm membranes at the postacrosomal and mid-piece regions and have stimulatory effects on the mitochondrial activity of spermatozoa (Einspanier et al., 1995). Hunter also discussed a local influence of adrenergic factors in activating sperm in the isthmic storage site (Hunter, 1995).

Another important factor(s) originates from oviductal fluid. Smith and Yanagimachi (1989) reported that the hamster oviduct apparently controls the timing of sperm capacitation and thus synchronizes the state of the spermatozoa with ovulation, thereby maximizing the chances of fertilization. A combination of factors in the oviduct probably optimizes the preparation of spermatozoa for fertilization. Higher calcium concentrations were found in isthmic nonluteal oviductal fluid, which may increase the

occurrence of the acrosome reaction in spermatozoa incubated in isthmic nonluteal oviductal fluid. Increased concentrations of lysophosphatidylcholine were also found in nonluteal ampullary oviductal fluid. This fusogenic lysophospholipid may serve to maximize the number of acrosome-reacted spermatozoa in the ampulla at fertilization. Estrous-associated protein is present in oviductal fluid primarily during the nonluteal stage and at a higher concentration in ampullary-derived fluid than in isthmic fluid. Estrous-associated protein is associated with the sperm membrane, and stimulates increased capacitation and fertilization of bovine spermatozoa (see Grippo et al., 1995).

Sperm characteristics may be a modulating factor in sperm transport from the isthmus to the fertilization site. Two kinds of evidence support this hypothesis. Firstly, results of heterospermic insemination experiments showed that when equal numbers of motile sperm from two boars (two populations) are mixed and used for artificial insemination, one population will usually prevail, fertilizing most of the oocytes (Berger et al., 1996). By using genetic markers in the offspring or labelling populations of sperm with x-irradiation and/or fluorescent markers prior to AI, the results suggest a genetic basis for fertility differences among males (Parrish and Foote, 1985; Berger et al., 1996). Secondly, a significant influence of boar on sperm distribution in the UTJ and the isthmus at the same ovulation stage has been reported (Mburu et al., 1996). The boar which had lower sperm numbers in the oviductal segments, showed a lower post-thaw motility than other boars studied, although there were no significant differences in initiated sperm concentrations among these boars; this suggests that spermatozoa from particular boars may have a short survival time in the female reproductive tract.

The precise mechanism for release of the sperm from the isthmic reservoir is not known. However, there are at least four possible mechanisms that could account for the release of a sufficient number of sperm to fertilize available oocytes: (a) the sperm-binding or immobilizing properties of the mucosal epithelium change at the time of ovulation; (b) capacitation and/or hyperactivation enable sperm to release themselves; (c) the binding of sperm to the epithelial surface is weak enough to allow for a gradual ascent of sperm to the ampulla, and (d) at ovulation, sperm could obtain a "push" toward the ampulla by adovarian beating of cilia lining the lumen, or by peristaltic contractions of the oviductal smooth muscle (see Suarez et al., 1990).

In conclusion, the isthmic environment may serve to facilitate capacitation, and the acrosome reaction, and thus prepare a reservoir or population of sperm available for gradual release to the site of fertilization.

2.2.4 Capacitation

Successful mammalian fertilization is the result of a complex series of interactions between complementary molecules located on the surfaces of the mature oocyte and spermatozoa (Wassarman, 1991). Although this concept was proposed more than 80 years ago (Lillie, 1913), progress in understanding the molecular basis of these events has been slow. An important contribution was that realization that spermatozoa in the testis are

unable to fertilize eggs (Austin, 1952; 1985). As discussed earlier, in many mammalian species, the sperm initially acquire the ability to fertilize eggs during passage through the epididymis (epididymal maturation) (Chang, 1951; 1984; Austin, 1985). However, upon mixing with seminal plasma at ejaculation, spermatozoa subsequently become coated with different factors, some of which prevent the occurrence of premature acrosome reactions and thus arrest their fertilizing capability (Cross, 1993). These "decapacitation" factors are released during sperm residence in the uterus permitting the expression of endogenous or acquired positive regulators of zona pellucida-induced exocytosis of the acrosome contents (Cross, 1993). These sperm plasma membrane remodeling events, together with other changes that prepare sperm to respond adequately to an acrosome reaction inducer, are collectively termed *sperm capacitation.* The changes in spermatozoa during capacitation, including changes in adenylate cyclase/ protein kinase systems, in metabolism, in intracellular ions, in the acrosome, in the nucleus and in the plasma membrane, have been comprehensively reviewed by Yanagimachi (1994).

Parrish and First (1991) have proposed a mechanism for the induction of capacitation by heparin. During epididymal maturation as well as at ejaculation, heparin-binding (like BSP proteins) and decapacitation proteins (like aSFP and/or caltrin) are added to the surface of the spermatozoa. As the sperm travel through the female genital tract they are exposed to heparin-like GAGs that bind to specific sperm proteins. This interaction would result in the release of decapacitation or acrosome-stabilizing factors, triggering the activation of proton channels that may then provoke cytoplasmic alkalinization and ultimately stimulate Ca^{2+} uptake. The rise in Ca^{2+} and pH could activate adenylate cyclase or could inhibit phosphodiesterases, either of which event may increase intracellular cAMP concentration. This increase would then set up a series of events resulting in the activation/inactivation of target cellular proteins through protein kinase pathways.

Other proposed mechanisms involved in the capacitation process include removal of surface-associated components, efflux of membrane cholesterol, and a decrease in the membrane cholesterol/phospholipid ratio. The BSP proteins bind to choline phospholipids of the sperm plasma membrane upon ejaculation and also bind to isolated apoA-1 as well as apoA-1 associated with high-density lipoproteins (HDL). The HDL present in follicular and oviductal fluids has been implicated in sperm capacitation and the AR (Langlais et al., 1988). In view of these observations, Bleau and Manjunath (1995) proposed another mechanism of action for BSP proteins in sperm capacitation. It involves the binding of BSP proteins to the sperm surface upon ejaculation (through a specific interaction with choline phospholipids), where they would mediate the exchange of cholesterol and phospholipids between the sperm membrane and HDL. This may result in a decreased cholesterol/phosolipid ratio leading to capacitation (Desnoyers and Manjunath, 1992).

The lipid changes in the plasma membrane of capacitating spermatozoa has been summarized by Gadella (1995). They include lateral redistribution of lipid, transverse redistribution of phospholipids across the lipid bilayers, changes in the mobility of lipids,

capacitative modifications of the lipid composition by lipoproteins, alterations in lipid metabolism and lipid signalling, and an increase in membrane fusogenicity (Gadella, 1995). Using chlortetracycline (CTC) fluorescence techniques, Tardif and his colleagues (1996) observed the increased phosphorylation of a 42 kDa protein and the decreased phosphorylation of a 32 kDa protein concomitant with porcine sperm capacitation. This suggested that the capacitation of porcine sperm is regulated by a mechanism that includes both protein phosphorylation and dephosphorylation. The lipid signalling mechanism could be involved in these processes since the lipid signalling molecules produced by phospholipases may effect specific protein kinases and phosphatases and intracellular ion levels (Gadella, 1995).

2.2.5 Sperm/oocyte interaction

Once the sperm have completed capacitation and oocytes have completed maturation to the second metaphase, either in the oviduct following ovulation or in vitro, the stage is set for their interaction and completion of fertilization. The fertilization process involves the following sequence of events: sperm penetration of the cumulus mass, sperm attachment to the zona pellucida of the oocyte, binding to the zona, the acrosome reaction, penetration of the zona, fusion of the sperm and oocyte plasma membranes, activation of the oocyte to complete meiosis II, the cortical reaction to block polyspermy, the zona reaction involving hardening the zona in response to cortical granule release, sperm head swelling, sperm chromatin decondensation in synchrony with oocyte chromatin decondensation, deposition of a pronuclear envelope around the sperm chromatin and finally syngamy of the two pronuclei and entry into the first mitotic cell cycle. Only a few of these steps are well studied in domestic species because the tools of in vitro oocyte maturation and fertilization which are necessary for such study, have only recently been developed (Parrish and First, 1993). Fertilization has been characterized most extensively in the mouse model. However the general paradigm of gamete interaction developed from the mouse system is believed to be applicable to other mammalian systems (Burks and Saling, 1992).

2.2.5.1 Cumulus cell penetration

In most mammalian species, sperm approaching the oocyte encounter a substantial layer of cumulus cells embedded in an extracellular matrix rich in hyaluronic acid. This cumulus cell complex forms a barrier that sperm must penetrate before they can approach the oocyte. Studies of hamsters (Yanagimachi, 1994) showed that sperm that are acrosome-reacted before reaching the cumulus remain stuck on the outer edge of the cumulus complex. Only acrosome-intact spermatozoa can penetrate the cumulus complex (Myles and Primakoff, 1997). It is proposed that a testis originated protein, PH-20 plays a required role in penetrating the cumulus and binding spermatozoa to the zona. PH-20 has two related forms with molecular weights of 64 kDa and 56 kDa and is present on the plasma membrane of sperm from different species studied (guinea pig, mouse, monkey and human) (reviewed by Myles and Primakoff, 1997). The hyaluronidase activity of plasma membrane PH-20 is required for sperm to pass through the cumulus layer, since

antibodies directed against mouse PH-20 that inhibit its hyaluronidase activity prevent sperm penetration through the cumulus, whereas antibodies that recognize mouse PH-20 but do not affect hyaluronidase activity have no effect on cumulus penetration (Lin et al., 1994).

Since cumulus oophorus cells are surrounded by a glycosaminoglycan-rich matrix and the zona pellucida is constructed with N-acetyllactosamine-rich glycoproteins, there is the possibility that sperm glycosidases contribute to dispersion of the cumulus cells or to solubilization of the zona after the acrosome reaction (Takada et al., 1994). Boar β-N-acetylhexosaminidase (β-Hex) present in the acrosome as well as in seminal plasma, is active during passage of sperm through cumulus oophorus cells surrounded with glycosaminoglycans (Takada et al., 1994). This cumulus dispersion activity of β-Hex could be inhibited by PUGNAC, a specific inhibitor of β-Hex. In a pig IVF system, addition of PUGNAC to the oocyte maturation medium and fertilization medium barely reduced the fertilization rate in cumulus-free oocytes but reduced the fertilization rate to 70-75% in cumulus-enclosed oocytes (Takada et al., 1994). These observations support the argument that the acrosome reaction occurs at least partially before the binding of sperm to the zona in the pig.

On the other hand, the cumulus may affect penetrating spermatozoa. A study in golden hamsters showed that the mature cumulus positively affects fertilization, whereas the immature cumulus adversely affects penetration (Legendre and Stewart-Savage, 1993). The positive effect of mature cumulus is achieved either by orienting sperm for the acrosome reaction on the zona surface (Drobnis et al., 1988) or by initiating the acrosome reaction before the sperm reach the zona (Boatman and Robbins, 1991).

2.2.5.2 Sperm binding to the zona pellucida

Zona proteins: All mammalian oocytes are surrounded by an acellular extracellular matrix, the zona pellucida (zp). The mouse zp has been well characterized and is known to consist of three sulfated glycoproteins designated ZP1 (2000 kDa), ZP2 (12 kDa) and ZP3 (83 kDa) (Bleil and Wassarman, 1980a, b). ZP proteins are coordinately synthesized in the growing oocyte and secreted to form a filamentous matrix in which dimers of ZP2 and ZP3 are cross-linked by ZP1, giving the structural appearance of 'beads-on-a-string' (Greve et al., 1982; Shimuzu et al., 1983). Of these three glycoproteins, ZP3 is the best characterized. A cDNA encoding mouse ZP3 has been cloned and shown to cross-hybridize with DNA from rat, rabbit, dog, pig, cow and human, suggesting that similar genes are present in these animals (Ringuette et al., 1988). ZP3 is believed to be the primary ligand for the homologous sperm receptor. Evolutionary conservation of ZP3 suggests that the mechanisms of ZP3-mediated sperm activation will likewise be conserved across species.

The porcine oocyte zona pellucida is comprised of three glycoproteins: pZPA, pZPB and pZPC. pZPB is a homologue of mouse ZP1 and pZPC is a homologue of mouse ZP3 (Yurewicz et al., 1987), therefore pZPC is also called pZP3 by some

investigators. The pZP3 displays sperm receptor activity. It is found that the pig ZP1 and ZP3 associate and the resultant high molecular weight heterocomplexes bind with higher affinity to boar sperm membrane-associated zona receptors than does pZP1 alone (Yurewicz and Sacco, 1996). Recently, a 100 kDa recombinant pZP1 has been produced in mammalian cells (Tsubamoto et al., 1996). The activities of the three bovine zp proteins, bZP1, bZP2 and bZP3 in solubilized zp have also been reported (Rahil and Hunter, 1996).

Sperm-zona binding: In the discussion of binding, zp molecules are normally designated as ligands and the corresponding sperm plasma-membrane proteins are termed receptors (Burks and Saling, 1992). The mouse zp glycoprotein involved in mediating binding to sperm plasma membrane protein (s) is ZP3 (Bleil and Wassarman, 1980a, b). When its *O*-linked oligosaccharides are removed chemically, ZP3's ligand activity is abolished, whereas removal of *N*-linked oligosaccharides has no effect on ligand activity (Florman and Wassarman, 1985). Thus, *O*-linked oligosaccharides of ZP3 are thought to mediate sperm binding. Following the acrosome reaction, which is also mediated by ZP3, sperm remain associated with zp, presumably through interaction of the acrosomal matrix with ZP2. In contrast to mouse ZP3 which binds to acrosome-intact sperm, ZP2 binds exclusively to acrosome-reacted sperm heads (Bleil et al., 1988). These two binding events, utilizing first ZP3 and then ZP2 as ligand, have been termed primary and secondary binding, respectively (Wassarman, 1988; Saling, 1989).

It has been reported that acrosomal status of spermatozoa that bind to ZP varies according to species. In pigs, the results of studies on acrosomal status of spermatozoa are controversial. Jones (1990) reported that FITC-labeled pig zona glycoproteins do not bind to intact boar sperm but do bind to the acrosomes of permeabilized spermatozoa. The study of Yonezawa et al. (1995) was consistent with this finding in showing that solubilized porcine zp binds only to the acrosomal region of acrosome-damaged or partially acrosome-reacted spermatozoa and did not bind to acrosome-intact or fully acrosome-reacted spermatozoa. They suggest that the binding site of the ZP is localized mainly in the acrosomal matrix and on the membranous compartments in the acrosome. However, Fazeli et al. (1997) indicate that acrosome-intact boar spermatozoa initiate binding to the pig zp and suggest that a gradient of sperm binding sites exists, decreasing from the outside to the inside of the zp.

Sperm proteins with zp binding properties have been identified in a number of species (see Töpfer-Pertersen et al., 1995). These proteins include enzymes and lectin-like molecules. They can be integral components of the sperm membrane or peripherally associated with the sperm surface. In the mouse, only two sperm proteins have been reported to bind selectively to ZP3, and are considered as candidates for the sperm receptor(s) involved in primary binding. They are p95 (95 kDa protein) and a 56 kDa sperm protein (see Burks and Saling, 1992). p95 has the characteristics of a protein tyrosine kinase (PTK) and binds ZP3 but not ZP2. The activation of p95 in response to ZP3 binding may be an important initial step in signal transduction events necessary for fertilization (Burks and Saling, 1992). In the pig, a boar sperm plasma membrane protein,

Ap_z, has been shown to be involved in the adhesion of uncapacitated and capacitated sperm to the porcine zp (Peterson and Hunt, 1989). Furthermore only the alpha group of ZP3 binds to Ap_z in the intact cell (Peterson et al., 1991). Sperm surface proacrosin may play a role in sperm-zona binding in the pig, but is present in insufficient amounts to allow binding of acrosome intact sperm (Peterson et al., 1991). Recently more attention has been paid to the porcine spermadhesin family. As previously mentioned, spermadhesins are carbohydrate-binding proteins which become sperm surface-associated at ejaculation (Sanz et al., 1993). AWN and AQN-3 bind to the zona pellucida with dissociation constants in the low micromolar range and the carbohydrate recognizing ability of AWN has been determined in the most detail (see Töpfer-Petersen, 1995). The carbohydrate structures (Galβ, GalNAc) in the *O*-linked glycoproteins of zp have been identified as the ligands for the AQN-3 and AWN molecules (Töpfer-Petersen, 1995).

Oviduct-specific glycoproteins (OGP) have been identified in some mammalian species including cow (Wegner and Killian, 1991), pig (Buhi et al., 1993) and human (O'Day-Browman et al., 1996), and they have been shown to associate with the zp and/or perivitelline space (PVS) of oviductal oocytes of these species in vivo. Sakai et al.(1988) were the first to demonstrate that OGP may play a role in sperm binding to the zp by using antibodies against hamster oviductal glycoprotein. Association of OGP with the zp of human oocytes also enhances sperm binding and this enhancement appears to be species-specific (O'Day-Browman et al., 1996). There is other evidence that bovine OGP facilitates sperm capacitation in vitro and significantly increases the fertilizing ability of bovine sperm in IVF (King et al., 1994); also hamster OGP (oviductin) enhances the penetration rate of hamster sperm into hamster ovarian oocytes (Boatman and Magnoni, 1995). These results suggest that there is a factor(s) synthesized and secreted by the oviduct that enhances fertilization.

The sperm-zp interaction is a species specific and complex phenomenon (Yanagimachi, 1994). Although zona proteins exhibit homology between species at the amino acid level, these molecules are highly glycosylated, and the carbohydrate moieties account for major differences between species and impart species specificity to the zona (Wassarman, 1990).

2.2.5.3 Acrosome reaction (AR)

Upon binding to the zona pellucida, a signal transduction pathway is activated leading to the influx of Na^+ and Ca^{2+} and the efflux of H^+ (elevation of pHi), which have been shown to initiate the fusion events of the acrosomal exocytosis (Yanagimachi, 1994). The exocytotic event is mandatory for fertilization since it enables sperm passage through the zp. Furthermore the AR is also essential for fusion with the oocyte: sperm that fail to undergo the AR are unable to fuse even with the plasma membrane of naked oocytes (Yanagimachi, 1994). However, as mentioned previously, sperm that undergo an AR prior to reaching the zp are unable to bind to this matrix and are thus excluded from the fertilization pathway (Saling et al., 1979).

In mouse sperm, it is well established that ZP3 triggers the AR (Bleil and Wassarman, 1983) although the molecular mechanism (s) by which this is mediated is not yet fully understood. It has been suggested that a guanine nucleotide regulatory protein may play a role in ZP3-induction of the AR (Kopf, 1990). It has been demonstrated that G_i protein subunits are present in acrosomal regions in mouse, guinea pig and human spermatozoa, suggesting the participation of a G_i-like protein in mediating the zp-induced AR (see Burk and Saling, 1992). Based on findings that anti-ZP3 antibodies cross-link ZP3 glycopeptides bound to sperm and lead to acrosomal exocytosis, another hypothesis is that the AR is triggered by aggregation of ZP3 receptors in the sperm plasma membrane (Leytonn and Saling, 1989). This proposed model explains the observation that glycopeptides of pronase-digested ZP3 are able to bind to the sperm but are unable to elicit the AR since they would be incapable of cross-linking sperm plasma membrane components (Burk and Saling, 1992).

More recently, Leclerc and Kopf (1995) proposed a working model that incorporates their observations on mouse sperm adenylyl cyclase (AC) activity into the AR mechanisms. Combining together their ideas with a mechanism proposed by Yanagimachi (1990), the events of the AR could be summarized as follows. Capacitated spermatozoa bind to the zp, allowing ZP3 to interact with its specific receptor(s) on the sperm surface; this stimulates the sperm membrane AC, resulting in an increase in cAMP concentration; such an increase in sperm cAMP concentrations can produce Ca^{2+} uptake in different ways; the massive influx of Ca^{2+} inactivates Na^+-K^+ ATPase, resulting in a rapid increase in intracellular Na^+. This causes an efflux of H^+ (through Na^+/H^+ antiport), resulting in a rise in intracellular pH. The Ca^{2+} that has penetrated through the plasma membrane may act with participation of calmodulin on both the plasma membrane and the outer acrosomal membrane. Ca^{2+} facilitates the fusion between these two membranes by binding with anionic phospholipids and inducing both phase transition and phase separation of membrane phospholipids. Ca^{2+} also activates membrane bound phospholipase A2 and phospholipase C. Activated phospholipases attack nearby phospholipids to yield fusogenic lysophospholipids, fatty acids and diacylglycerols. When the membranes are fused with each other, Ca^{2+} enters and H^+ leaves the acrosome matrix. This causes the conversion of proacrosin to enzymatically activate acrosin, which disperses the acrosomal matrix containing various enzymes or lysins. The acrosome-reacted spermatozoa have a limited life span and once acrosome-reacted, the sperm must penetrate through the zona pellucida and fuse with the oocyte without delay (Yanagimachi, 1990).

Progesterone, a steroid hormone secreted by the cells of the cumulus oophorus surrounding the ovulated oocyte, is a putative physiological initiator of the AR. It has been shown to initiate the in vitro AR in human, golden hamster, pig, and mouse spermatozoa (Osman et al., 1989; Meizel et al., 1990; Melendrez et al., 1994; Roldan et al., 1994). Melendrez and Meizel (1995) demonstrated that AR initiation by porcine zona and by progesterone requires Cl^- but involves different receptor/ Cl^- channels, and provide the first evidence that a sperm glycine receptor/ Cl^- channel (GlyR) plays a role in the zona-initiated acrosome reaction. Glycine can initiate the acrosome reaction in vitro, and

is present in the porcine oviduct at estrus, at a concentration higher than the lowest effective acrosome reaction initiation concentration in vitro (Melendrez and Meizel, 1995). However, the in vivo role of glycine is not clear.

2.2.5.4 Sperm-oocyte fusion

Sperm oocyte membrane fusion begins in the equatorial and/or posterior head regions and then continues to occur throughout the length of the tail, so that these regions of the sperm plasma membrane become incorporated into the plasma membrane of the new zygote. Because fusion is limited to acrosome-reacted sperm, the inner acrosomal membrane is the only region of the sperm membrane that does not fuse with the oocyte membrane. Instead, the inner acrosomal membrane becomes incorporated into the oocyte cytoplasm in a membrane vesicle (see Myles and Primakof, 1997). As the sperm plasma membrane is divided into separate domains and membrane proteins are localized to these separate domains, it is probable that more than one sperm surface protein is involved in this series of events.

The mechanism of how sperm fuse with the oocyte is still not known. Studies in the mouse suggest that fertilin, a protein that is localized to the equatorial region of the sperm plasma membrane, is in an appropriate domain to be involved in the initial steps of sperm-oocyte fusion (Myles and Primakoof, 1997). One subunit of fertilin, fertilin α, is shown to play a required role in sperm-oocyte fusion (Yuan et al., 1995), whereas the function of the other subunit, fertilin β, is not clear. Preliminary evidence indicated that in addition to fertilin, at least one other family member, cyritestin may participate in a binding step that precedes sperm fusion (Yuan et al., 1995). These results suggest that sperm-oocyte fusion may be a multistep process and that the disintegrin binding sites of different fertilin family proteins may participate in more than one of these binding sites.

2.2.5.5 Oocyte activation

There is only limited information available on oocyte activation in mammals. Initial responses of the oocyte to sperm activation include changes in membrane permeability, with a rise in pH_i, oscillatory increases in intracellular Ca^{2+}, cortical granule exocytosis and resumption of meiosis (Burks and Saling, 1992). Ca^{2+} is thought to be the primary intracellular signal mediating initiation of development at fertilization. There is evidence to suggest that in some species the Ca^{2+} rise is mediated by a guanine-nucleotide binding protein, subsequent production of inositol, 1,4,5-triphosphate and release of Ca^{2+} from internal stores (Jaffe, 1990). Since it has been reported recently that sperm extracts are also capable or reproducing the effects of intact sperm in oocyte activation, an alternative explanation is that, upon fusion, sperm introduce a factor into the oocyte that directly stimulates Ca^{2+} release (Swann, 1990). However, mammalian oocytes can be activated by many external stimuli such as Ca^{2+} ionophores, ethanol and electric voltage and all these events appear to alter intracellular Ca^{2+}.

2.2.5.5.1 Cortical granule exocytosis and zona reaction

The block to polyspermy is the crucial early event during oocyte activation. The block to polyspermy occurs at the zona pellucida, the oocyte plasma membrane or both, and the relative contribution of the zona reaction and the plasma membrane block to polyspermy differs among species (Wolf, 1981). The zona block to polyspermy results from fusion of cortical granules (CGs) in the cytoplasm with the oocyte plasma membrane and exocytotic dumping of the contents of these granules into the perivitelline space. Cortical granules are small, dense organelles found near the plasma membrane of mature unfertilized oocytes (see Parrish and First, 1993) and immature mammalian oocytes have a higher incidence of polyspermic fertilization than oocytes matured in vivo or in vitro (see Cherr and Ducibella, 1990). Fertilization of immature pig oocytes has been reported to result in greater polyspermy rates (Polge and Dziuk, 1965). After administration of hCG and precocious ovulation of immature oocytes in the pig, about 90% polyspermic fertilization occurs (Hunter et al., 1976). The development of competence to undergo the cortical reaction has been studied in mice with the calcium ionophore A23187. Both in vivo and in vitro studies indicate that the competence arises between Metaphase I and II about 10h post-hCG and just prior to ovulation (Ducibella et al., 1989).

Centrifugal migration of CGs in farm animal oocytes appears to be a common feature during the late stage of oocyte maturation (Cran et al., 1980; Cran and Cheng, 1985). It has been reported that there are variations and delays in the time of CG spread in bovine oocytes matured in vitro compared with those matured in vivo (Hyttel et al., 1989). In pig oocytes, the distribution of CGs during in vivo maturation in relation to hCG administration is well documented, and the migration that takes place between 20 and 30 h post hCG corresponds to the time of the completion of germinal vesicle breakdown and formation of the first metaphase plate (Cran and Cheng, 1985; Yoshida et al., 1993).

In mammals, CG release appears to require at least some specificity in sperm-oocyte interactions, because hamster spermatozoa that are frozen/thawed or capacitated and have not undergone the normal process of penetration through the zona pellicida do not elicit a cortical reaction in zona-free eggs (Gwatkin et al., 1976) and heterologous sperm induced a delayed and incomplete cortical reaction in zona-free hamster eggs (Fukuda and Chang, 1978). These data suggest that a signal from homologous sperm is required for a complete cortical reaction.

2.2.5.5.2 Possible mechanism of the zona block

The zona reaction involves modification of zona pellucida sperm receptor molecules, causing reduced sperm binding and penetration, which is associated with exocytosis of cortical granule molecules. The mechanism of the zona reaction has been reviewed by Cherr and Ducibella (1990). They propose that fertilization results in an increase in oocyte inositol 1,4,5-trisphosphate (IP_3) by virtue of either a sperm-egg

receptor interaction or actual membrane fusion of the two gametes; IP_3 initiates the release of intracellular free calcium in a wave-like fashion from the point of fertilization; calcium activates CG secretion which occurs by fusion with the oocyte plasma membrane; the cortical granule contents cause changes in the zp, including reduced sperm binding, the ZP2-to ZP2f conversion, and reduced levels of acrosome reaction-inducing activity in solubilized ZP (Cherr and Ducibella, 1990). The increased insolubility ('Hardening') of the zp and inactivation of its sperm binding activity produce a block to polyspermy (Wassarman, 1988). It is found that concomitant with hardening of the zp, ZP2 is converted to ZP2f through the action of a protease that appears to be released from the oocyte during the cortical reaction (Moller and Wassarman, 1991). A study in bovine zona hardening revealed that one of the three bovine zona proteins, bZPA, has thiol residues and this component of bZPA cleaves at fertilization. During fertilization cysteine residues of bZPA of the unfertilized oocyte are oxidized to cystine, forming a more rigid structure and this structural change may be responsible for the zona hardening (Nakano et al., 1996). The role of cortical granules in early fertilization and the nature of their contents have yet to be fully solved. It is possible that the changes in both bovine and murine zp are under the same enzymatic regulatory mechanism.

2.2.5.5.3 The plasma membrane block to polyspermy

Austin and Braden (1956) first suggested the existence of a membrane block to polyspermy. The plasma membrane block is present in some mammals and certainly in invertebrates (see Parrish and First, 1993). In the rabbit, the major block is at the egg plasma membrane since 10 to 20 sperm in the perivitelline space are not uncommon (Hunter et al., 1976). In the cow, pig and sheep, the major block to polyspermy is at the level of the zona (Parrish and First., 1993). A relatively incomplete plasma membrane block to polyspermy has been observed in many species, including hamster (Stewart-Savage and Bavister, 1988), and human (Sengoku et al., 1995). Although definitive mechanisms regulating the oocyte plasma membrane block have not been completely identified, alterations in membrane fluid and the lateral diffusion of membrane constituents may be involved (Wolf and Ziomek, 1983). The masking or disappearance of putative sperm receptor sites and the fusion property of the plasma membrane are likely to be involved (Blobel et al., 1992). Another possible mechanism could be the presence of cortical granule exudate on the cell surface and its contact with sperm in the perivitelline space. The oocyte plasma membrane could be altered by the insertion of cortical granule membrane components, resulting in a new mosaic membrane which could be refractory to spermatozoa (Cherr et al., 1988).

2.3 CURRENT STATUS OF PIG IN VITRO FERTILIZATION

The history of porcine oocyte in vitro maturation and fertilization only extends back about 20 years. In 1974, Motlik and Fulka first reported in vivo fertilization of pig oocytes that had been matured in vitro. Two years later Baker and Polge (1976) summarized experiments on in vitro and in vivo fertilization of porcine and bovine

oocytes and predicted that if in vitro maturation and fertilization (IVM-IVF) techniques could be developed in domestic animals, a significant portion of the vast number of follicular oocytes could be used instead of them going atretic in the ovaries. Important steps toward realizing this prediction were made by Iritani et al. (1978) who reported the first successful IVM-IVF in pig using spermatozoa incubated in isolated female genital tracts. Later, Cheng (1985) established an in vitro sperm capacitation procedure and obtained piglets from oocytes matured in vivo and fertilized in vitro. Mattioli and his colleagues first reported offspring obtained with IVM-IVF procedures in the pig (Mattioli et al., 1989). Successful pregnancies and piglets born from in vitro matured and fertilized pig oocytes were also reported by Rath (1992) and Yoshida et al. (1993b). However the pregnancy rate and the litter size obtained from IVM-IVF oocytes are much lower compared to in vivo results. In recent years, work in porcine oocyte in vitro maturation and fertilization has been reviewed by several experts in this research area who summarized the progress and the problems encountered in pig IVM-IVF from different points of view; in regard to the relationships between oocyte maturation and male pronuclear formation (Moor et al., 1990), polyspermy (Hunter, 1990, 1991), methodology for in vitro fertilization (Parrish, 1991), factors for the improvement of porcine IVF (Niwa, 1993), maturation of porcine gametes and their interaction (Sirard et al., 1993), and experimental strategies to improve porcine IVM-IVF (Nagai, 1994, 1996). As they pointed out, the limitations of in vitro production of porcine embryos and of in vitro maturation protocols are mainly related to high levels of variation between boars and ejaculates, a high incidence of polyspermy and a low rate of male pronucleus formation after IVF. These continuing problems encountered in vitro are mainly due to the lack of knowledge of the corresponding in vivo fertilization events. Thus the replication of in vivo maturation and fertilization environments will improve porcine IVM-IVF procedures and lead to an increase in the viable embryo production rate. The optimization of oocyte IVM-IVF methods will, in turn, give us a better understanding of the in vivo maturation and fertilization process.

2.3.1 Limiting factors from the male

In porcine IVM-IVF experiments, its is common to observe large variations in semen quality among individual boars and even variations between ejaculates from the same boar (Sirard et al., 1993). A significant boar effect has been shown when fresh ejaculated semen was used to test the capacity of spermatozoa (Nagai, 1996), to fertilize in vivo matured pig oocytes (Yoshida, 1987), to penetrate germinal vesicle stage pig oocytes (Martinez et al., 1993) and to fertilize in vitro matured pig oocytes (Sirard et al., 1993; Rath and Niemann, 1997). The boar effect was also observed when frozen-thawed boar sperm were used to inseminate in vitro matured pig oocytes (Wang et al., 1991; Rath and Niemann, 1997). A better understanding of these variations between ejaculations and between boars will help us to standardize the semen preparation procedure and reduce the variations in IVM-IVF outcomes.

2.3.1.1 Standardization of sperm preparation protocol for IVF

Standardization of semen collection methods seems a necessary step to obtain stable IVF results. Einarsson (1971) conducted experiments comparing the composition of semen fractions and whole semen from a numbers of boars, in which the ejaculates were split into 7 fractions; fractions I, II, III and IV were sperm-rich fractions and V, VI and VII were sperm-free fractions. Besides changing sperm concentration in different fractions, there were also differences in osmotic pressure, total protein content, calcium, potassium, magnesium, glutamic-oxaloacetic transaminase (GOT) and alkaline phosphatase (Einarsson, 1971). The characteristics of fractions within the sperm-rich part of an ejaculate may indicate the different sperm quality in each fraction. Therefore the routine collection of what generally called the 'sperm-rich fraction' may not be specific enough to avoid the variation among different sperm-rich fractions in the in vitro fertilization experiments.

Sperm from different sources can result in variations in porcine IVF (see reviews by Sirard et al., 1993; Niwa, 1993). Rath and Niemann (1997) were the first to compare the in vitro fertilizing capacity of fresh or frozen-thawed ejaculates or of frozen-thawed epididymal semen obtained from identical boars. The frozen-thawed epididymal semen samples showed motility rates (72%) similar to those of fresh semen (76%), however motility in frozen-thawed ejaculated semen was decreased (40%). Higher rates of pronuclear formation were found with frozen-thawed epididymal semen and the better fertilizing capacity of frozen-thawed epididymal semen was confirmed by the cleavage rates and embryo development. They suggested that epididymal semen is superior for IVF because the standard freezing protocol leads to high motility and fertilization rates after thawing and consistent IVF rates with minimal variability have been obtained (Rath and Niemann, 1997).

The quality of frozen semen is also influenced by the freezing procedures used. Variations in cooling velocity, thawing velocity and cryoprotectant concentration may greatly influence the survival of spermatozoa after thawing (Sirard et al., 1993). Different glycerol contrations, separation procedures and sperm concentrations were compared by Zheng et al. (1992). It was shown that the glycerol concentration did not influence the normal apical ridge rate after thawing, but the separation procedure did. Among the three sperm separation methods (swim up, upper fraction and percoll), the upper fraction procedure seemed to be the most effective method with respect to oocyte penetration (Zheng et al., 1992). By using new percoll separation procedures, a similar penetration rate for frozen-thawed spermatozoa compared to the fresh spermatozoa was obtained when using similar sperm concentrations (Zheng et al., 1992). These results indicate that boar spermatozoa are very heterogeneous; however, the proper separation procedures can minimize this variation.

Different sperm concentrations used in pig IVF have been reported from different laboratories. Rath (1992) plotted the relationship between sperm concentrations reported by different groups and the polyspermy rates they obtained, and showed no significant

correlations between these two characters (r=0.111). However, when the concentrations were converted to sperm/oocyte ratio, they were highly correlated to the polyspermy rates (r=0.959). Therefore, the use of sperm/oocyte ratio in the IVF experiments will not only improve the standardization of procedures, but also make IVF results from different groups more comparable.

2.3.2 In vitro capacitation of porcine spermatozoa

Successful in vitro capacitation of mammalian sperm has made it possible to study physiological mechanisms and factors causing capacitation in vivo. In the boar, in vitro methods of capacitation induction have not been fully established, although involvement of sperm concentration (Pavlok, 1981; Nagai et al., 1984), temperature and pH (Cheng, 1985), and Ca^{2+} (Toyoda, 1985) have been suggested. In vitro-induced sperm capacitation initiates a sequence of changes in the whole sperm population which are essentially irreversible under the conditions presently used for IVF. The consequence is that there is a finite window of time post capacitation when oocytes can be fertilized by this population (Nagai, 1996). It has been shown that capacitated porcine spermatozoa are capable of inseminating oocytes in vitro within 2.5 h of their exposure to the oocytes (Nagai et al., 1993) and they could maintain fertilizing capacity for at least an additional 2 h (Mori et al., 1995). Coincidentally, 2.5 h is approximately the time they need to penetrate the zona pellucida and fuse with the oocyte. Spermatozoa that lose their motility within 2.5 h following capacitation may not have sufficient time to penetrate oocytes (Nagai, 1996).

These observations raise a question: Are all the sperm undergoing capacitation at the same time or do some of them get capacitated faster and some more slowly? Recently there has been evidence that capacitation is a positive process that involves alterations in dynamic sperm plasma membrane functions. Within the whole sperm population, individuals respond to capacitating conditions at widely different rates (Harrison et al., 1996). Evidence for this has been obtained by the application of flow cytometry to living sperm suspensions to detect changes in individual cells, which enables the observation of trends within the overall population in response to the 'capacitating' treatment (Harrison et al., 1993). This technique involves use of different sorts of fluorescent probes, fluo-3 to detect changes in intracellular calcium levels (Harrison et al., 1993), fluorescein-conjugated lectins to detect changes in surface glycoproteins (Ashworth et al., 1995) and merocyanine to detect changes in plasma membrane lipid architecture (Harrison et al., 1996). Bicarbonate specifically induces major alterations in these parameters within the sperm population, and enhances considerably the ability to bind solubilized zona proteins to the sperm head plasma membrane (Harrison et al., 1996). Bicarbonate has been shown to be a key effector of capacitation and fertilization in vitro (Suzuke et al., 1994; Harrison, 1996). It is suggested that the sperm population consists of two broad groups, those sperm that respond rapidly to bicarbonate and those sperm that respond at slower rates (Harrison et al., 1996). A recent study of the exposure of specific zona-binding sites on the sperm surface under capacitating conditions also showed the presence of two sub-populations of spermatozoa with different response speeds (Harrson et al., 1996).

onsidering sperm in vivo capacitation in the female oviduct, the release of spermatozoa from the isthmic reservoir in response to ovarian signaling must be coupled with the need for a sufficient supply of capacitated sperm throughout the period of oocyte release at ovulation (2 to 3 h) (Hunter, 1995), and the sperm population response to full capacitating conditions should be diverse rather than rapid (Harrison et al., 1996). This supports the fertilization 'window' theory proposed by Nagai (1996). Therefore the slowly responding sub-population might represent the spermatozoa which would be more likely to fertilize oocytes in vivo.

Semen samples from different ejaculates or from different boars may therefore consist of sub-populations of sperm with different capacitation speeds, which could account for the large variations observed in IVF results between ejaculations and between boars.

2.3.2.1 Commonly used sperm capacitation methods

To obtain consistently high fertilization rates in vitro, use of standardized frozen semen with proven fertilizing ability in vitro would be a considerable advantage. When frozen-thawed epididymal and ejaculated spermatozoa were preincubated for 4 h in the modified TCM199 supplemented with 2 mM caffeine at pH 7.8, only epididymal spermatozoa penetrate oocytes in BO solution (Nagai et al., 1988). The first successful in vitro fertilization of in vivo matured pig oocytes using ejaculated spermatozoa was reported by Cheng (1985). The method includes storing semen at 20°C for 16 h, washing spermatozoa with saline three times and preincubation of spermatozoa (2×10^8 cell/ml) for 4-5 h at 37 °C in TCM199 (pH7.8) supplemented with glucose, calcium lactate, sodium pyruvate, dibekacin sulfate (antibiotic) and 12% fetal calf serum for inducing sperm capacitation, and fertilizing oocytes with $0.1\text{-}1\times10^6$ sperm/ml at 39°C in the TCM199 (pH 7.4) supplemented with 2 mM caffeine /L. This procedure is also effective for fertilization of in vitro matured oocytes (Mattioli et al., 1988a, b). The same method with minor modifications has been used by different laboratories.

Alternative methods for inducing capacitation of ejaculated spermatozoa were reported by Hamano and Toyoda (1986), who observed that sperm concentration during preincubation is an important factor, as was observed in epididymal spermatozoa (Nagai et al., 1984). When ejaculated spermatozoa were preincubated for 4 h in mKRB solution at a concentration of 40×10^8 cell/ml and used to inseminate matured oocytes at 5×10^5 sperm/ml in mKRB with 2 mM caffeine/ml, a significantly higher proportion (100%) of oocytes was penetrated than after preincubation at $2.5\text{-}10\times10^5$ sperm/ml (25-57%) (Hamano and Toyoda, 1986).

The first successful in vitro fertilization of pig oocytes matured in vivo and in vitro with frozen-thawed spermatozoa (Nagai, et al., 1988) was performed using almost the same procedures as those reported by Cheng (1985). The results showed that when oocytes were inseminated with frozen-thawed epididymal spermatozoa in modified Tyrode' solution (Brackett and Oliphant, 1975) containing 10 mg BSA/ml and 2 mM

caffeine/ml, penetration was observed; however, none of the oocytes inseminated with frozen-thawed ejaculated spermatozoa were penetrated (Nagai et al., 1988). Wang et al. (1991) successfully fertilized in vitro matured pig oocytes with frozen-thawed ejaculated semen. The methods used were essentially the same as described by Cheng (1985) except that the medium (TCM199) was supplemented with 10% FCS rather than 12% FCS, and with penicillin and streptomycin rather than dibekacin. These results suggest that for successful fertilization of in vitro matured oocytes with frozen-thawed ejaculate boar semen, (a) the preincubation step should be omitted from the frozen-thawed sperm treatment to avoid the great loss of sperm motility; (b) a higher concentration of spermatozoa 25-100$\times 10^6$/ml) needs to be added in the fertilization medium; (c) heparin inhibits the efficacy of caffeine to promote sperm penetration of oocytes, which is different from the heparin effect in cattle; (d) cumulus cells of matured oocytes support sperm capacitation; and (e) the use of sperm selection techniques can increase the penetration rates to the level seen with fresh sperm and reduce the incidence of polyspermy at the same time (Wang et al., 1991; Zheng et al., 1992).

2.3.3 Reducing polyspermic penetration of oocytes in vitro

A high incidence of polyspermy is still the major problem during fertilization of pig oocytes in vitro. Under normal physiological conditions, fertilization in the pig takes place shortly after ovulation and is monospermic (Hunter, 1972). Polyspermy will generally only occur when abnormal conditions are imposed upon the oocyte (see Cran and Cheng, 1986). In contrast to normal in vivo fertilization, a high polyspermy rate was observed by many investigators in vitro (see reviews by Sirard et al., 1993; Niwa, 1993). The major polyspermy block is at the zona level in the pig as described earlier. It is suggested that polyspermy in vitro may be due to a delay in the cortical reaction or an insufficient cortical reaction rate (Sathananthan and Trounson, 1982). Cran and Cheng (1986) observed that the contents of the granules remained in situ, lying immediately above the plasma membrane, whereas at a comparable stage following in vivo fertilization, dispersal had taken place. It seems likely that, following IVF in the pig, vital constituents of the cortical granules remain unavailable for interaction with the zona pellucida. If this is the case, the zona hardening process is probably retarded.

Ca^{2+} plays a key role in the cortical reaction (Guraya, 1983). It has been showed that the availability of calcium is a factor involved not only in exocytosis but also in subsequent reactions of the cortical granules with oocyte investments (Cran and Cheng, 1986). However, artificial activation of oocytes and the resulting Ca^{2+} release could not induce sufficient exocytosis of cortical granules to prevent polyspermy (Funahashi et al., 1993a). Nagai and Moor (1990) suggested that macromolecules secreted from pig oviductal epithelial cells may reduce the incidence of polyspermy in pig oocytes fertilized in vitro by means of some interaction with fertilizing spermatozoa. It is known that follicular fluid will promote capacitation and the acrosome reaction of spermatozoa in vitro more effectively than does oviductal fluid in many species (see Funahashi and Day, 1993). Again, as discussed earlier, follicular fluid as well as oviductal secretions may affect sperm penetration and the incidence of polyspermy in the pig in vitro. Results of

Zheng and Sirard (1992) and Funahashi and Day (1993a) supported this hypothesis by providing evidence that follicular fluid or follicle explants added to the medium reduced polyspermy rates. It is suggested by these two groups that BSA, but not FCS has beneficial effects on blocking polyspermic penetration of pig oocytes in vitro (Zheng and Sirard, 1992; Funahashi and Day, 1993b). Reducing sperm:oocyte ratio in the fertilization medium also proved to be an effective method for reducing polyspermy in pig oocytes (Rath, 1992). In our laboratory, high penetration rates (mean 100%), high polyspermy rates (mean 100%) and high sperm number per penetrated oocyte (mean 8.5 sperm/oocyte) have been repeatedly observed when a sperm:oocyte ratio of 50,000:1 was used in IVF with semen collected from some fertile boars. In a heavily polyspermic oocyte, over 20 sperm heads at different decondensation stages could be seen and some such oocytes have numerous sperm attached to their plasma membrane (see Figures 2-L, 2-M and 2-N). When a sperm:oocyte ratio 3000:1 was used for IVF, penetration rates were still high (mean 90%), but polyspermy rates were greatly reduced (mean 30%) and more monospermic oocytes were observed (see Figure 2-I, 2-J and 2-K) (Xu, unpublished data).

2.3.4 Improving male pronuclear formation in IVM oocytes

It is clear that sperm decondensation during fertilization requires a reversal of at least some of the changes undergone by sperm nuclei during spermiogenesis and epididymal maturation (see Perreault, 1990). Mammalian spermatid nuclei become physically stable and biochemically inert following removal of somatic histones and their replacement, first by transitional nucleoproteins and finally by protamines (Poccia, 1986; Hecht, 1989). As a result, the highly condensed chromatin of mature spermatids is resistant to disruption by physical means such as sonication (Perreault, 1990). During subsequent epididymal transit, cysteine sulfhydryls in the sperm protamine are oxidized to disulfides, and the mature sperm nuclei become resistant to chemical disruption as well (Zirkin et al., 1985). This unique packaging apparently serves to protect the chromatin and maintain it in a transcriptionally inactive form during its storage and during transport through the female reproductive tract.

During fertilization, the incorporation of the sperm nucleus into the oocyte cytoplasm triggers a reaction between cytoplasmic factors and sperm chromatin resulting in molecular changes, including breakdown of sperm nuclear membrane, cleavage of disulfide bonds and partial enzymatic degradation of nuclear proteins, and release of chromatin-associated protamines and their immediate replacement by oocyte-derived histones (Zirkin et al., 1989).

2.3.4.1 Oocyte controlled male pronuclear formation

In vivo and in vitro studies have demonstrated that sperm nuclear decondensation is controlled by the oocyte and oocyte maturational state, although there is possibility that sperm-associated proteinase may be involved (Usui and Yanagimachi, 1976; Perreault et al., 1984; Zirkin et al., 1985). It has been found that immature, germinal vesicle intact

oocytes do not support decondensation of fertilizing or micro-injected sperm nuclei, but they acquire this ability subsequent to GV breakdown (Niwa and Chang, 1975; Usui and Yanagimachi, 1976; Perreault et al., 1984). Sperm decondensing activity appears to be maximal in mature, metaphase II oocytes. This capability of oocytes to decondense a sperm nucleus is lost again shortly after fertilization and sperm nuclei introduced at the pronuclear stage do not decondense (Usui and Yanagimachi, 1976).

The oocyte cytoplasmic factor that controls male pronuclear formation has been defined as the Male Pronucleus Growth factor (MPGF) by Thibault and Gerard (1973). A likely candidate for MPGF is glutathione (γ-glutamylcysteinylglycine; GSH). Glutathione is a major intracellular free thiol that has important biological functions during cellular proliferation, amino acid transport, synthesis of protein and DNA, and reduction of disulfides and other chemicals; it also protects cells against oxidation (Kosower and Kosower, 1978; Meister and Anderson, 1983). Mature oocytes contained relatively high levels of GSH (1.8 pmol/oocyte) compare to those found in GV oocytes (0.9pmol/oocyte) (Perreault, 1990). In hamster oocytes, glutathione synthesis inhibitor buthionine sulfoximine (BSO) has no effect on the already high GSH levels in mature, metaphase II oocytes; however it reduces GSH concentrations significantly when oocytes are cultured with BSO shortly after GVBD, suggesting that GSH synthesis is occurring during oocyte maturation and that GSH synthesis and degradation rates are low in metaphase II oocytes (Perreault et al., 1988). Yoshida (1993) further indicated that in the pig, GSH synthesis during initial and mid phases of oocyte maturation is related to the acquisition of sperm nuclear decondensing ability of pig oocytes. Glutathione is regenerated continually in order to maintain protamine sulfhydryls in the reduced state long enough for the decondensation factors to gain access to the sperm chromatin (Perreault, 1990).

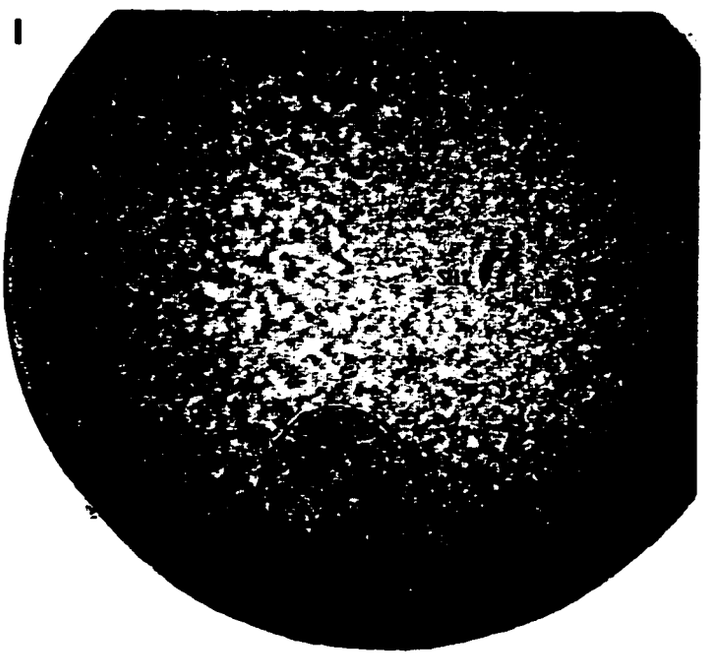
As oocyte GSH levels decline after fertilization, they may act as a limiting factor controlling sperm nuclear decondensation within a critical window of time. In some species (hamster, human, mouse and monkey), sperm nuclei decondense fast (< 60 min); however in other species (rat, bull, pig), the sperm nuclei appear to be more stable and undergo decondensation and male pronuclear formation slowly (up to 6 h) (see Perreault, 1990; Yoshida, 1993). Perreault (1990) summarized the interspecies differences in sperm protamines and the timing of sperm nuclear decondensation in hamster oocytes, and proposed that the presumptive determinant of decondensation timing in these heterologous species was the type of protamine present in the sperm nucleus. A first class of protamine, protamine 1 or protamine P1, has been found in all mammalian spermatozoa and a second class of protamine, protamine 2 or protamine P2, has been described in humans, mice, hamsters and stallions (see Rousseaux and Rousseaux-Prevost, 1995). The sperm nucleus decondenses completely in less than 1 h in species that express protamine P2 (Perreault et al., 1988). There are six cysteines in protamine P1 and five cysteines in protamine P2 (Perreault et al., 1988). The cross linking is tight in protamine P1 since both inter- and intra-protamine cross-links are formed, however the cross-linking could be less tight in protamine P2 due to the lower number of cysteines (Perreault et al., 1988; Balhorn et al., 1991).

Figure 2-3 I-J Photomicrographs of in vitro matured and fertilized porcine oocytes showing asynchrony of female and male pronuclear formation.

I: A monospermic penetrated oocyte with a full size female pronucleus (FPN) excluded 2nd polar body (PB) and a condensed sperm head (SH1) with the tail detached.

J: A monospermic penetrated oocyte with a completely formed female pronucleus (FPN) and a swelling sperm head (SH2) and a detached tail.

I



J

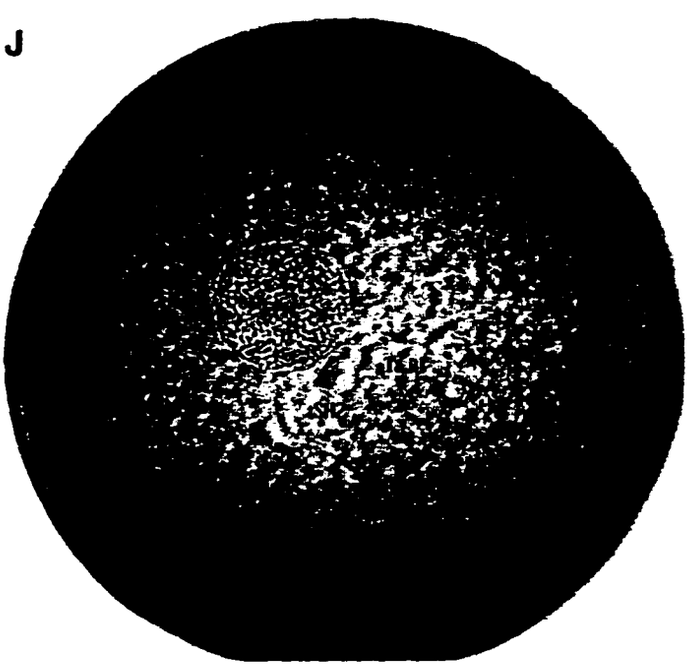


Figure 2-4 K Photomicrograph showing an in vitro matured and fertilized porcine oocyte with both full sized male and female pronuclei (MPN & FPN) after 14 h incubation with spermatozoa (400×).

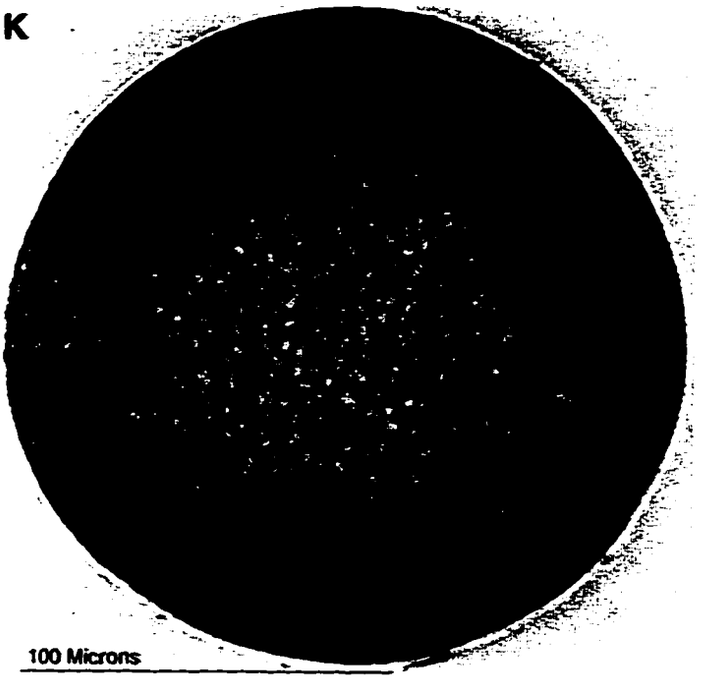
Figure 2-5 L-N Photomicrographs showing polyspermic penetration of IVM-IVF porcine oocytes when a high sperm:oocyte ratio is used for fertilization (400×).

L: A polyspermic penetrated porcine oocyte with over 15 sperm penetrated. Most sperm heads are condensed (SH1) or just starting to decondense (SH2) and no male pronuclues can be seen.

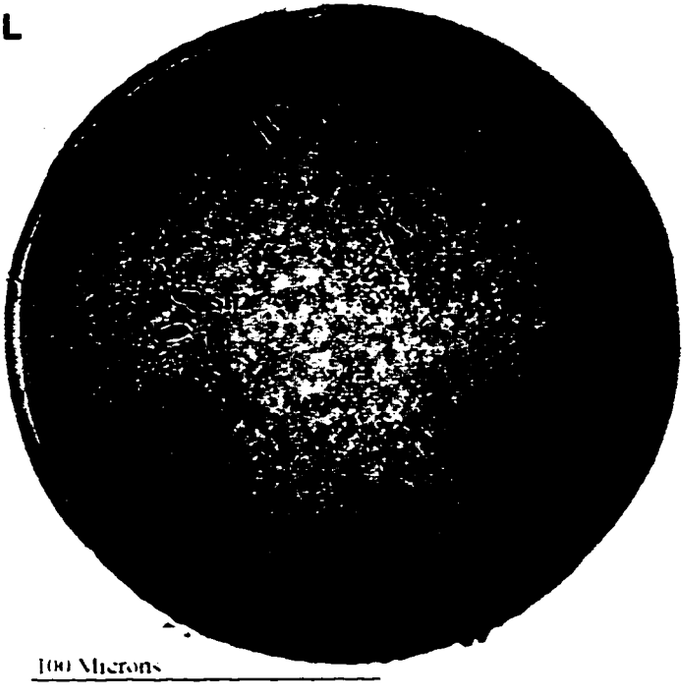
M: A polyspermic penetrated oocyte with the female pronucleus (FPN), decondensing sperm heads (SH2) and several male pronuclei (MPN) visible.

N: A heavily polyspermic penetrated oocyte with several sperm heads at different status of decondensation and many sperm attached to the plasma membrane.

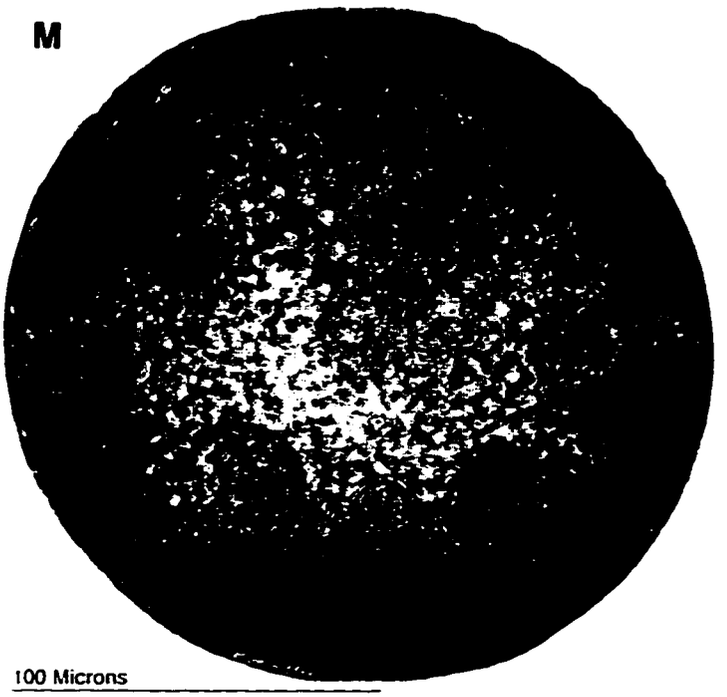
K



L

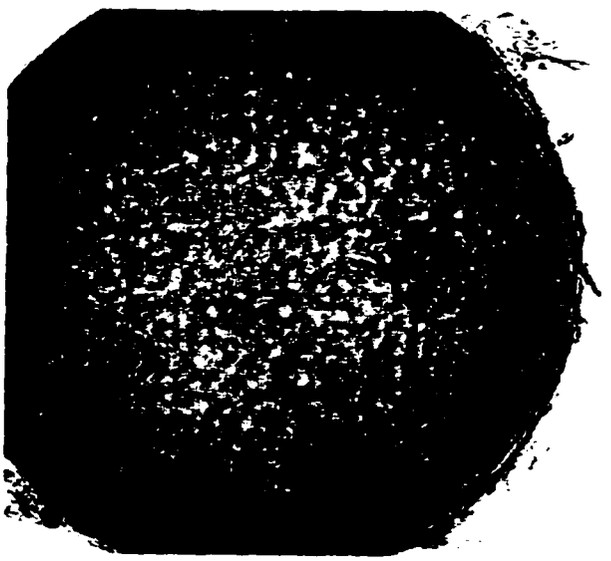


M



100 Microns

N



2.3.4.2 Male pronuclear formation in IVM pig oocytes

Low rates of male pronucleus formation in pig oocytes matured in different culture media and fertilized in vitro, and a high level of asynchronous development of male and female pronuclei have been reported (Iritani et al., 1978; Nagai et al., 1984; Mattioli et al., 1988a; Yoshida et al., 1990, 1992a, b). Male pronuclear formation in vivo can be observed in the cytoplasm as early as 6 h after mating (Hunter, 1972), but it is usually delayed by at least 3 h in porcine ooocytes matured and fertilized in vitro (Ocampo et al., 1994). Figures 2-I and 2-J show the delayed decondensation of sperm nucleus and asynchronous development of pronuclei. An insufficient male pronucleus growth promoting effect of oocytes matured in these media in vitro was proposed by Yoshida (1993). Yoshida (1992b) showed that the composition of the maturation medium has a significant impact on the ability of pig oocytes for form the male pronucleus: medium containing a high concentration of GSH substrate, L-cysteine (CySH), greatly promotes this ability. Supplementing the media with cysteine or related -SH donor substrates is important in maintenance of GSH synthesis during oocyte in vitro maturation (Yoshida 1993; Yoshida et al., 1993a,b). Waymouth medium contains several substrates of glutathione, in particular a high concentration of cysteine (Waymouth, 1959), and proved to be an effective medium in supporting the functional maturation of pig oocytes (Yoshida, 1993). However, CySH readily oxidizes to cysteine (CySS) under the culture conditions of 5% CO_2 in air and oocytes can take up only a limited amount of cystine for glutathione synthesis (Takahashi and Yoshida, 1993). In contrast, cumulus cells can readily incorporate cystine and utilize it in the synthesis of GSH. Therefore, GSH produced in the cumulus cells may enter the oocytes through the corona-oocyte junction complexes and create a high GSH concentration in metaphase II oocytes (Motlik et al., 1986). Glutathione transport may therefore be an important function of couplings between oocytes and follicular cells during normal maturation.

We have observed that in pig oocytes matured in vitro, the percentage of oocytes with fully expanded cumulus cells (see Figure 2-A) is highly correlated to male pronuclear formation rate, but not metaphase II rate: this suggests that nuclear maturation may occur before the total disassociation of cumulus cells from the oocyte, and earlier than the cytoplasmic maturation, which occurs when cumulus cells fully expand and lose connection with the oocyte (Xu and Foxcroft, unpublished data). This suggestion is consistent with the GSH transport hypothesis and the intracellular profile of GSH levels during oocyte maturation described above.

A recent study showed that the inclusion of low-molecular-weight thiol compounds, such as cysteamine and β-mercaptoethanol, in the culture medium aids the in vitro development of IVM-IVF bovine embryos in the absence of feeder cells; the effect is mediated through an increase of intracellular GSH levels. Further, Grupen et al. (1995) reported that addition of 500 μM cysteamine to TCM199 promotes the synthesis of GSH, significantly increased the incidence of synchronous pronuclear development in both monospermic and polyspermic oocytes (see Figure 2-K), and significantly enhanced

embryonic development. To explain this cysteamine effect, it has been proposed that under normal in vitro culture conditions, the cysteine (0.62 μM in TCM199) may be completely oxidized to form cystine. The addition of an excess of cysteamine may prevent the complete oxidation of cysteine to cystine (Ishii et al., 1981), or cysteamine may be directly involved in the reduction of protamine disulfide bonds in the sperm nuclear chromatin (Grupen et al., 1995). Increased levels of thiol compounds within the oocyte cytoplasm, accumulated during maturation with cysteamine, may persist in the cleaving embryo and assist mitotic cell division (Chance et al., 1979).

Funahashi et al. (1994a, b) showed different effects of oocyte maturation media on male pronuclear formation. They suggest that the difference in the concentration of sodium chloride between modified Whitten medium (68.45 mM) and TCM199 (116.4 mM) may cause differences in the intracellular GSH concentration and subsequently the incidence of male pronuclear formation (Funahashi et al.,1994a, b). However, the mechanism is not clear. Effects of other oocyte maturation factors, such as follicular cells, follicular fluid, serum and hormones, have been reviewed by Niwa (1993) and Sirard et al. (1993) and also discussed previously in this review.

In conclusion, a big gap still exists between the effectiveness of in vitro and in vivo maturation and fertilization of pig oocytes, although incorporation of a great deal of knowledge accumulated by many investigators has greatly improved IVM-IVF techniques. However, further basic research on gamete maturation both in vitro and in vivo, and interactions at cellular and molecular levels are needed to bridge the gap and establish an optimal in vitro maturation and fertilization protocol in pigs.

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3. IN VITRO FERTILIZATION OF IN VITRO MATURED PIG OOCYTES: EFFECTS OF BOAR AND EJACULATE FRACTION[1]

3.1 INTRODUCTION

The formation of a successfully fertilized oocyte which has the potential to develop into a healthy embryo requires the contribution of both the matured oocyte and the spermatozoon. Therefore, for in vitro fertilization systems, effective maturation of the spermatozoa is of the same importance as maturation of the oocyte. In the pig, fresh caudal epididymal spermatozoa (Iritani et al., 1978; Nagai et al., 1984), fresh ejaculate semen (Cheng et al., 1986; Ding and Foxcroft., 1992, 1994; Funahashi and Day, 1993; Mattioli et al., 1989; Yoshida, 1987), frozen caudal epididymal spermatozoa (Nagai et al., 1988) and frozen ejaculate semen (Wang et al., 1991) have been reported to fertilize porcine oocytes in vitro. Because of the difficulty of cryopreservation of boar semen, fresh ejaculate semen is still the main source of spermatozoa used for in vitro fertilization. However, large variations among individual males have been noted in fertilization rates (Sirard et al., 1993). The variability in semen quality is one of the major problems in establishing reliable IVM-IVF procedures in the pig. Previous experiments in our laboratory have also suggested that the use of a standardized sperm-rich fraction of boar ejaculates for IVF produced more consistent results (Ding et al., unpublished observations). Removal of these sources of variability (within and between boars) would lead to improved porcine IVF procedures and would standardize techniques for assessing potential boar fertility.

Therefore the present study was designed 1) to determine the effect of variability of different fractions of a single ejaculates on boar semen used for IVF, and 2) to standardize and improve techniques for evaluating fresh ejaculated semen from different boars using IVM-IVF systems.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Oocytes and Follicular Shells

Ovaries were collected from prepubertal gilts with the body weights of approximately 100 kg within 15 min of slaughter at a local abattoir. The ovaries were

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transported to the laboratory within 40 min in a polystyrene box to prevent rapid temperature changes. Follicles with a diameter of 3 to 6 mm were dissected from the ovaries. Dissection was carried out in PBS at a room temperature of 24 to 26 °C. The dissected follicles were first washed with PBS and then with Hepes buffered Medium-199 (containing 10% new born calf serum). Oocyte-cumulus-granulosa cell complexes (oocyte-complexes) were harvested from healthy follicles and used as the source of immature oocytes. Follicle shells from 5- to 6-mm healthy follicles with oocytes and follicular fluid removed were selected for oocyte IVM co-culture. Detailed dissection procedures have been described by Ding and Foxcroft (1992).

3.2.2 Oocyte Maturation

Pooled oocyte-complexes were randomly divided into groups of 10 to 15 oocytes which were cultured with 2 everted follicular shells in 2 ml maturation culture medium in a 35 × 15 mm plastic Petri dish (Falcon 1008). The oocyte maturation culture medium was TCM199 (with Earles' salts and L-glutamine, Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco Grand Island, NY), 100μg/ml glutamine (BDH, Toronto, Canada), 70μg/ml L-ascorbic acid (BDH, Toronto, Canada), 35μg/ml insulin (Sigma, St. Louis, MO), gonadotropins (2.5μg/ml NIADDK-oLH-26, AFP-5551b; 2.5μg/ml USDA-pFSH-B-1, AFP-5600), and prolactin (20 ng/ml USDA-pprl-B-1, AFP-5000). Maturation culture was carried out with gentle rocking agitation in an atmosphere of 5% CO_2 in humidified air at 39 °C for 47 ± 1 h.

3.2.3 Semen Collection

Three 9-month-old Lacombe boars were selected for study and semen collection at the Alberta Swine AI Centre. For at least 2 weeks before, and during the 6 weeks of the experiment, ejaculate collection was standardized to twice a week by an experienced operator using the gloved-hand method. Ejaculates used for evaluation in vitro were collected from all 3 boars on 4 occassions, with 1week between these collections. Each total ejaculate was collected into a series of numbered 15-ml graduated plastic conical tubes (sterilized, Falcon 2099) while removing the gel component by filtering through gauze. The concentration of spermatozoa in each tube was tested with a Spectrophotometer (Spectronic 301, Milton Roy Company, USA), and the motility and morphology of spermatozoa were assessed at 37 °C under a microscope. Based on the concentration of spermatozoa in each tube, 3 fractions from each ejaculate were selected for IVF, as shown in the typical profiles of boar ejaculates (Figure 3-1). Fractions 1 and 3 were identified as those tubes from the first and second sperm-rich portions of the ejaculate which contained maximal sperm numbers. The tube with the lowest sperm numbers between the 2 sperm-rich portions of the ejaculate was taken as Fraction 2. The concentrations of spermatozoa in the 3 selected tubes were equalized by adjusting the volume of seminal plasma after centrifugation at 550 g for 15 min. The 9 tubes of semen from the 3 boars were then tightly capped and held at 20 °C for 16 h (Cheng et al., 1986).

3.2.4 In Vitro Fertilization

One millilitre of semen from each tube was diluted with 4 ml 0.9% NaCl solution containing 1 mg/ml BSA (Fraction V, Sigma) and centrifuged at 550 g for 5 min. The supernatant was discarded and the sperm pellet was washed twice more with the above solution. The sperm pellet was then resuspended at 4×10^8 sperm/ml in preincubation medium at pH 7.8 (Cheng, 1985) and incubated for 90 min at 39 °C in an atmosphere of 5% CO_2 in air before being co-cultured with oocytes. In vitro matured oocytes were pooled and washed twice with fertilization medium (modified medium M199 supplemented with 10 mM caffeine sodium benzoate and 10% FCS, pH 7.4). Then 10 to 15 oocytes were randomly transferred to each well of a 4-well culture dish (Nunclone 176740) containing 0.95 ml fertilization medium per well. Preincubated spermatozoa were diluted to 1×10^7 cells/ml with fertilization medium, and 50µl of this dilution were added to a fertilization well. Oocytes in 2 or 3 wells were randomly assigned for fertilization with spermatozoa from the same fraction. Sperm concentration was therefore the same in all the wells, and the final fertilization concentration of spermatozoa was 5×10^5 cells/ml. Oocytes were co-cultured with spermatozoa for 6 h at 39 °C in an atmosphere of 5% CO_2 in air and then transferred to sperm-free modified Krebs'-Ringer-bicarbonate medium for a further 4 to 6 h of culture (Petters et al., 1990).

3.2.5 Assessment of Sperm Penetration

At the end of culture, oocytes in each treatment were denuded of cumulus cells, rinsed in DPBS, mounted on a slide with a whole mount technique, and fixed in an acetic acid-ethanol solution (1:3) for 48 h or more. The oocytes were then stained with 1% lacmoid in 45% aquatic acetic acid for about 10 min and then washed with the 45% aquatic acetic acid to clear the background. Sperm penetration was examined under a phase-contrast microscope at 200× and 400× magnification. Sperm penetration and male pronuclear development were determined as described by Ding et al. (1992). Those oocytes with unswollen or slightly swollen sperm head(s) and/or further swollen sperm head(s) and/or male pronucleus/pronuclei with detached sperm tail(s) were classified as being penetrated. Those penetrated oocytes with full size male pronucleus/pronuclei were considered to have undergone normal male pronuclear development. Oocytes with more than one sperm head or a male pronucleus were considered to be polyspermic.

3.2.6 Statistical Analysis

The data were analyzed using ANOVA for a split-plot design (Steel and Torrie, 1980). The effects of boar were tested against the interaction term between replicate (block) and boar (main plot). The effects of semen fraction (sub-plot) and the interaction between boar and fraction were tested against the interaction term between replicate and fraction and among replicate, boar and fraction. Statistical analysis of the experiment was carried out using the General Linear Model procedures of the Statistical Analysis System (version 6.08, SAS Institute, Cary, NC). The Student-Newman-Keul test was used for multiple comparisons among treatment means, and the Student t-test was used for

comparing variances of 2 treatment data with equal numbers. Data are expressed as least squares means (LSM), and standard errors of LSM are given in each case.

3.3 RESULTS

3.3.1 Typical Profiles of Boar Semen Ejaculates

The ejaculates collected for study in vitro averaged 180 ml in volume and contained a number of recognizable fractions. The first part of the ejaculate was mainly seminal plasma with very low sperm numbers. Next there was usually a pronounced sperm-rich component that contained the greatest proportion of viable spermatozoa. After a marked reduction in sperm concentration in the next part of the ejaculate, a second sperm-rich portion was present. A third sperm-rich portion was observed in a few cases. The typical profiles of semen ejaculates from the 3 young boars are shown in Figure 3-1. There were no changes in the percentage of progressively motile spermatozoa (above 85%) and percentage of morphologically abnormal spermatozoa in the 3 fractions (less than 8%), although there were small differences in these values among the 3 boars. More spermatozoa in Fraction 2 had a cytoplasmic droplet in the distal site of the middle piece (20%) compared with those in Fractions 1 and 3.

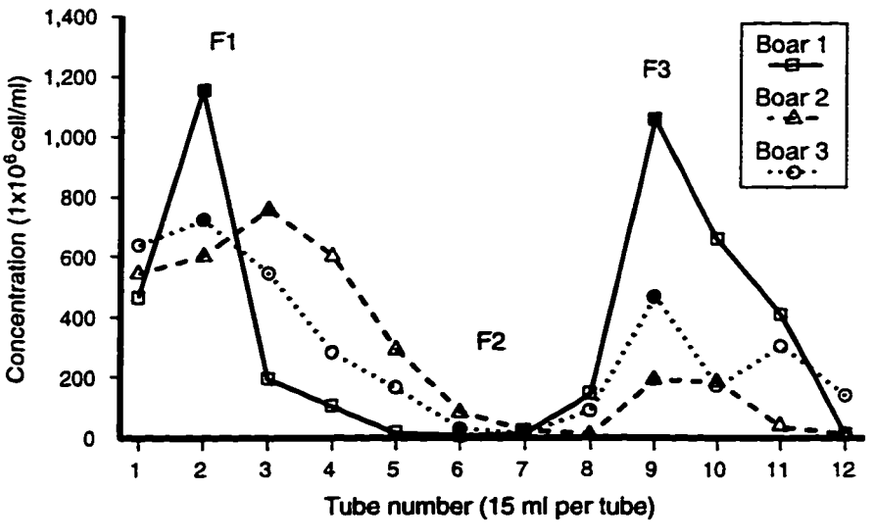

Figure 3-1 Typical profiles of semen ejaculates from the three young boars. The curves represent sperm concentration changes in the ejaculates. The solid points indicate the three sperm fractions selected for IVF from sequences of 15-ml tubes of each ejaculate. Fraction 1 (F1): maximum sperm concentration in the first sperm-rich component; Fraction 2 (F2): minimum sperm concentration following the first sperm-rich component; and Fraction 3 (F3): maximum sperm concentration in the second sperm-rich component.

3.3.2 Effects of Ejaculate Fraction on IVF

Six parameters were used to study the ejaculate fraction effect and the results of the 4 main parameters are summarized in Figure 3-2. There were differences among fractions in oocyte penetration rate (P=0.001), in male pronuclear formation rate (P=0.028) and in the rate of polyspermy (P=0.0001). The average number of penetrated spermatozoa per oocyte was also different among fractions (P=0.002). There were also significant differences among fractions in average numbers of swollen sperm head and unswollen or slightly swollen sperm head (both P<0.01); Fractions 1 and 3 were not different but both had more swollen sperm heads and unswollen or slightly swollen sperm heads than Fraction 2 (mean swollen sperm head: 0.81, 0.23 and 0.64 for Fractions 1, 2 and 3, respectively, SEM 0.11; mean unswollen or slightly swollen sperm head: 4.47, 1.54 and 4.07 for Fractions 1, 2 and 3, respectively; SEM 0.55). The results indicate superior fertilizing ability of Fraction 1 and Fraction 3 spermatozoa.

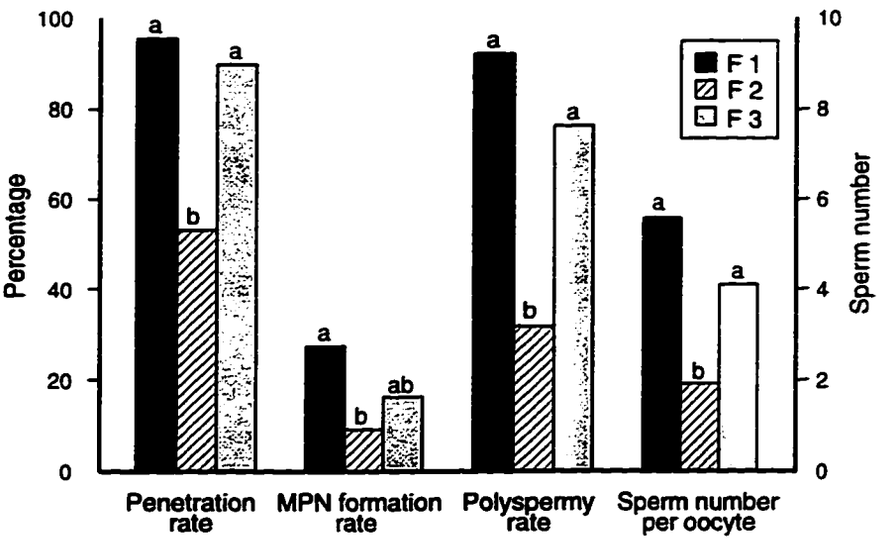

Figure 3-2. Effect of ejaculate fraction (F) on IVF. Bars represent least squares means (LSM) of treatments in four replicates (with a minimum of 240 oocytes per treatment). For each parameter, LSM with different subscripts differ significantly (P<0.05). Standard errors of LSM (SEM) for penetration rate, male pronuclear (MPN) formation rate, polyspermy rate and average sperm number per oocyte were 5.82, 4.42, 7.21 and 0.62, respectively.

Variation in oocyte penetration rate for the 3 ejaculate fractions is shown in Figure 3-3. For each fraction, data represent the results from the 3 boars in 4 replicate experiments. Spermatozoa of Fraction 2 gave the largest variation in penetration rate. The variation in Fraction 3 was also significantly greater (P<0.05) than in Fraction 1. The

spermatozoa of Fraction 1, therefore, provide a high oocyte penetration rate, with the least variation from experiment to experiment.

3.3.3 Effects of Boar on IVF

Boar effect on IVF results were summarized in Figure 3-4. Overall, the effect of boar on IVF results was significant ($P<0.05$). By multiple comparisons, use of semen from Boar 1 resulted in a significantly higher oocyte penetration rate ($P<0.05$) than from Boar 2 or Boar 3, and higher male pronuclear formation rate ($P<0.05$) and polyspermy rate ($P<0.05$) than Boar 3. More spermatozoa from Boar 1 penetrated each oocyte than from Boar 2 or Boar 3 ($P<0.05$) when the same sperm:oocyte ratio was used for IVF. Similar differences were observed for the average number of swollen sperm head per penetrated oocyte ($P<0.05$, mean 0.88, 0.45 and 0.35 for Boars 1, 2 and 3, respectively; SEM 0.24), whereas there were no significant differences among the 3 boars in the average number of unswollen or slightly swollen sperm head per penetrated oocyte ($P>0.05$, mean 4.17, 3.45 and 2.43 for Boars 1, 2 and 3, respectively; SEM 0.98).

There were no interaction effects between fraction and boar ($P>0.05$) for any of the IVF parameters tested in this experiment.

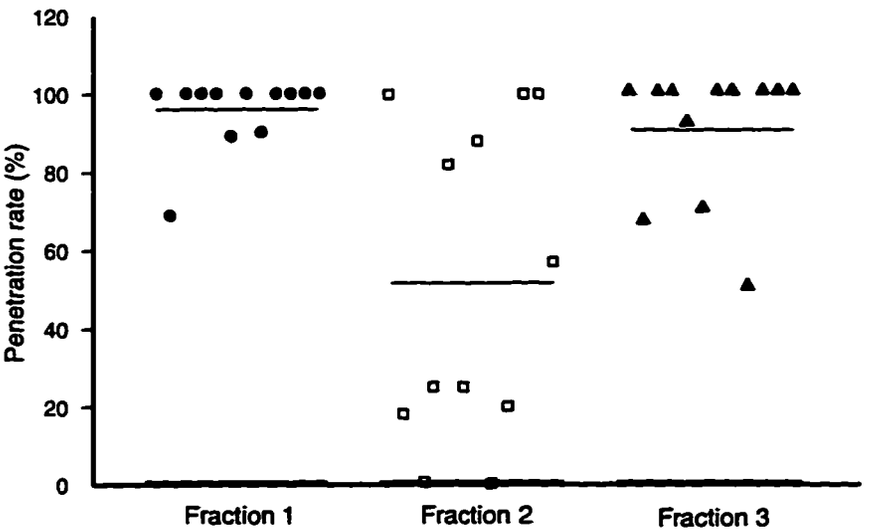

Figure 3-3. Comparisons of variances of penetration rate of the three semen fractions. The three short lines represent LSM of penetration rates of the three ejaculate fractions. Fraction significantly affected variance in penetration rate.

3.4 DISCUSSION

Ejaculates from the 3 young boars were characterized as having 2 sperm-rich components separated by a seminal plasma-rich part of the ejaculate containing few spermatozoa. The source and function of the second sperm-rich component is uncertain.

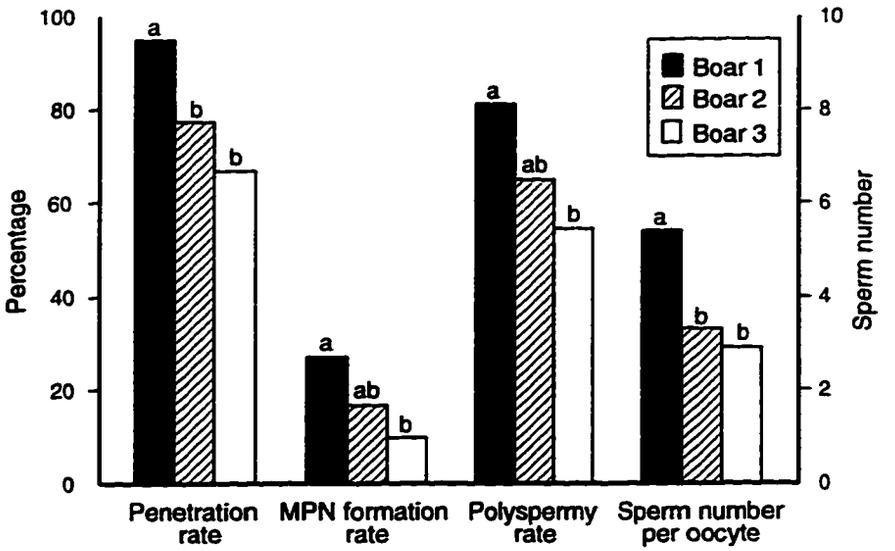

Figure 3-4. Effect of boar on IVF. Bars represent LSM of treatments in the four replicate experiments. a-b: For each parameter, LSM with different letters were significantly different ($P<0.05$). Standard errors of LSM of penetration rate, male pronuclear (MPN) formation rate, polyspermy rate and average number of spermatozoa per oocyte were 7.09, 4.94, 11.68 and 0.83, respectively.

Usually all the sperm-rich components of an ejaculate are collected and pooled for AI use. For IVF, a few papers have mentioned that the semen used was selected from the sperm-rich fraction of the ejaculate (Funahashi and Day, 1993a, b and c) but use of a standardized collection of a specific sperm-rich fraction has not been reported. In this study 3 semen fractions representing the 2 sperm-rich components and the seminal plasma-rich component were selected for investigating the effect of fraction on IVF. The results demonstrate a very significant fraction effect and spermatozoa from different fractions behave differently in IVF (Figure 3-2). Spermatozoa from the seminal plasma-rich fraction (Fraction 2) showed significantly lower fertilizing ability than the sperm-rich fractions. Between the 2 sperm-rich fractions (Fractions 1 and 3) no significant differences were observed in any of the parameters used to determine sperm fertilizing ability, although values tended to be lower in the second (Fraction 3) than the first

(Fraction 1) sperm-rich fraction. However, the data shown in Figure 3-3 suggest that using spermatozoa from the first sperm-rich fraction for IVF results in less variation from experiment to experiment than from the other 2 fractions. Since sperm concentrations were equalized immediately after collection, the semen of the 3 fractions was treated the same in vitro, and no difference in sperm motility was observed among the three fractions immediately after collection, factors which modified sperm function in vitro must have been present in the spermatozoa or the seminal plasma before or during ejaculation. According to Nagai et al. (1984), epididymal spermatozoa become infertile once they are exposed to seminal plasma. The seminal plasma may decapacitate the spermatozoa by modifying the sperm plasma membrane. The decapacitated (ejaculated) spermatozoa must then be recapacitated before they become capable of fertilizing oocytes (Yanagimachi, 1988). Other in vitro studies have shown some factors that affect capacitation and cause individual variation in sperm quality. Spermatozoa from some males can be capacitated more easily than those from others of the same species (Shalgi et al., 1981; Yanagimachi, 1984). We suggest that the observation of the variable fertilizing ability in vitro among spermatozoa from different fractions within an ejaculate in this study could be due to decapacitation factors in the seminal plasma. Since the statistical analysis showed no interaction between fraction and boar, an important practical outcome of this study was to demonstrate that the standardized use of specific sperm-rich fractions of an ejaculate in IVF reduce the variability among different ejaculates from the same boar. A standardized IVM/IVF technique would make comparisons of boar fertility more precise.

Although the most valid test of sperm fertilizing ability would be to obtain viable pregnancies and normal offspring following in vivo insemination, this is not a viable test of sperm fertilizing ability. With the widespread of use of AI in animal breeding, a variety of in vitro procedures have been devised for assessing sperm quality; but only methods involving interactions with living ova can provide unequivocal evidence of sperm fertilizing ability (Bavister, 1990). In the pig, sperm penetration tests using zona-free hamster ova (Almlid et al, 1989; Clark and Johnson, 1987) and germinal vesicle-stage pig oocytes (Martínez et al., 1993) have been reported to be useful for providing information on fertility characteristics of frozen-thawed boar semen and fresh ejaculated semen. However, there is still disagreement about how well the test results correlated with actual fertility of spermatozoa (Aitken, 1988; Almlid et al, 1989). Recently, a zona-intact binding bioassay made it possible to determine semen parameters directly associated with the events required for physiological competence of spermatozoa to bind and penetrate homologous, zona-intact pig oocytes (Ivanova and Mollova, 1993). The penetration rate of fertile and subfertile boar spermatozoa in vitro is related to the in vivo fertility rate of males, and can therefore be used as a significant marker of their fertilizing ability (Ivanova and Mollova, 1993). In the current study, in vitro-matured pig oocytes were fertilized in vitro by spermatozoa from 3 young boars. Each of the parameters was useful for demonstrating boar effects on IVF results. However, the 4 main parameters used discriminated differently among the boars. Penetration rate and the average sperm number per penetrated oocyte discriminated between Boar 1, and Boars 2 and 3, whereas the other two measured characteristics were not able to discriminate between Boar 1 and Boar 2.

Further work is needed to determine which pattern of discrimination is more closely related to proven fertility in vivo. Large variations among different boars in terms of penetration rates and rates of polyspermy in pig IVF have also been observed in other laboratories (Sirard et al., 1993; Wang et al., 1991). In other species, bull-specific effects on fertilization and embryo development have been studied extensively, and both the ability of spermatozoa to penetrate oocytes in vitro and subsequent embryo development in vitro were related to in vivo fertility (Eyestone and First, 1989; Hillery et al., 1990; Shi et al., 1990). In these experiments oocytes used in IVF were all homologous in vitro matured oocytes. The presence of the intact zona pellucida, and preferably the cumulus/corona layers as well, provides the major challenge to spermatozoa attempting to reach the oocyte plasma membrane, and in an extensive review of the literature existing at that time, Bavister (1990) concluded that IVF of zona-intact homologous oocytes is the most informative in vitro test of sperm fertilizing ability.

In our present study, the high rates of penetration were accompanied by high incidences of polyspermy. ability of boar spermatozoa in vitro (Ivanova and Mollova, 1993; Martínez et al., 1993; Wang et al., 1991), high penetration rates are usually also associated with a high incidence of polyspermy during the fertilization of pig oocytes in vitro. It is still not known whether or not this abnormality is due to inadequate conditions during the maturation of oocytes or during fertilization in vitro (Niwa, 1993). However, the polyspermy rate could still be used to evaluate the ability of spermatozoa to penetrate in oocytes in vitro. Similarly, an estimate of the average sperm number per penetrated oocyte does not reflect the normal fertilization events in vivo, but may provide a useful estimate of the population of spermatozoa with high fertilizing ability. The high polyspermy rate may be due to the high sperm:oocyte ratio (50,000:1) applied in the fertilization medium. A high correlation between the incidence of polyspermy and the absolute number of spermatozoa and oocytes present at fertilization in vitro has been reported (Rath, 1992). This suggests that the sperm:oocyte ratio should be optimized to a point that can minimize the polyspermy rate but still keep a high penetration rate in order to obtain more viable in vitro produced embryos. Another important parameter used to assess the IVF results in this study was the male pronuclear formation rate. In the pig, male pronuclear formation rate is a common criterion for measuring cytoplasmic maturation of in vitro matured oocytes, since it is closely related to oocyte cytoplasmic maturation (Mattioli et al., 1988). Cytoplasmic glutathione has been implicated as playing a key role in sperm chromatin decondensation, and thus in male pronuclear formation (Niwa, 1993). In the current study, all the oocytes were cultured and fertilized in vitro under identical conditions, and, therefore, it might be assumed that male pronuclear formation rates would not be different among treatments. However, male pronuclear formation rates were different between fractions (Figure 3-2) and between boars (Figure 3-4), and male pronuclear formation rates showed similar trends as penetration rates for both fraction and boar effects. These observations imply that male pronuclear formation may not only be determined by the maturational state of the oocyte. Because there is considerable interaction between the sperm and oocyte components throughout the fertilization process, the characteristics of the spermatozoa may also have an impact on the successful male pronuclear formation.

In vitro maturation of follicular oocytes has been described as an effective means of obtaining large numbers of uniform and fertilizable oocytes (Ivanova and Mollova, 1993). When IVM/IVF systems are used to assess sperm function, it is desirable to reduce any female effect on variability. In our study standardized procedures of follicle selection, oocyte collection and maturation, randomizing IVM oocytes for IVF, and use of duplicate or triplicate fertilization dishes for each semen sample overcame problems of oocyte variability and minimized the female effect on the IVF results. Previous experiments using the same oocyte in vitro maturation system reported a maturation rate as high as 94% (Ding and Foxcroft, 1992; Ding et al., 1992). The present study proved the consistency of this follicle shell co-culture system with a mean maturation rate of 92%. However, the use of follicle dissection as a means of oocytes collection is very time-consuming compared with follicle aspiration. Use of aspiration as a more efficient method of oocyte recovery will be necessary to meet the need for a large number of oocytes for pig IVF and for using the IVF technique to assess boar fertility in future studies.

In conclusion, our results show that the IVM/IVF systems can be adopted for comparing boar semen quality in vitro, and the criteria used in this study allowed for discrimination between boars in terms of apparent semen quality. Because of the effect of ejaculate fraction, but no fraction by boar interaction, use of a sperm-rich part of the standardized fraction from an ejaculate would provide better comparisons between boars. The data suggest that a standardized aliquot of the first fraction would provide the best semen for use in pig IVF.

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4. SEMEN DILUTION FOR ASSESSMENT OF BOAR EJACULATE QUALITY IN PIG IVM AND IVF SYSTEMS[2]

4.1 INTRODUCTION

The most valid assessment of boar semen quality is to obtain viable pregnancies and normal offspring following in vivo insemination. For obvious reasons this is not practicable as a routine assay of sperm fertilizing ability in AI units. A variety of in vitro procedures have therefore been devised for predicting male fertility and sperm fertilizing ability. Assessment of the testes was a valuable predictor used in AI (Gabor et al., 1995; Rathje et al., 1992) and computer-assisted sperm analysis allowed for determination of specific motion characteristics of sperm cells in vitro (Ellington et al., 1993; Jasko et al., 1991; O'Connor et al., 1981). Among the in vitro assays, evaluation of sperm cellular and motility profiles, and oocyte penetration tests, were reviewed as two main procedures by Bavister (1990). Examination of semen characteristics, such as sperm morphology, concentration and progressive motility, are routine procedures for obtaining information about potential male fertility in AI centres. However, these semen traits were often not significantly correlated to fertility (Barth, 1992; Buchner et al., 1954; Linford et al., 1976). In the pig, sperm penetration tests using zona-free hamster ova (Almlid, 1989; Clark and Johnson, 1987), zona-intact rat oocytes (Kiehm et al., 1991), germinal vesicle stage pig oocytes (Martínez et al., 1993) and matured zona-intact pig oocytes (Ivanova and Mollova, 1993) have been reported to be useful for providing information about fertility characteristics of frozen-thawed and freshly ejaculated boar semen.

Our previous data established that use of a standardized sample of the sperm-rich fraction of freshly ejaculated boar semen for in vitro fertilization of in vitro matured pig oocytes provided the most consistent in vitro estimate of semen quality (Xu et al., 1996). However, use of a sperm concentration of 5×10^5 sperm /ml in our established IVM/IVF system resulted in high rates of penetration but also polyspermy (Ding and Foxcroft, 1992; Xu et al., 1996).

The objectives of the present study were 1) to examine the effect of using decreasing sperm concentrations (sperm:oocyte ratios) of standardized ejaculate fractions from different boars on penetration rate, polyspermy rate, male pronuclear (MPN) formation rate and average number of spermatozoa penetrated per IVM porcine oocytes; 2) relate the results to standard AI laboratory characterization of the same semen samples;

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and 3) to provide an improved method for discriminating between boars and semen samples and thereby predicting relative fertility.

4.2 MATERIALS AND METHODS

4.2.1 Semen Preparation and Optical Evaluation

One boar each from the Lacombe, Landrace and Yorkshire breeds were selected for study and semen collection at the Alberta Swine AI Centre for a period of 6 wk. Ejaculate collection was standardized to twice a week by an experienced operator using the gloved-hand method. The sperm-rich fraction of the ejaculate was collected into a sterilized warm thermos flask while filtering out the gel component. Fresh semen characteristics measured as routine procedures included ejaculate volume, concentration of spermatozoa tested with a spectrophotometer (Spectronic 301, Milton Roy), and progressive motility and morphology of spermatozoa assessed under a microscope at 37°C. Semen were diluted in Extender BL-1 to 6×10^7 cell/ml and kept in the dark at room temperature for further motility analysis. Extended semen was warmed up to 37°C for 30 min and re-evaluated Day 7 after collection. During this period, ejaculates from the 3 boars were collected for IVF on 4 occasions as described by Xu et al. (1996). A 15-ml sample representing the high sperm concentrations in the first sperm-rich fraction of an ejaculate was selected and held in tightly capped tubes at 20°C for 16 h (Cheng et al., 1986).

4.2.2 Oocyte Collection and In Vitro Maturation

Ovaries were collected from prepubertal gilts with body weights of approximately 100 kg within 15 min of slaughter at a local abattoir and transported to the laboratory within 40 min in a polystyrene box to prevent rapid temperature changes. Oocytes were aspirated through an 18-gauge needle into a disposable 10-ml syringe from follicles with a diameter of 3 to 6 mm. Collection and processing of porcine follicular fluid (pFF) for use in IVM and the washing and assessment of oocyte-cumulus complexes were carried out according to Funahashi and Day (1993a). Pooled oocyte-cumulus complexes were randomly divided into groups of 30 to 40 and cultured in 2 ml maturation culture medium in a 35×15 mm plastic petri dish (Falcon 1008). The oocyte maturation culture medium was TCM 199 (with Earles' salts and L-glutamine, Gibco, Grand Island, NY) supplemented with 100 μg/ml glutamine (BDH, Toronto, Canada), 70 μg/ml L-ascorbic acid (BDH, Toronto, Canada), 35 μg/ml insulin (Sigma, St. Louis, MO), gonadotropins (2.5 μg/ml NIADDK-oLH-26, AFP-5551b; 2.5 μg/ml USDA-pFSH-B-1, AFP-5600), and prolactin (20 ng/ml USDA-pprl-B-1, AFP-5000) according to previous experiments (Ding and Foxcroft, 1992; Ding et al., 1992; Mattioli et al., 1988). Ten percent pFF was added to the culture medium in the present study instead of 10% FCS. Maturation culture was carried out in an atmosphere of 5% CO_2 in humidified air at 39°C for 44 ± 1 h.

4.2.3 In Vitro Fertilization

One ml semen from each tube was diluted with 4 ml 0.9% sodium chloride solution containing 1 mg/ml BSA (Fraction V, Sigma, St Louis, MO) and centrifuged at 550g for 5 min. The supernatant was discarded and the sperm pellet was washed twice more with the above solution. The sperm pellet was thereafter resuspended at 4×10^8 sperm/ml in preincubation medium at pH 7.8 (Cheng, 1985) and incubated for 90 min at 39°C in an atmosphere of 5% CO_2 in air before being serially diluted.

In vitro matured oocytes were pooled and washed twice with fertilization medium (modified medium TCM 199 supplemented with 10 mM caffeine sodium benzoate (Sigma, St Louis, MO) and 10% FCS (Sigma, St Louis, MO) (pH 7.4). Then 10 oocytes were randomly transferred to each well of a 4-well culture dish (4-well multidish, Nunclone 176740) containing 0.95 ml fertilization medium per well.

Pre-incubated spermatozoa were diluted to 1×10^7, 5×10^6, 2.5×10^6, 1.25×10^6 and 6.25×10^5 cells/ml with fertilization medium, and 50µl of each dilution was added to a fertilization well. Duplicate or triplicate groups of 10 matured oocytes were co-cultured with each sperm concentration. Therefore the ratio of sperm:oocyte for IVF groups were 50,000, 25,000, 12,500, 6,250 and 3,125. Oocytes were co-cultured with spermatozoa for 6 h at 39°C in an atmosphere of 5% CO_2 in air and then transferred to sperm-free modified Krebs'-Ringer-bicarbonate medium for a further 6 h culture (Petters et al., 1990).

4.2.4 Assessment of Sperm Penetration

At the end of culture, oocytes were denuded of cumulus cells, rinsed in phosphate buffer solution, mounted on a slide with a whole mount technique and fixed in an acetic acid-ethanol solution (1:3) for 48 h or more. Oocytes were then stained with 1% lacmoid in 45% aqueous acetic acid for about 10 min and then washed with the 45% aqueous acetic acid to clear the background. Sperm penetration was examined under a phase-contrast microscope at 200× and 400× magnification. Sperm penetration and male pronuclear development were determined as described by Ding et al. (1992). Those oocytes with unswollen or slightly swollen sperm head(s) and/or further swollen sperm head(s) and/or male pronuclei (MPN) with detached sperm tail(s) were classified as being penetrated. Those penetrated oocytes with full size MPN were considered to have undergone normal MPN development. Oocytes with more than one sperm head or MPN were considered to be polyspermic. Average number of spermatozoa (ANS) penetrated per oocyte (total number of spermatozoa penetrated/ total number of oocytes inseminated) was recorded.

4.2.5 Statistical Analysis

Characteristics of fresh semen samples were analysed using one-way ANOVA, while the IVF data were subjected to ANOVA for a split-plot design (Steel and Torrie, 1980). The effects of boar and therefore breed were tested against the interaction term between replicate (block) and boar (main plot). The effects of semen dilution (sub-plot) and the interaction between boar and semen dilution were tested against the interaction term between replicate and dilution and among replicate, boar and dilution. Statistical analysis of the fresh semen characteristics and IVF data was carried out using the General Linear Model procedures of the Statistical Analysis System (version 6.08, SAS Institute, Cary, NC). Where appropriate, multiple comparisons among treatment means were made using the Student-Newman-Keuls (SNK) test. Correlation analysis was used to reveal relationships between fresh semen characteristics and IVF data for the same ejaculates. Data shown in the table and figures are expressed as least squares means (LSM), and standard errors of LSM are given in each case.

4.3 RESULTS

4.3.1 Optical Evaluation of Semen Quality

Mean values of ejaculate sperm concentration, ejaculate volume, total sperm number per ejaculate, percentage of progressively motile spermatozoa on the day of collection (Day 0) and on Day 7, and the difference in motility between Days 0 and 7 (Days 0 to 7) are shown in Table 4-1.

Table 4-1. Fresh semen characteristics of ejaculates collected from the three boars during a 6-week period

Boar	No. of collections	Concentration ($\times 10^6$ cell/ml) of ejaculate	Volume (ml) per ejaculate	Total no. of sperm ($\times 10^9$) per ejaculate	Motility (%)		
					Day 0	Day 7	Days 0 to 7
A	12	474.55	166.8^a	79.17^a	88	77^a	11^a
B	12	451.71	141.8^b	64.16^a	85	71^a	14^a
C	12	435.00	91.3^c	40.88^b	81	45^b	36^b
SEM		44.71	7.4	6.98	1.2	2.4	2.5

$^{a-c}$: Among boars, values with different superscripts were significantly different ($P<0.05$).

There were no significant differences in ejaculate concentration among the 3 boars ($P>0.05$), although the volume of the ejaculate varied from boar to boar ($P<0.001$). As a result, Boar C ejaculated less spermatozoa than Boars A and B ($P<0.002$). Progressive

sperm motility of the 3 boars was above 80% and not different (P>0.05). On Day 7, semen of Boar C showed significant lower motility compared with semen from the other 2 boars (P<0.001). Compared with the motility on Day 0, motility dropped 10.9, 14.3 and 35.8%, respectively, for Boars A, B and C, and Boar C semen underwent bigger motility change than did semen for Boars A and B (P<0.001). The percentage of morphologically abnormal spermatozoa in all ejaculates from these boars was very low (less than 5%), and no obvious changes existed between Days 0 and 7.

4.3.2 Semen Dilution and Boar Effects on IVF

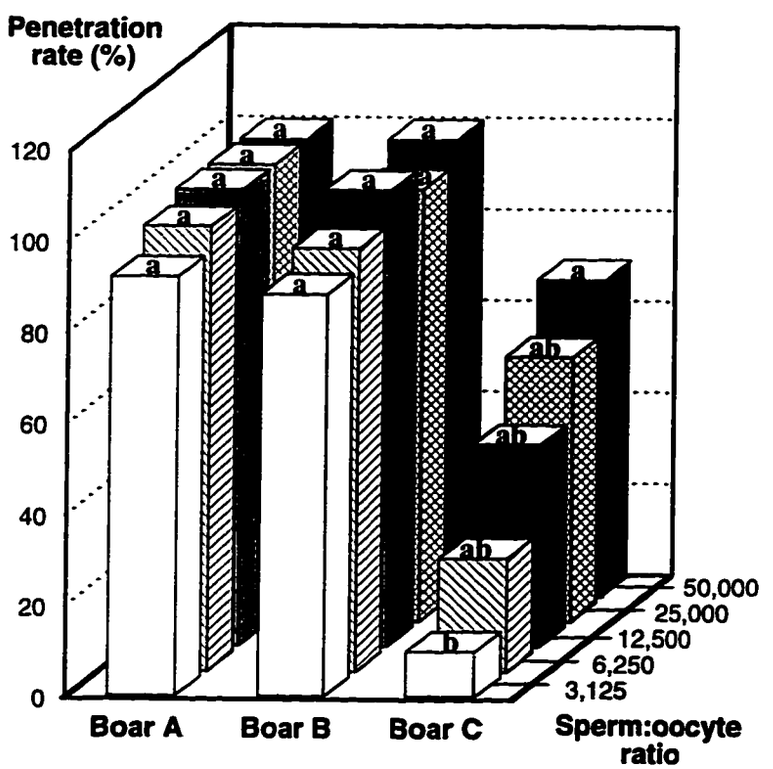

Figure 4-1. Semen dilution and boar effects on oocyte penetration rates. Bars represent LSM of penetration rates in four replicate experiments (with a minimum of 240 oocytes per treatment). a-b: Within boars, LSM with different letters were different (P<0.05); Within all sperm:oocyte ratios among boars, penetration rates for Boars A and B were different from that of Boar C (P<0.05). Standard errors (SE) of LSM for Boars A, B and C were 2.46, 3.55 and 12.03, respectively; SE of LSM for sperm:oocyte ratio 1, 2, 3, 4 and 5 were 8.38, 8.29, 8.65, 4.85 and 5.93, respectively.

Oocyte penetration, polyspermy, MPN formation rates and average number of spermatozoa penetrated per oocyte were significantly affected by boar (all P<0.001), semen dilution (all P<0.001) and their interactions (all P<0.05). Multiple comparisons established

lower penetration rates in Boar C compared to Boars A and B ($P<0.05$) and semen dilution in Boar C resulted in a significant decrease in penetration rate but had no effect in Boars A and B over the range of dilution used (Figure 4-1).

The semen dilution and boar effects on polyspermy rates are shown in Figure 4-2. As sperm:oocyte ratio reduced from 50000 to 3125, the polyspermy rates of Boars A and B decreased significantly (from 100 to 32.2% for Boar A and from 100 to 34.5% for Boar B); however, semen dilution had no significant effect on Boar C.

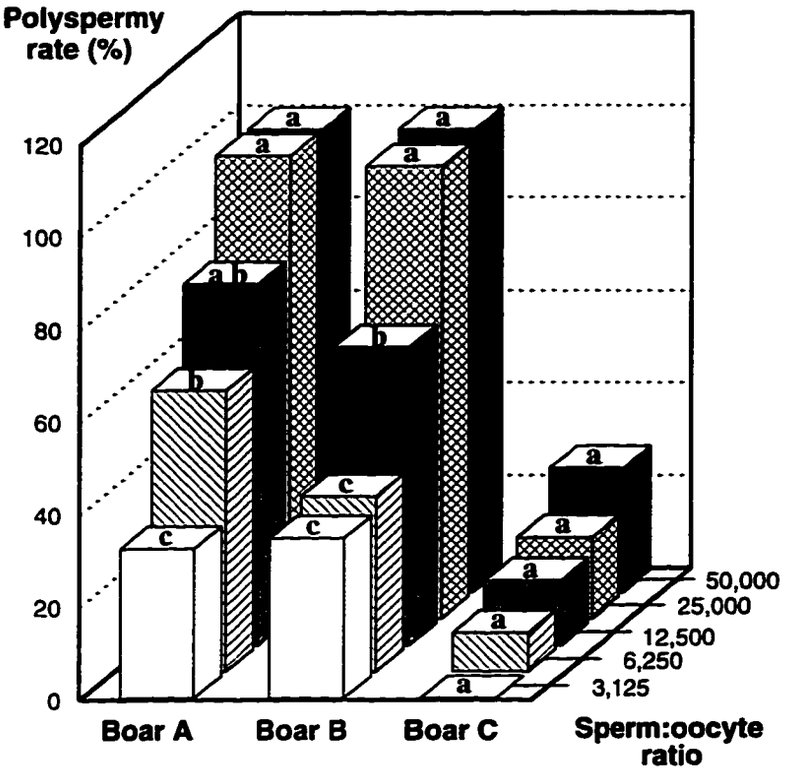

Figure 4-2. Semen dilution and boar effects on polyspermy rate. Bars represent LSM of polyspermy rates in four replicate experiments (with a minimum of 240 oocytes per treatment). a-c: Within boars, LSM with different letters were different ($P<0.05$). Within sperm:oocyte ratios among boars, polyspermy rates for Boars A and B were different from that of Boar C ($P<0.05$). SE of LSM for Boars A, B and C were 7.02, 5.97 and 10.07, respectively; SE of LSM for sperm:oocyte ratio 1, 2, 3, 4 and 5 were 8.31, 8.29, 5.75, 7.00 and 19.19, respectively.

The semen dilution and boar effects on average number of spermatozoa penetrated per oocyte are shown in Figure 4-3. As sperm:oocyte ratio decreased from 50000 to 3125, average number of spermatozoa per oocyte of Boars A and B but not Boar C decreased significantly. Moreover, the initial change in sperm:oocyte ratio from 50000 to 25000

resulted in a reduction ($P<0.05$) in average number of spermatozoa per oocyte in Boars A and B.

Semen dilution, boar and interaction effects on MPN formation rates are shown in Figure 4-4. Overall, MPN formation rates increased as the sperm:oocyte ratio became smaller and then started to drop once a critical sperm:oocyte ratio had been reached. However, the optimal sperm:oocyte ratio for maximal MPN formation rate was different among boars.

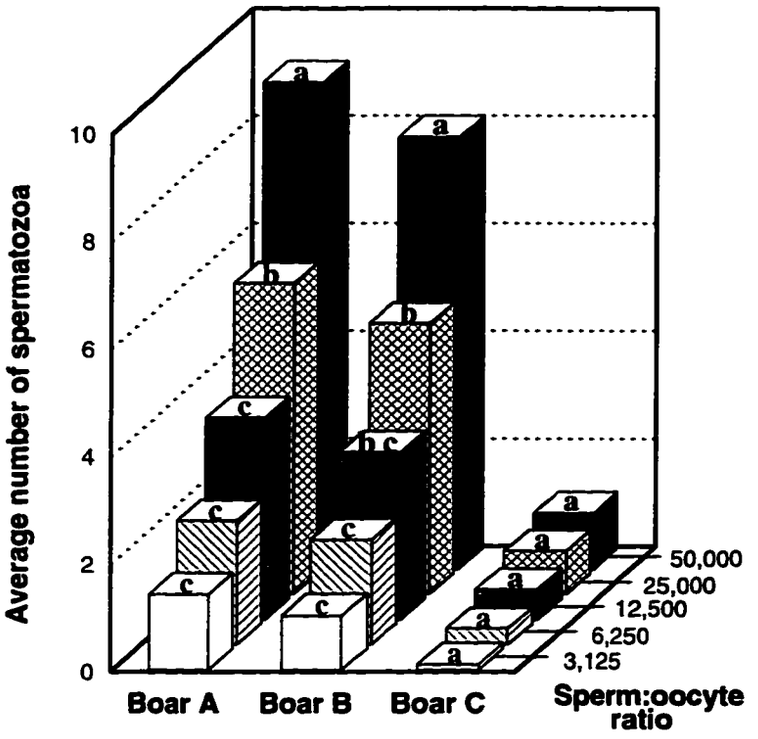

Figure 4-3. Semen dilution and boar effects on average number of spermatozoa (ANS) penetrated per oocyte. Bars represent LSM of ANS per oocyte in four replicate experiments (With a minimum of 240 oocytes per treatment). a-c: Within boars, LSM with different letters were different ($P<0.05$); For all sperm:oocyte ratios among boars, ANS per oocyte for Boars A and B were different to Boar C ($P<0.05$). SE of LSM for Boars A, B and C were 0.65, 0.68, and 0.24, respectively; SE of LSM for sperm:oocyte ratio 1, 2, 3, 4 and 5 were 1.14, 0.35, 0.26, 0.25 and 0.19, respectively.

Using the current IVM/IVF system, the number of potential viable embryos produced from all the oocytes cultured in vitro could be predicted for each treatment groups. Only those oocytes, which were penetrated by a single spermatozoa and had developed to form the full-size female and male pronuclei at the time of examination, were considered having

the potential to develop to normal embryos. Percentage of such potential viable embryo production for each treatment is shown in Figure 4-5. For a given sperm:oocyte ratio, embryo production rate for Boar C was always the lowest among the three boars. The optimal sperm:oocyte ratio was 6250 for both Boars A and B.

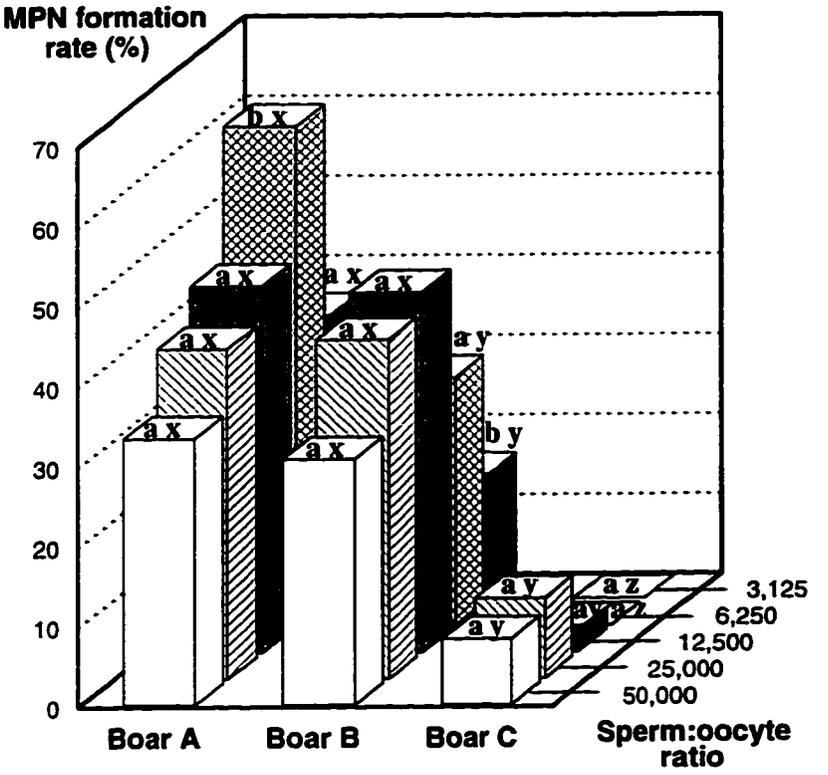

Figure 4-4. Semen dilution and boar effects on MPN formation rates. Bars represent LSM of MPN formation rates in four replicate experiments (with a minimum of 240 oocytes per treatment). a-b: Within boars, LSM with different letters were different ($P<0.05$); x-z: Within sperm:oocyte ratios, LSM with different letters were different ($P<0.05$). SE of LSM for Boars A, B and C were 3.65, 4.08 and 5.96, respectively; SE of LSM for sperm:oocyte ratio 1, 2, 3, 4 and 5 were 5.55, 6.07, 3.74, 3.59 and 3.83, respectively.

4.3.3 Correlation Analysis

Correlation coefficients of oocyte penetration rate to the concentration of ejaculate, volume of ejaculate, total number of spermatozoa per ejaculate, sperm progressive motility on Day 0, sperm progressive motility on Day 7 and the difference of motility of these 2 days are shown in

Table 4-2. Oocyte penetrability was highly correlated with sperm motility of Day 7. Consequently, penetration rate was highly and negatively correlated with the motility

difference between Days 0 and 7. Penetrability and the other semen characteristics were not highly correlated.

During the same period of time the IVM/IVF experiment was carried out, ejaculated semen of the 3 boars were also used for commercial artificial insemination and limited field data provided estimates of farrowing rate (number of sow farrowed / number of sows inseminated × 100%). For Boars A, B and C the farrowing rates were 87.5% (7/8), 86.2% (25/29) and 69.2% (9/13), respectively.

Table 4-2. Correlation coefficients of penetration rates to other semen values of the three boars

n=12	Concentration of ejaculate	Volume per ejaculate	Total no. of sperm per ejaculate	Motility		
				Day 0	Day 7	Days 0 to 7
r	0.2476	0.5355	0.5205	0.5848	0.8985	-0.8464
p	0.4378	0.0728	0.0827	0.0558	0.0001	0.0005

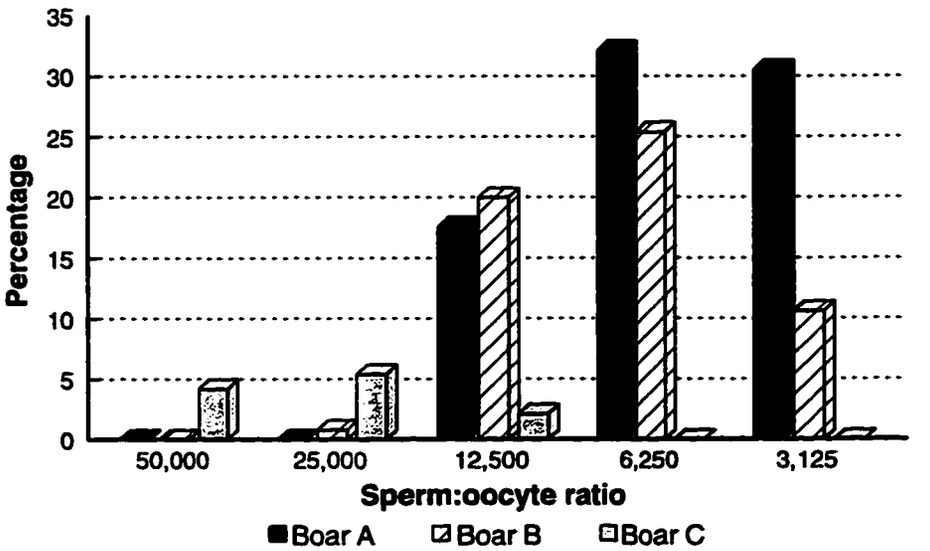

Figure 4-5. Potential viable embryo production using in vitro matured oocytes fertilized with semen from the three boars at different semen dilution.

4.4 DISCUSSION

As discussed by Whitfield and Parkinson (1992) in their paper, the most commonly used laboratory tests for assessing semen quality are volume of ejaculate, concentration, motility and percentage of abnormal spermatozoa. Also evaluation of acrosome integrity of spermatozoa is used as a routine procedure for analysing fresh and frozen-thawed semen quality in bull (O'Connor et al., 1981; Pace and Sullivan, 1978; Pace et al., 1981). However the published data concerning the relationship between these parameters and sperm fertilizing ability are controversial (O'Connor et al., 1981; Saacke and White, 1972; Salisbury et al., 1978). In the current study, we analyzed several characteristics of ejaculates from individual boars of 3 breeds and found significant differences among boars in the volume of ejaculate, total number of spermatozoa per ejaculate, sperm motility on Day 7 and the difference of motility between Days 0 and 7. However, there were no significant differences in sperm motility on Days 0 among boars. It seems that semen of Boars A and B was of better quality from the gross examination. The correlation analysis of IVF oocyte penetration rates with these gross parameters established a very high correlation between sperm penetration and sperm motility on Day 7, suggesting that the retention of sperm motility in extended semen, rather than the motility on the collection day, is more important an indicator of sperm fertilizing ability.

In vitro fertilization of homologous, zona-intact oocytes is the most informative method for assessing sperm fertilizing ability in vitro (Bavister, 1990). Penetration rate is the most commonly used marker of the boar sperm fertilizing ability in vitro (Ivanova and Mollova, 1993; Martínez et al., 1993; Wang et al., 1991). The IVF results showed that oocyte penetration was significantly affected by boar, semen dilution and their interaction. Over the sperm:oocyte dilution range used, penetration rate decreased greatly in Boar C, but no changes were observed for Boar A and Boar B. Even when the sperm:oocyte ratio was reduced to 3125, the penetration rate of these two boars was still very high (above 88%). This implies that further dilutions were needed to allow any difference in sperm penetrability between Boars A and B to be established.

A high incidence of polyspermy is one of the major unsolved problems in pig IVF (Niwa, 1993). From a physiological perspective, the number of viable spermatozoa reaching the site of fertilization in the oviducts must be strictly controlled, at and immediately after ovulation, in order to reduce polyspermic penetration of the oocytes (Hunter, 1993). The sperm:oocyte ratio at the time of initial penetration of the oocyte membrane is close to unity under in vivo conditions (Hunter, 1993; Niwa, 1993). High polyspermy rates were observed when both in vivo (Yoshida, 1989) and in vitro (Funahashi and Day, 1993b; Xu et al., 1996; Yoshida, et al., 1990) matured oocytes underwent IVF. Many factors may be involved in this phenomenon (Hunter, 1993). Under in vitro conditions the oocyte defense mechanism against polyspermy seems to be delayed and incomplete: Excessive numbers of spermatozoa are present at the site of fertilization and increase the chance of multiple penetration. Rath (1992) summarized and analyzed the existing IVF data and concluded that a high correlation exists between polyspermy rate and the absolute number of spermatozoa and oocytes, but not between polyspermy

rate and sperm concentration. Therefore, the use of sperm:oocyte ratios could be critical for evaluating semen quality in IVM/IVF systems.

In this study, the high polyspermy rates obtained for Boars A and B were similar to those of other reports using similar sperm:oocyte ratios (Iritani et al., 1978; Rath, 1992). In Boars A and B, the serial dilution of semen used in IVF resulted in a decrease in the polyspermy rate, whereas polyspermy rates in Boar C were low and were not affected by semen dilution. Although the same trend was seen among boars in the average number of spermatozoa per oocyte, the response to semen dilution in Boars A and B was more pronounced (Figure 4-3). Average number of spermatozoa per oocyte may therefore be more useful than the polyspermy rate in characterizing semen quality, although this characteristic still did not discriminate between Boars A and B.

Male pronuclear formation rate, which proved to be an important criterion for assessing IVF results in previous experiments (Xu et al., 1996), provided effective discrimination among all boars at specific sperm:oocyte ratios (Figure 4-4). The overall MPN rate of Boar C was very low compared with that of Boars A and B. However, generally, as the sperm:oocyte ratio declined, MPN formation rates initially increased and then fell once an ideal sperm:oocyte ratio had been reached. Because of the interaction effects seen, the highest MPN rate was obtained at different sperm:oocyte ratio for the 3 boars, indicating that MPN rate can be used to discriminate between boars when evaluating semen quality in vitro.

The results also support our assumption that quality of spermatozoa as well as the maturational state of the oocyte contribute to successful MPN formation. The extent of morphological transformation of the sperm head into a male pronucleus during the first few hours after penetration seemed to be associated with the degree of polyspermy (Hunter, 1976). It has been proposed that mature ooplasm contains a component that promotes the formation of the male pronucleus, referred to as male pronuclear growth factor (MPGF; Thibault, 1977), and Niwa (1993) suggested that glutathione functions like a MPGF in pig oocytes. A spermatozoon may need a certain amount of MPGF to process the decondensation of sperm head chromatin and develop further to a full-size male pronucleus. We postulate that in a polyspermic penetrated oocyte, more than one spermatozoon may compete for MPGF to form a male pronucleus. Therefore, MPN formation rate may be affected by the number of sperm in the oocyte when a high sperm:oocyte ratio is applied.

Of the total number of penetrated oocytes, the proportion of monospermic oocytes with both full-size female and male pronuclei was compared among boars as an important index of successful IVF (Figure 4-5), as these monospermic oocytes were considered to have the potential to develop into normal viable embryos. The results indicate the potential yield of normal embryos from the initial number of oocytes matured in vitro and inseminated with different numbers of spermatozoa from the three boars. Again the optimal sperm:oocyte ratio varied from boar to boar, and using our current IVM/IVF systems, the maximum rate of normal embryo production would be predicted as 32, 25

and 5.5% for Boars A, B and C, respectively. Additionally, although the field data are limited, they show the same trend as the IVF results, and Boar C would certainly be considered subfertile compared with the other two boars.

Overall, the IVF parameters discussed above revealed that considerable variability exits between boars in the ability of their spermatozoa to fertilize oocytes in vitro and suggested the possibility of quantifying this ability as a method of predicting fertility when semen is used in AI programs. If the semen quality can be quantified, then in AI practice the number of effective inseminations that could be achieved from more fertile boars could be increased by more extensive extension of the semen. Conversely, for boars assessed as producing lower quality semen (e.g., Boar C), the number of spermatozoa per insemination dose could be increased to achieve acceptable levels of fertility. This would have important economic benefits to the industry and improve the impact of superior boars in genetic improvement programs.

In conclusion, among the fresh semen characteristics studied in vitro, progressive sperm motility at Day 7 seems to be a better indicator of sperm fertilizing ability and was highly correlated to estimates of oocyte penetration rate. However, these characteristics did not discriminate between Boars A and B, for which acceptable farrowing rates were also obtained. In contrast, in our IVM/IVF evaluations using different sperm:oocyte ratios, the characterization MPN formation rates and possibly the rate of polyspermy and the average number of spermatozoa per oocyte allowed us to discriminate between all the 3 boars studied. We suggest, therefore, that serial dilution of semen used in IVM/IVF systems could provide even better discrimination of semen quality among boars than existing laboratory procedures. The optimization of the sperm concentration used in the IVM/IVF systems would improve not only IVF and pig embryo production but also the evaluation of semen quality.

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5. IVM-IVF TECHNIQUE FOR ASSESSMENT OF SEMEN QUALITY AND PREDICTING BOAR FERTILITY IN VITRO[3]

5.1 INTRODUCTION

Artificial insemination is still greatly under utilized in the pig industry yet it is generally accepted that high levels of AI use can be an essential component of successful genetic improvement programs. To enhance the use of AI, the AI industry needs to be seen as an initiator of new programs aimed at using biotechnological approaches to improve fertility.

Examination of semen characteristics, such as sperm morphology, concentration and progressive motility, are routine procedures for obtaining information about potential male fertility in AI centers. However, several studies on domestic animals showed that these semen characteristics were often not significantly correlated to fertility (Barth, 1992; Buchner et al., 1954; Linford et al., 1976). Computer-assisted semen analysis (CASA) systems have been used to measure the proportion of sperm exhibiting motility and motility characteristics, in order to provide information about the likely fertility of an individual (Holt, 1995). Many tests have been developed for examination of the gross damage to semen (a population of the spermatozoa that have lost their capacity to fertilize) during storage. One of these sperm viability tests is to employ SYBR-14 and propidium iodide to distinguish between intact and damaged spermatozoa (Garner and Johnson, 1995); it is useful for direct observations of sperm in ice during cryomicroscopy and as a long acting detector of sperm viability for assessing cryopreservation protocols (Medrano and Holt, 1996). Another approach is the hypoosmotic swelling test (HOST); it has been proved to be a sensitive and reproducible test to assess the functional integrity of boar sperm membranes after incubation under hypoosmotic stress conditions and may be a useful tool for detecting sub-populations of sub-viable spermatozoa when used in conjunction with another type membrane integrity test (Vazquex et al., 1997). As morphological assessment of sperm allows the recognition of infertile sperm, it is more applicable to the diagnosis of infertility than to semen assessment from normal males (Holt, 1995). The application of a fluorescent antibiotic, chlortetracycline (CTC) makes it possible to distinguish three patterns of sperm: uncapacitated (F pattern), acrosome reacted (AR) or capacitated (B pattern) (Wang et al., 1995). The CTC method is very informative in evaluation of sperm status in vitro; however, the CTC technique needs

[3] Experiments in this chapter were a collaboration with Pig Improvement Canada (PIC), Ltd.

fixation of the sperm prior to the evaluation, and this necessarily precludes the simultaneous assessment of motility and capacitation status (Holt, 1995). Sperm penetrating tests are well accepted as giving information about potential sperm function during sperm-oocyte interactions. In the pig, sperm penetration tests using zona-free hamster ova (Almlid et al., 1989; Clark and Johnson, 1987), zona-intact rat ooocytes (Kiehm et al., 1991), germinal vesicle stage pig oocytes (Martínez et al., 1993), and matured zona-intact pig oocytes (Ivanova and Mollova, 1993) have been reported to be useful for providing information about sperm fertilizing ability of frozen-thawed and freshly ejaculated boar semen.

The most valid assessment of boar semen quality is to obtain viable pregnancies and normal offspring following artificial insemination; however, for obvious reasons this is not practicable as a routine assay of sperm fertilizing ability. There is an urgent need to improve laboratory-based techniques for evaluation of boar fertility and semen quality. Such techniques should 1) accurately predict fertility when compared to fertility estimates based on breeding records, 2) provide a quantitative measure of the fertilizing capacity of sperm rich fractions of semen, thereby allowing the further dilution of semen from superior sires, and 3) incorporate methods for identifying particular sperm populations within the sperm rich fraction of an ejaculate, with the aim improving the fertilizing capacity of dispatched semen.

Methods for improved in vitro maturation (IVM) and in vitro fertilization (IVF) of porcine oocytes have been established in our laboratory (Ding et al., 1992; Xu et al., 1996a, b). Previous results showed that the IVM-IVF systems can be adopted for comparing boar semen quality in vitro, and the criteria used for assessment of IVF results allowed discrimination between boars in apparent semen quality (Xu et al., 1996a). By using a standardized sperm-rich fraction, serial dilution of semen used in IVM-IVF systems provide even better discrimination of semen quality among boars than existing laboratory procedures (Xu et al., 1996b).

The objectives of this study were: 1) To assess boar semen quality in vitro using large numbers of boars and to discriminate potential boar fertility using serially diluted semen in our IVM-IVF systems; and 2) to correlate field data (farrowing rate and litter size) with laboratory semen characteristics and IVM-IVF results to discover the best in vitro parameters for predicting sperm fertilizing ability and boar fertility. A preliminary experiment was carried out to further develop the methodologies required and to establish the complex logistics of co-ordinating the in vitro and in vivo components of the study in collaboration with staff of Pig Improvement (Canada) Ltd (PIC). On the basis of these results and experience gained, the major experiment described in this chapter was then carried out using a proportion of boars from the preliminary experiment, to provide substantial data with which to compare in vitro and in vivo estimates of boar fertility and semen quality.

5.2 MATERIALS AND METHODS

5.2.1 Boar Semen Evaluation In Vitro

5.2.1.1 *Ejaculate Collection for IVF*

In a preliminary experiment, 12 boars (R5114, R52128, R1801, R1984, T3136, T2437, D830, V709, V707, Z160 and Z177) of two different breeds in active AI use were selected from the (PIC) boar stud in Acme, Alberta. They were males with consistently high levels of fertility in the period before the start of the experiment. Semen collection was standardized to once a week for all boars during the period of the experiment and semen samples from a rotation of three boars were assessed every 4 weeks for IVF. This four-week rotation allowed the evaluation of a total of 12 boars in a four-week period and each group of boars was evaluated three times during the course of the experiment. In the main experiment, six boars were selected from the initial group of boars used in the preliminary IVF trial on the basis that they would be available for a sufficient period to permit the collection of field data from up to 50 inseminations at each of two sperm doses used. Although the initial intention had been to utilize those boars with the highest and those with the lowest apparent fertility from the preliminary experiment for further study, the genetic constraints of the PIC nucleus program led us to modify this strategy. The inclusion of inseminations at two semen doses was however incorporated in the hope that this would permit the identification of the relative fertility of boars used for in vivo AI. Semen was collected three times from the six boars for IVF assessment during the experiment. The first collection was the week before the start of recorded breedings, the second collection was in the middle of the breeding period and the third collection was the week after the end of the 20-week breeding period.

For IVF purposes, fresh ejaculates were collected into a series of 15 ml pre-warmed, graduated, conical tubes (Falcon 2095) as described by Xu et al., (1996a) by an experienced operator using the gloved-hand method. Sperm concentration in each tube was tested with Spermacue (Minitube). The tube with highest sperm concentration from the first sperm-rich fraction was selected for use in IVF and transported in a polystyrene box to the University in vitro laboratory on the same day of collection. Semen characteristics, such as the total volume of an ejaculate, sperm density of the ejaculate, percentage of normal sperm morphology (normality) and motility on Day 0, 5 and 7 in extended semen, were recorded for analysis at the site of collection, using the same trained technicians whenever possible.

5.2.1.2 *Oocyte In Vitro Maturation, Fertilization and Assessment of Penetration*

Porcine ovaries were collected from slaughtered prepubertal gilts at the local Gainer's Inc. slaughterhouse and oocytes were prepared for IVM and IVF as described previously (Xu et al., 1996b).

5.2.2 Boar Fertility Evaluation In Vitro

During the preliminary experiment, semen from the same boars was also used for AI in a PIC nucleus herd. A minimum of 20 sows for each boar were bred using triple inseminations with 3 billion total spermatozoa in each dose following routine procedures. The extender "AndroHEP" (Minitub) was consistently used in both the preliminary and main AI trials for semen dilution and preservation. Semen was collected at a frequency of three times in two weeks. Fresh semen was routinely processed as follows: firstly, sperm concentration was tested using a spectrophotometer (Spermacue, Minitube) and the total number of sperm in each ejaculate was calculated; secondly, semen was diluted with warm "AndroHEP" (which was adjusted to the same temperature as that of the semen) to make a sperm concentration of 30 billion /L in the extended semen; finally, the extended semen was aliquoted into 100 ml plastic AI bottles which were shipped immediately to the breeding farm for AI. Boars V707, Z160 and Z177 were culled for various reasons within the first three weeks of the experiment. Breeding data, including farrowing rate and litter size, from the remaining eight boars were therefore finally available for analysis.

During the main breeding trial, ejaculates from boars R5114, R1801, T2437, T3136, D830 and V709 were collected with the same frequency. Fresh semen from each boar was diluted to provide multiple bottles of two different sperm doses (3 billion and 2 billion) for use in AI. The purpose of using two different doses of semen for breeding was to obtain better comparisons of in vivo data among boars and to demonstrate the potential to optimize the use of semen from boars with higher fertility for AI. Pairs of sows were inseminated three times within two days with each sow receiving one or other dose of the same day 0 and day 1 extended semen from the same boar. The target of this breeding trial was to breed a minimum of 50 sows per dose per boar.

5.2.3 Statistical Analysis

Differences in characteristics of fresh semen samples among boars (Preliminary experiment: b=8; Main experiment: b=6) were analyzed using one-way analyses of variance. Analyses of variance of the IVF data were computed as split-plot designs with boar (Preliminary experiment: b=8; Main experiment: b=6) and replicates nested within boar (n_i = 2 to 4) as the main plots and semen dilution (d=3) and boar by semen dilution as the sub-plots. The effect of boar was tested against the term replicates within boar and the effects of semen dilution and boar by dilution interaction were tested against the residual error. All IVF percentage data were subjected to arcsine transformation. Statistical analyses of the fresh semen characteristics and IVF data and least square means (LSM) and standard errors of LSM were carried out using the General Linear Model (GLM) procedure of the Statistical Analysis System (version 6.08, SAS Institute, Cary, NC). Where appropriate, multiple comparisons among treatment means (boars, semen dilution and their interaction) were made using the repeated t-test (P-DIFF function of SAS). In the preliminary experiment, breeding data were analyzed using one-way analyses of variance to determine differences among boars (b=8). During the four months

of the study, the IVF values measured on each of 2 to 4 replicates (collections) of the eight boars were paired with each boar's corresponding breeding values, computed as the average across three weeks (one week before, during, and one week after the replicate). Relationships between these paired values of IVF measurements and breeding values were determined using stepwise regression. When using the stepwise linear regression procedure to analyze the correlation between in vivo data (farrowing rate and litter size) and in vitro parameters (IVF data and laboratory semen characteristics), parameters which met the 0.15 significance level were entered into the regression model. In the main experiment, the whole breeding period (5 month) was divided into three-week blocks (replicates). Analyses of variance of the breeding data were computed as split-split-plot designs with boar (b=6) and replicates nested within boar (n_i = 2 to 5) as the main plots, sperm number per dose (SPN=2) and boar by SPN interaction as the split-plots, and semen age (Day 0 and Day 1; SA=2), boar by SA interaction, SPN by SA interaction and boar by SPN by SA interaction as the split-split plots. The effect of boar was tested against the term replicates within boar and the effects of sperm number per dose were tested against the boar by SPN interaction and boar by SPN interaction were tested against the residual error; the effects of semen age were tested against boar by SA interaction and boar by SA interaction, SPN by SA interaction and boar by SPN by SA interaction were all tested against the residual error. Relationships between the IVF measurements and the breeding values were determined using stepwise regression. The SAS POLYNOMIAL contrast and SUMMARY options were used to reveal the polynomial relations between semen dilution and each of the IVF characteristics for both trials.

5.3 RESULTS

5.3.1 Preliminary Experiment

5.3.1.1 Optical Evaluation of Semen Quality

Mean values of ejaculate volume, total sperm number per ejaculate and percentage of morphologically normal spermatozoa on the day of collection (Day 0), and the percentage of progressively motile spermatozoa on Day 0, Day 5 and Day 7 are shown in Table 5-1. There were differences in ejaculate volume (P=0.004) and in the total number of spermatozoa per ejaculate (P=0.011) among the eight boars. However, there were no differences in sperm normality and motility on either Day 0, Day 5 and Day 7 among boars (all P>0.05). Additional analysis of overall effects on motility over days showed that motility on the three days differed significantly across all boars (P<0.05).

5.3.1.2 IVF Data

The boar and semen dilution effects on penetration rate, polyspermy rate, average number of sperm per penetrated oocyte and average number of sperm attached to the ooplasm membrane are shown in Table 5-2 and Table 5-3. Both boar and semen dilution

affected oocyte penetration rate (all P<0.05). There were semen dilution effects (all P<0.05), but no boar effects and boar by dilution interaction (all P>0.05) for polyspermy rate, monospermy rate, average number of sperm per penetrated oocyte and average number of sperm attached to the ooplasm membrane, and potential embryo production rate. Furthermore, the three dilutions differed significantly across all eight boars for all these IVF characteristics except potential embryo production rate (Table 5-3).

Table 5-1. Fresh semen characteristics of ejaculates collected for IVF from the eight boars during a 4-month period*

Boar	No. of collections	Volume (ml) per ejaculate (LSM±SE)	Total no. of sperm ($\times 10^9$) per ejaculate (LSM±SE)	Sperm normality (%) (LSM±SE)	Motility (%) (LSM±SE)		
					Day 0	Day 5	Day 7
		P=0.004	P=0.011	P=0.48	P=0.79	P=0.48	P=0.95
R5114	4	118±14.1 ^{cd}	73±7.8 ^{ab}	78±5.9	78±6.2	63±8.7	30±12.9
R5128	3	178±14.1 ^{ab}	45±7.8 ^{c}	74±4.8	73±6.2	50±10.1	37±10.6
R1801	3	171±16.2 ^{ab}	88±9.0 ^{a}	74±5.9	67±7.2	47±10.1	33±10.6
R1984	3	182±19.8 ^{ab}	63±11.0 ^{abc}	87±5.9	75±8.8	60±12.4	25±12.9
T3136	3	149±16.2 ^{bc}	68±9.0 ^{abc}	73±4.8	70±7.2	30±12.4	27±10.6
T2437	3	143±16.2 ^{bc}	46±9.0 ^{c}	82±4.8	80±7.2	50±12.4	20±12.9
D830	3	85±16.2 ^{d}	55±9.0 ^{bc}	72±4.8	67±7.2	40±10.1	30±10.6
V709	3	176±14.1 ^{a}	85±7.8 ^{a}	73±4.2	68±6.2	43±8.8	38±10.6
$\bar{x}$					71.9 ^{x}	48.3 ^{y}	30.9 ^{z}

* Values in the table are least squares means (LSM) and standard errors of LSM (SE) of treatments (boars). SEs represent appropriate pooled standard errors of LSMs. The differences of SEs among boars for each measurement are associated with uneven numbers of data for these boars; the contributory factors to these differences were the number of replicated evaluations in specific boars (Replicates for R5114 are 4 compared to 3 in all other animals) and missing data on a few occasions. $^{a-d}$: LSM±SE with different superscripts within each column were different (P<0.05). $^{x-z}$: Mean motility values in the bottom row with different superscripts denote overall effect of sperm motility of different days (P<0.05).

For male pronuclear formation (MPN) rate, there was no boar effect (P>0.05), but there was a semen dilution effect (P=0.002) and a boar by dilution interaction (P=0.048). The statistical analyses of MPN rate using either polyspermy rate, or average number of sperm per penetrated oocyte as covariant, showed similar results as the analyses using

MPN alone. The results of multiple comparisons of the differences among semen dilutions within each boar, and the differences among boars within each semen dilution, are shown in Table 5-4. The semen dilution effects in boars R1801, T2437 and T3136 contributed to the significant boar by semen dilution interactions.

Table 5-2. Boar effects on IVF*

Boar	Penetration rate (%)	Polyspermy rate (%)	Monospermy rate (%)	No. of sperm per oocyte	No. of sperm attached	Potential viable embryo(%)
	P=0.042	P=0.256	P=0.256	P=0.296	P=0.156	P=0.089
R5114	52.9±8.5 ^{ab}	37.0±13.1	63±13.1	2.3±0.5	11.8±2.3	13.2±3.3
R5128	72.1±9.8 ^{a}	63.5±14.0	36±14.0	2.8±0.5	7.3±2.6	10.2±3.5
R1801	42.1±9.8 ^{bc}	23.9±14.0	76.1±14.0	1.7±0.5	5.4±2.6	14.0±3.5
R1984	45.2±9.8 ^{abc}	32.8±14.0	67.2±14.0	1.6±0.5	2.0±3.2	17.4±3.5
T2437	29.2±9.8 ^{bc}	26.7±19.8	73.3±19.8	1.3±0.7	4.5±2.6	11.4±3.5
T3136	23.7±9.8 ^{c}	3.9±19.8	96.1±19.8	1.1±0.7	1.8±2.6	6.9±3.5
D830	25.2±9.8 ^{c}	10.3±19.8	89.7±19.8	1.2±0.7	2.1±3.2	0.0±3.5
V709	35.8±12.0 ^{bc}	20.1±21.0	79.9±21.0	1.3±0.7	4.0±3.2	8.7±4.3

* Values in the table are least squares means (LSM) and standard error of LSM (SE) of treatments (boars) in two to four replicates, with a total 1,400 oocytes evaluated and a minimum of 120 oocytes per treatment. SEs represent appropriate pooled standard errors of LSMs for specific comparisons. The differences of SEs among boars for each IVF parameter are associated with uneven numbers of data for these boars; the contributory factors to these differences were the number of replicated evaluations in specific boars (Replicates for R5114 and V709 are 4 and 2, respectively, compared to 3 in all other animals) and missing data on a few occasions. $^{a-c}$: LSM±SE with different superscripts within each column were different ($P<0.05$).

Table 5-3. Semen dilution effects on different IVF parameters*

Sperm: oocyte ratio	Penetration rate (%)	Polyspermy rate (%)	Monospermy rate (%)	No. of sperm per oocyte	No. of sperm attached	Potential viable embryo (%)
	P=0.0001	P=0001	P=0.0001	P=0.0001	P=0.0001	P=0.119
50000:1	57.3±1.8 [a]	48.6±3.1 [a]	51.4±3.1 [a]	2.7±0.2 [a]	10.5±1.1 [a]	8.3 ±1.8
12500:1	41.9±1.8 [b]	29.6±3.1 [b]	70.4±3.1 [b]	1.6±0.2 [b]	3.2±1.1 [b]	13.4±1.8
3125:1	23.1±1.8 [c]	3.7±4.7 [c]	96.3±4.7 [c]	0.7±0.3 [c]	0.9±1.1 [b]	8.9±1.9

*Values in the table are presented as least squares means (LSM) and the appropriate standard error (SE) of LSM of treatments (semen dilutions) in two to four replicates, with a total of 1,400 oocytes evaluated and 480 oocytes per treatment. SEs represent pooled standard errors of LSMs for specific comparisons. Differences of SEs among dilutions for some IVF parameters are related to uneven numbers of data for the three sperm:oocyte ratios due to missing data on a few occasions. [a-c]: LSM±SE with different superscripts within each column were significantly different (P<0.05).

Table 5-4. Boar by semen dilution effects on male pronuclear (MPN) formation rates(%)*

Boar / Sperm: oocyte ratio	R5114	R5128	R1801	R1984	T2437	T3136	D830	V709
50000:1	45.6± 6.4 [x ab]	61.1± 7.3 [x a]	49.1± 7.3 [x ab]	52.8± 7.3 [x ab]	28.9± 7.3 [x b]	31.7± 7.3 [x b]	0± 7.3 [x c]	32.9± 9.0 [x b]
12500:1	40.6± 6.4 [x ab]	57.9± 7.3 [x a]	61.9± 7.3 [x a]	49.8± 7.3 [x ab]	26.7± 7.3 [x cd]	11.7± 7.3 [xy cd]	0± 7.3 [x d]	33.9± 9.0 [x bc]
3125:1	30.2± 7.8 [x ab]	45.0± 7.3 [x a]	0.0± 7.3 [y c]	36.2± 7.3 [x ab]	44.1± 4.7 [y a]	0.0± 14.7 [y c]	0± 14.7 [x c]	11.6± 14.2 [x bc]

* Values in the table are least squares means (LSM) and the appropriate standard errors (SE) of LSM of treatments (boar × semen dilution) in two to four replicates, with a total of 1,400 oocytes evaluated and a minimum of 40 oocytes per treatment. SEs represent pooled standard errors of LSMs. The differences of SEs among boars for each LSM are related to uneven numbers of data for these treatments. The contributory factors to these differences were the number of replicated evaluations in specific boars (Replicates for R5114 and V709 are 4 and 2, respectively, compared to 3 in all other animals) and missing data on a few occasions. [x-y]: LSM±SE with different superscripts within each column were different (P<0.05). [a-d]: LSM±SE with different superscripts within each row were different (P<0.05).

5.3.1.3 Results of Polynomial Analyses

The relationships between different sperm:oocyte ratios (semen dilutions) and each of the IVF parameters for the eight boars are listed in Table 5-5. Penetration rate (Figure 5-1), polyspermy rate, number of sperm per penetrated oocyte and potential embryo production rate showed quadratic relationships with sperm:oocyte ratio (all $P<0.005$) and for these characteristics there were no quadratic by boar interactions. The relationship between sperm:oocyte ratio and MPN formation rate was also significant at a quadratic level ($P<0.005$); however for this parameter, the quadratic by boar interaction was also significant ($P<0.05$), indicating that changes in sperm:oocyte ratios affect MPN formation rate differently in individual boars (Figure 5-2). The number of sperm attached on the ooplasm membrane showed a linear relationship with sperm:oocyte ratios ($P<0.005$) but no linear by boar interaction (Figure 5-3).

Table 5-5. Polynomial analyses of relationships between sperm:oocyte ratio and different IVF parameters for the eight boars

	Penetration rate (%)	Polyspermy rate (%)	MPN formation rate (%)	No. of sperm per oocyte	No. of sperm attached	Potential viable embryo (%)
Degree of polynomial contrast	Quadratic P=0.0001	Quadratic P=0.0003	Quadratic P=0.004	Quadratic P=0.045	Linear P=0.0005	Quadratic P=0.035
Quadratic by boar interaction	P=0.553	P=0.483	P=0.045	P=0.305	P=0.176*	P=0.130

*Linear by boar interaction.

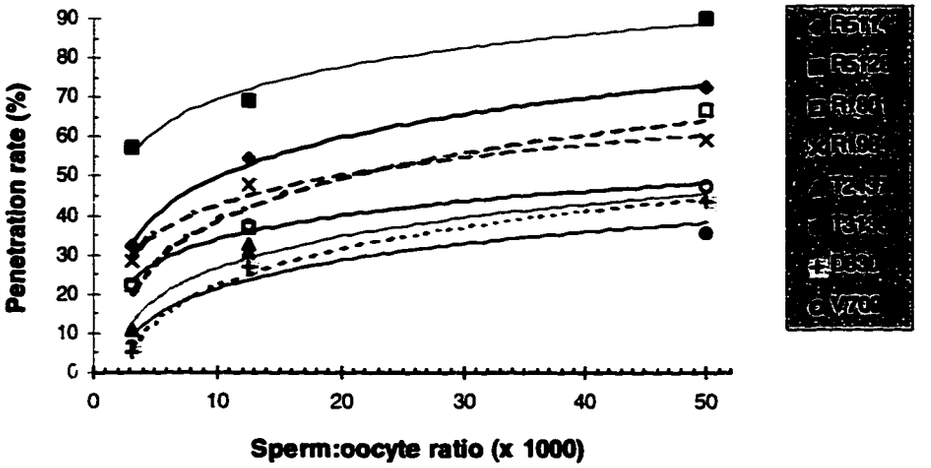

Figure 5-1. Quadratic relationship of sperm:oocyte ratio (semen dilution) and oocyte penetration rate for the eight boars.

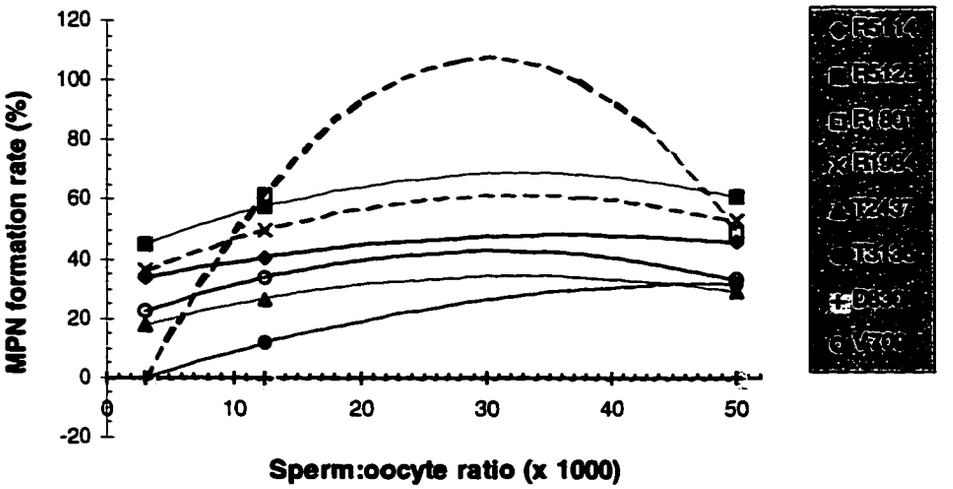

Figure 5-2. Quadratic relationship of sperm:oocyte ratio (semen dilution) and MPN formation rate for the eight boars. There was a significant interaction effect between these two measurements.

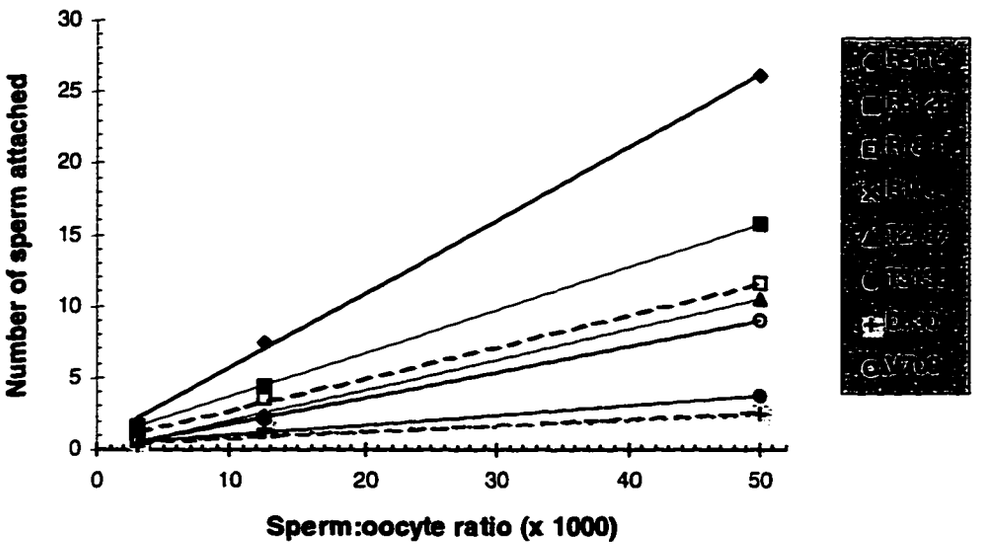

Figure 5-3. Linear relationship of sperm:oocyte ratio (semen dilution) and average number of sperm attached per oocyte for the eight boars.

Table 5-6. Breeding data of the eight boars during a period of four months

Boar	Number of sows bred	Farrowing rate (%) (LSM±SE)	Litter size (LSM±SE)
		P=0.0001	P=0.0001
R5114	20	61.3±9.1 [b]	12.6±1.4 [ab]
R5128	24	67.5±9.1 [b]	8.6±1.4 [b]
R1801	29	78.1±11.2 [ab]	10.3±1.7 [ab]
R1984	21	63.1±9.1 [b]	13.5±1.4 [a]
T2437	36	100±9.1 [a]	11.5±1.4 [ab]
T3136	37	48.0±11.2 [b]	10.0±1.7 [ab]
D830	28	74.0±9.1 [ab]	10.9±1.4 [ab]
V709	31	79.0±11.2 [ab]	8.4±1.7 [b]

[a-b]: Within each column, values with different superscripts were different (P<0.05).

5.3.1.4 In Vivo Results

Breeding data from the eight boars were collected during a four month AI trial. There were significant differences among boars in both farrowing rate and litter size (Table 5-6).

5.3.1.5 Stepwise Linear Regression Analyses of In Vitro Parameters on In Vivo Data

Since there were significant boar effects on farrowing rate and litter size, the relationships of in vitro parameters to both of the in vivo measurements were analyzed.

Correlation between IVF parameters and in vivo data: For Dilution 1, no IVF parameters correlated to the in vivo data. For Dilution 2, only potential embryo production rate (EmbryoR) correlated to farrowing rate. For Dilution 3, no parameters correlated to farrowing rate but the number of sperm per penetrated oocyte (SpermN) and the number of sperm attached per oocyte (AttachN) correlated to litter size. The relevant regression equations for these relationships are listed below:

Dilution 2: Farrowing rate = 57.35 + 0.81 EmbryoR; R^2 =0.17 (P=0.08)

Dilution 3: Litter size = 3.86 + 7.29 SpermN - 1.1 AttachN; R^2 =0.41 (P=0.07)

Correlation between laboratory semen characteristics and in vivo data: No semen characteristics showed correlations with farrowing rate and only percentage normal sperm (Normality) was positively correlated to litter size. The relevant regression equation for this relationship is listed below:

Litter size = 0.06 + 0.15 Normality; R^2 = 0.26 (P=0.036).

5.3.2 Main Experiment

5.3.2.1 Optical Evaluation of Semen Quality

Mean values of ejaculate volume, total sperm number per ejaculate and percentage normal spermatozoa, and percentage of progressively motile spermatozoa on the day of semen collection (Day 0), Day 1, Day 4 and Day 8 are shown in Table 5-7. There were differences in ejaculate volume (P=0.0001), in the total number of spermatozoa per ejaculate (P=0.0002), sperm normality (P=0.0001) and sperm motility at different days (all P<0.005) among the six boars. However, there was no overall day effect on sperm motility across all boars (all P>0.05) and the day by boar interactions were not significant (P>0.05).

Table 5-7. Motility and normality of semen collected from the six boars during the period of breeding*

Boar	No. of collec -tion	Volume (ml) per ejaculate (LSM±SE)	Total no. of sperm (×109) per ejaculate (LSM±SE)	Normality (%) (LSM±SE)	Motility (%) (LSM±SE)			
					D0	D1	D4	D8
		P=0.0001	P=0.0002	P=0.0001	P=0.0017	P=0.0001	P=0.0001	P=0.0001
R5114	14	181.3±24.8 ^{c}	402.0±25.0 ^{b}	74.7±2.3 ^{a}	75.1±1.2 ^{a}	73.9±1.5 ^{a}	73.2 ±1.7 ^{a}	72.5±2.4 ^{a}
R1801	14	384.9±24.8 ^{a}	444.2±25.0 ^{ab}	75.6±2.3 ^{a}	70.0±1.2 ^{ab}	67.8±1.5 ^{b}	67.8 ±1.7 ^{bc}	65.3±2.4 ^{b}
T2437	13	285.0±25.8 ^{b}	321.7±25.9 ^{c}	74.3±2.5 ^{a}	70.8±1.2 ^{ab}	68.5±1.6 ^{b}	66.9 ±1.8 ^{bc}	60.0±2.5 ^{bc}
T3136	13	285.1±25.8 ^{b}	397.6±25.9 ^{b}	60.3±2.5 ^{b}	70.4±1.2 ^{ab}	66.9±1.6 ^{b}	65.0 ±1.8 ^{c}	53.8±2.5 ^{c}
D830	14	236.5±37.9 ^{bc}	482.8±25.0 ^{a}	77.5±2.3 ^{a}	68.6±1.2 ^{b}	68.9±1.5 ^{b}	70.0 ±1.7 ^{ab}	68.9±2.4 ^{a}
V709	6	308.8±37.9 ^{ab}	311.0±38.2 ^{c}	61.7±3.6 ^{b}	62.5±1.8 ^{c}	53.3±2.3 ^{c}	47.5±2.6 ^{d}	25.0±3.6 ^{d}

*Values are least squares means (LSM) and standard errors (SE) of LSM of treatments (boars) for specific comparisons. SE represent appropriate pooled standard errors of LSM. The differences of SE among boars for each measurement are associated with uneven numbers of data for these boars; the contributory factors to these differences were the number of replicated evaluations in specific boars and missing data on a few occasions. $^{a-d}$: LSM±SE with different superscripts within each column were significantly different (P<0.05).

5.3.2.2 IVF Data

There were effects of boar (Table 5-8) and dilution (Table 5-9) on all the IVF parameters measured (all P<0.05). However, boar by dilution interactions only affected male pronuclear formation rates (P<0.05; Table 5-10). Compared to all other boars studied, boar V709 showed lower values for penetration rate, polyspermy rate, average number of sperm per penetrated oocyte and average number of sperm attached per oocyte and conversely a higher monospermy rate than all other boars.

Table 5-8. Boar effects on IVF*

Boar	Penetration rate (%)	Polyspermy rate (%)	Monospermy rate (%)	No. of sperm per oocyte	No. of sperm attached	Potential viable embryo (%)
	P=0.0001	P=0.0001	P=0.0001	P=0.0001	P=0.01	P=0.018
R5114	97.9 ^{a}	99.2 ^{a}	0.8 ^{a}	18.7 ^{a}	19.5 ^{b}	0.4 ^{b}
R1801	96.7 ^{a}	93.8 ^{a}	6.2 ^{a}	8.9 ^{c}	16.3 ^{bc}	4.5 ^{b}
T2437	80.0 ^{b}	63.2 ^{b}	36.8 ^{b}	6.6 ^{d}	13.9 ^{c}	13.2 ^{a}
T3136	73.0 ^{c}	58.9 ^{b}	41.1 ^{b}	3.3 ^{e}	3.4 ^{d}	13.7 ^{a}
D830	94.0 ^{a}	94.4 ^{a}	5.6 ^{a}	14.8 ^{b}	35.7 ^{a}	4.3 ^{b}
V709	6.9 ^{d}	1.1 ^{c}	98.9 ^{c}	0.8 ^{f}	0.3 ^{d}	2.1 ^{b}
SEM	3.7	4.9	4.9	0.7	2.7	2.1

*Values are least squares means (LSM) and pooled standard error of LSM (SE) of treatments (boars) in three replicates, with a total of 1,080 oocytes evaluated and 180 oocytes per treatment.
$^{a-f}$: LSM±SE with different superscripts within each column were significantly different (P<0.05).

The statistical analyses of MPN rate using either polyspermy rate or average number of sperm per penetrated oocyte as covariate showed similar results as the analyses of MPN alone. The results of multiple comparisons of the differences among semen dilutions within each boar, and the differences among boars within each semen dilution, are shown in Table 5-10. Boar V709 showed a significantly lower MPN formation rate compared to the other boars at the two lower sperm:oocyte ratios (all P<0.05).

Table 5-9. Semen dilution effects on different IVF parameters*

Sperm: oocyte ratio	Penetration rate (%)	Polyspermy rate (%)	Monospermy rate (%)	No. of sperm per oocyte	No. of sperm attached	Potential viable embryo (%)
50000:1	82.8 ^{a}	78.1 ^{a}	21.9 ^{c}	13.4 ^{a}	33.2 ^{a}	2.1 ^{b}
12500:1	75.3 ^{b}	68.2 ^{b}	31.8 ^{b}	8.7 ^{b}	8.8 ^{b}	8.7 ^{a}
3125:1	66.1 ^{c}	58.9 ^{c}	41.1 ^{a}	4.4 ^{c}	2.6 ^{c}	8.3 ^{a}
SEM	1.2	1.6	1.6	0.3	1.1	1.0

* Values are least squares means (LSM) and pooled standard error (SE) of LSM of treatments (semen dilutions) in three replicates, with a total of 1,080 oocytes evaluated and 360 oocytes per treatment. $^{a-c}$: LSM±SE with different superscripts within each column were significantly different ($P<0.05$).

Table 5-10. Boar by semen dilution effects on male pronuclear (MPN) formation rates (%)*

Boar / Sperm:oocyte ratio	R5114	R1801	T2437	T3136	D830	V709	SEM
50000:1	19.8 $^{x\,b}$	54.2 $^{x\,a}$	32.3 $^{x\,ab}$	28.7 $^{x\,ab}$	49.1 $^{x\,a}$	15.8 $^{x\,b}$	8.1
12500:1	64.5 $^{y\,b}$	87.1 $^{y\,a}$	70.8 $^{y\,b}$	63.4 $^{y\,b}$	63.2 $^{xy\,b}$	34.3 $^{x\,c}$	8.1
3125:1	53.8 $^{y\,ab}$	72.9 $^{xy\,a}$	45.8 $^{xy\,b}$	42.0 $^{xy\,b}$	75.2 $^{y\,a}$	0.0 $^{y\,c}$	8.1
SEM	8.1	8.1	8.1	8.1	8.1	8.1	

* Values are least squares means (LSM) and pooled standard error (SE) of LSM of treatments (boar by semen dilution) in three replicates, with a total of 1,080 oocytes evaluated and 60 oocytes per treatment. $^{x-y}$: LSM±SE with different superscripts within each column were significantly different ($P<0.05$). $^{a-c}$: LSM±SE with different superscripts within each row were significantly different ($P<0.05$).

5.3.2.3 Results of Polynomial Analyses

Results of polynomial analyses of the relationships between different sperm:oocyte ratios (semen dilutions) and each of the IVF parameters for the six boars are listed in Table 5-11. For penetration rate and polyspermy rate, their relationships with sperm:oocyte ratio were significant at a quadratic level (both $P<0.05$) (for example, see Figure 5-4) and there were no quadratic by boar interactions. The relationship between

sperm:oocyte ratio and number of sperm per penetrated oocyte was significant (P<0.05) at a quadratic level, and a quadratic by boar interaction was also observed (P<0.01). Number of sperm attached on the ooplasm membrane had a linear relationship with sperm:oocyte ratios (P<0.005) and there was also a linear by boar interaction such that the correlation lines for the six boars had different slopes (Figure 5-5). The relationship between sperm:oocyte ratio and MPN formation rate was significant at a quadratic level (P<0.05) and the quadratic by boar interaction was also significant (P=0.045; Figure 5-6). Potential embryo production rate had a quadratic relationship with sperm:oocyte ratio (P<0.01) and a significant quadratic by boar interaction (P<0.05; Figure 5-7)).

Table 5-11. Polynomial analyses of relationships between sperm:oocyte ratios (semen dilutions) and different IVF parameters for the six boars.

	Penetration rate (%)	Polyspermy rate (%)	MPN formation rate (%)	No. of sperm per oocyte	No. of sperm attached	Potential viable embryo (%)
Degree of polynomial contrast	Quadratic P=0.022	Quadratic P=0.041	Quadratic P=0.0007	Quadratic P=0.0001	Linear P=0.0001	Quadratic P=0.005
Quadratic by boar interaction	P=0.933	P=0.788	P=0.045	P=0.005	P=0.001*	P=0.033

* Linear by boar interaction.

5.3.2.4 In Vivo Data

The total number of sows bred to each boar using each dose of semen during the experimental period are shown in Table 5-12. Boar V709 was removed from the AI trial after the second month of breeding because sperm motility and normality were below acceptable levels.

Table 5-12. Total number of sows bred using two dilutions of semen from the six boars.

Number of sperm per AI	R5114	R1801	T2437	T3136	D830	V709
3 billion	38	54	45	42	33	13
2 billion	42	53	44	39	29	12

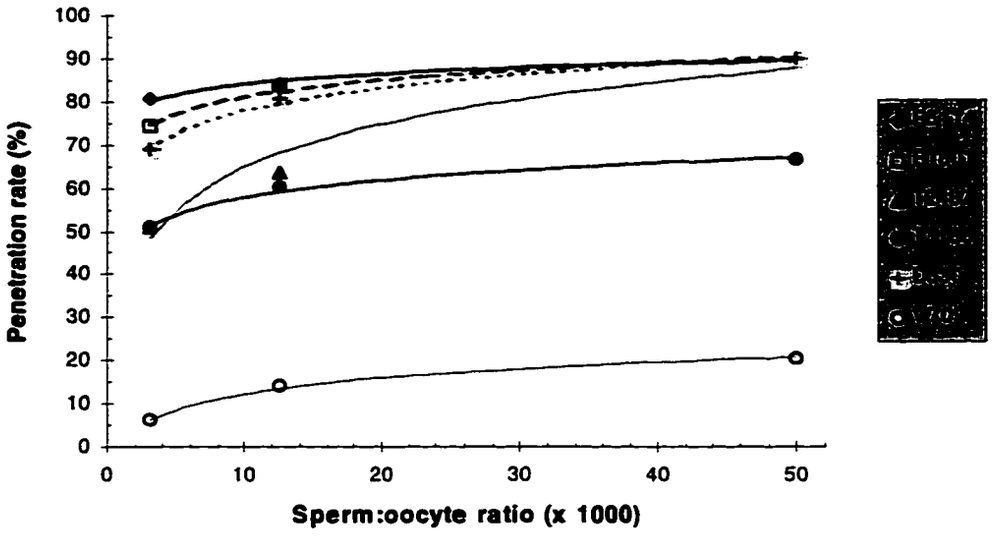

Figure 5-4. Quadratic relationship between sperm:oocyte ratio (semen dilution) and penetration rate for the six boars. No quadratic by boar interaction existed (P>0.05).

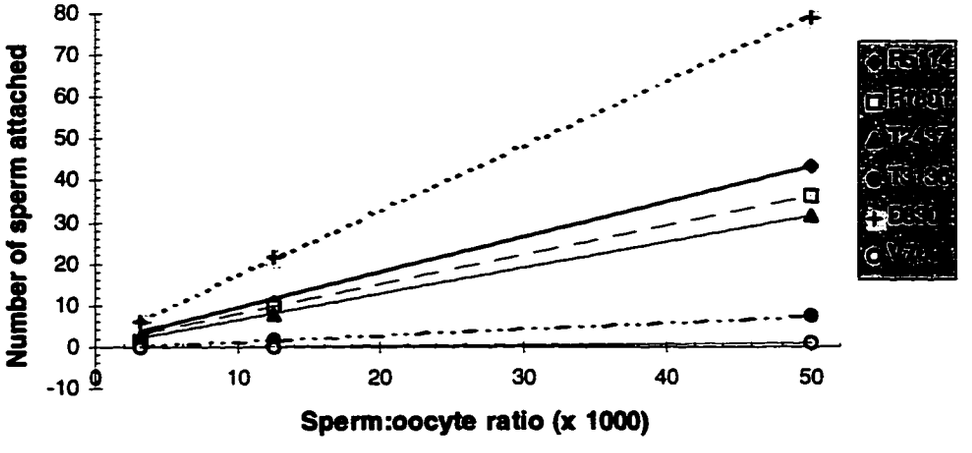

Figure 5-5. Linear relationships and the interactions between sperm:oocyte ratio (semen dilution) and average number of sperm attached per oocyte for the six boars.

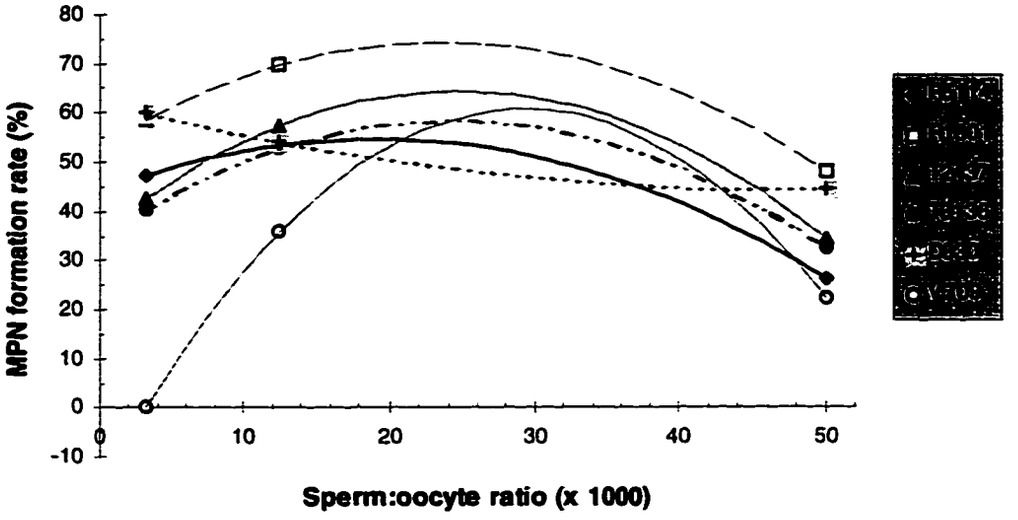

Figure 5-6. Quadratic relationship between sperm:oocyte ratio (semen dilution) and MPN formation rate for the six boars. The quadratic by boar interaction was significant (P=0.055).

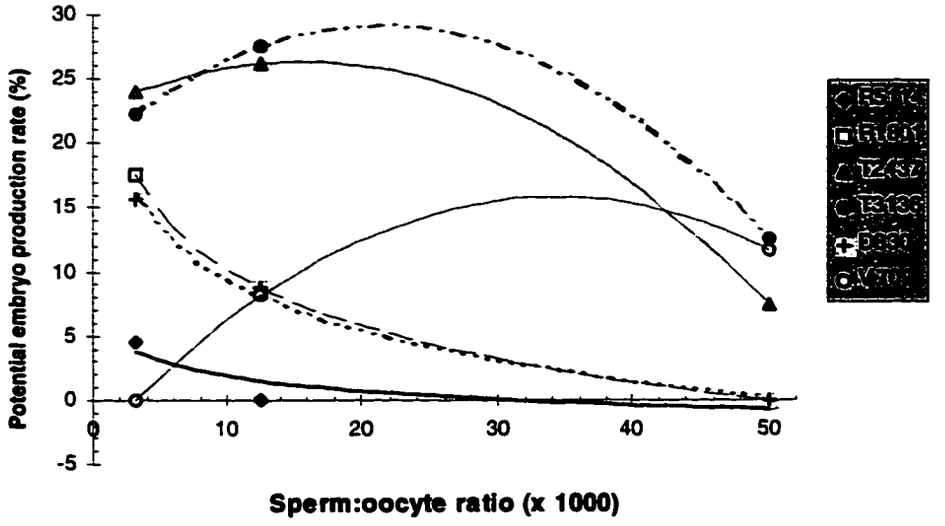

Figure 5-7. Quadratic relationships and the interactions between sperm:oocyte ratio (semen dilution) and potential embryo production rate for the six boars.

The in vivo breeding results are shown in Table 5-13. There were no differences among boars for farrowing rate and litter size (both P>0.05) and use of Day 0 or Day 1 extended semen for the first insemination did not affect breeding results (P>0.05). Reducing sperm number per dose from 3 billion to 2 billion did not affect farrowing rate, but reduced litter size from 10.8 to 10.0 (P=0.045). All interactions examined were not significant.

Table 5-13. Effects of boar, sperm number per dose (SPN) and sperm age (SPA) on farrowing rates and litter sizes

Source of factors	DF	Farrowing rate		Litter size	
		P value	Mean±SEM	P value	Mean±SEM
Boar	5	0.4089	74.6±5.9 (R5114) 69.1±5.9 (R1801) 82.5±8.6 (T2437) 90.1±9.4 (T3136) 78.5±5.9 (D830) 91.7±13.3 (V709)	0.9168	10.0±0.6 (R5114) 10.2±0.6 (R1801) 10.9±0.9 (T2437) 11.2±1.0 (T3136) 10.7±0.7 (D830) 10.4±1.4 (V709)
Replicates within boar	19	0.5047		0.0638	
SPN	1	0.7382	80.7±5.2 (3 billion) 81.5±5.0 (2 billion)	0.0451	10.8±0.3 (3 billion)[a] 10.0±0.3 (2 billion)[b]
Boar by SPN interaction	5	0.2875		0.8461	
SPA	1	0.4522	79.7±4.1 (Day 0) 82.5±6.0 (Day 1)	0.1502	10.1±0.4 (Day 0) 10.5±0.6 (Day 1)
Boar by SPA interaction	5	0.2904		0.4287	
SPN by SPA interaction	1	0.8197		0.2064	
Boar by SPN by SPA interaction	5	0.8674		0.6464	

5.3.2.5 Stepwise Linear Regression Analyses of In Vitro Parameters on In Vivo Data

Since there were no significant boar and semen dose effects on farrowing rate, only the relationship of in vitro parameters on litter size were analyzed.

Correlation between IVF parameters and litter size: IVF data were sorted by the three sperm:oocyte ratios (semen dilutions). Litter size obtained from the two doses of semen (3 billion and 2 billion) were expressed as litter size (H) and (L), respectively. On the basis of the results from the preliminary experiment, and to examine what were considered to be the most physiologically meaningful and directly comparable relationships, the in vitro parameters were expressed as average numbers of sperm per penetrated oocyte (SpermN), average numbers of sperm attached per oocyte (AttachN) and potential embryo production rate (EmbryoR). The regression equations for the significant relationships established are listed below:

Dilution 1: Litter size (H) = 10.62 + 0.08 EmbryoR; R^2=0.22 (P=0.046)

Litter size (L) = 8.91 + 0.12 AttachN - 0.02 SpermN; R^2 = 0.55 (P=0.026)

Dilution 2: Litter size (H) = 10.4 + 0.05 EmbryoR; R^2 = 0.70 (P=0.001)

Litter size (L) = 9.48 + 0.02 AttachN; R^2 = 0.36 (P=0.04)

Dilution 3: Litter size (H) = 10.55 + 0.05 EmbryoR -0.05 AttachN; R^2=0.55 (P=0.03)

Litter size (L) = 9.32 +0.03 EmbryoR +0.05 Attached; R^2 = 0.53 (P=0.03)

Correlation between laboratory semen characteristics and in vivo data: Laboratory semen characteristics were expressed as ejaculate volume (EjaVol), sperm normality (Normality), sperm progressive motility on Day 8 (D8), and total number of sperm per ejaculate (Total). The linear regression equation for the only significant relationship established is given below:

Litter size (L) = 7.26 + 0.04 Normality; R^2 = 0.59 (P=0.07).

5.4 DISCUSSION

Successful AI depends upon the production, preparation and delivery of consistently high quality semen. The accurate evaluation of boar fertility is of prime importance for AI centers to obtain optimal results in the pig industry. The monitoring of sperm production of individual boars and the selection of boars for sperm quality will

enable AI centers to produce sperm doses efficiently and economically (Colenbrander et al., 1993). Development of new in vitro tests for sperm quality assessment and for prediction of fertilizing capacity would be of great value for optimizing boar selection and semen handling procedures. The widely used in vitro tests for evaluation of sperm quality are associated with three qualitative semen traits: sperm viability, morphology (structure) and the functionality of sperm. In vitro fertilization of homologous porcine oocytes is potentially more suitable for assessing overall sperm fertilizing function. However, in vivo field trials are needed for validating such in vitro sperm quality tests.

In the current studies, a reasonable number of fertile boars were selected from the PIC boar stud and freshly ejaculated semen from these boars was used to inseminate sows in a nucleus herd in two breeding trials for collection of in vivo fertility data. Over the same periods, in vitro maturation and fertilization experiments were carried out using semen from the same boars for evaluation of semen quality in vitro at the beginning, in the middle, and at the end of the breeding trials. There appear to be no published studies which provide comparable information of laboratory semen characteristics, comprehensive IVF results, AI field data and their correlation in boars.

Our previous studies showed that use of a sperm-rich part of the standardized fraction from an ejaculate reduced variations between ejaculates and boars and provided the best semen for use in IVF (Xu et al., 1996a). A standardized aliquot of the sperm-rich fraction of all ejaculates was again used in the present experiments. Although very time consuming, the IVM-IVF systems have been shown to be adaptable for comparing boar semen quality in vitro, and the criteria used allowed for discrimination between boars in terms of apparent semen quality, especially when serially diluted semen was used for IVF (Xu et al., 1996b). Therefore the same IVF parameters were used at three semen dilutions (sperm:oocyte ratios) to maximize the chance of revealing variations in semen quality among boars. In the preliminary experiment, boar effects were significant only on oocyte penetration rate, whereas semen dilution effects were significant for all IVF parameters except potential embryo production rate. As the sperm:oocyte ratio decreased, values of penetration rate, polyspermy rate, average number of sperm per penetrated oocyte and average number of sperm attached per oocyte dropped significantly but the trend was similar in all the boars studied. However, a boar by semen dilution effect was seen on male pronuclear (MPN) formation rate and boars R1801, T2437 and T3136 in particular contributed to this interaction. Table 5-4 shows that the optimal sperm:oocyte ratio to obtain the highest MPN formation rate varied among boars, which supports our previous results that MPN formation rate is especially useful to discriminate between boars when evaluating semen quality in vitro (Xu et al., 1996b). The values of penetration rate and other parameters of V709 dropped dramatically compared to those of the preliminary experiment, indicating a change in semen quality of this boar and this animal was eventually withdrawn from service.

Semen dilution effects on IVF outcomes have been showed previously and a positive correlation between sperm:oocyte ratio and polyspermy rate was observed (Rath, 1992). In the current studies, relationships between sperm:oocyte ratio and a number of

different IVF parameters were established and described by different "best fit" trend lines (see Figures 5-1 to 5-7). Disregarding the absolute values of the IVF parameters in the two parts of the study, both penetration rate and polyspermy rate showed significant quadratic relationships, but no interactions with sperm:oocyte ratio. Since those 'best fit' trend lines are parallel to each other, any sperm:oocyte ratio chosen for IVF would produce a similar comparison in the case of penetration and polyspermy rates for these boars. In the preliminary experiment, the penetration rates for the eight boars ranged from 30 to 85% at the sperm:oocyte ratio 50,000:1, and from 5 to 58% at the sperm:oocyte ratio 3125:1 (Figure 5-1). However, in the main experiment, the penetration rates for all except boar V709 ranged from 62 to 85% at the sperm:oocyte ratio 50,000:1, and from 50 to 80% at the sperm:oocyte ratio 3125:1 (Figure 5-4) with less of a dilution effect than shown in Figure 5-1. The semen collected from boars of different ages may account for this discrepancy in sperm penetrability, since the eight boars used in the preliminary experiment were young boars (8-9 months old), whereas when six of these boars were used in the main experiment they were adult boars (16-17 months old). These results suggest that for penetration rate as well as polyspermy rate, sperm of young boars responded to semen dilution more sensitively than did sperm of adult boars. Therefore, when using penetration rate to achieve the best discrimination between boars in vitro, a sperm:oocyte ratio in the range from 3125:1 to 12500:1 is suggested for young boars, whereas a further dilution of semen (sperm:oocyte ratio < 3125:1) is suggested for adult boars. In both experiments, boar effects on oocyte penetration rates were significant, which offers the possibility for the application of this IVF parameter in the AI industry to compare semen quality among boars in vitro using a simplified IVM-IVF procedure.

It is interesting to note the different relationships between sperm:oocyte ratio and average number of sperm per penetrated oocyte and average number of sperm attached per oocyte, as these two parameters respond to the sperm:oocyte ratio change in different ways. It is not difficult to understand the linear relationship between sperm:oocyte ratio and average number of sperm attached per oocyte. During in vitro fertilization, only a certain proportion of sperm population will undergo capacitation and the acrosome reaction, penetrate the zona pellucida and then interact with the oocyte ooplasm (see Yanagimachi, 1994). As the sperm:oocyte ratio increases, the sub-population of sperm that is able to penetrate the zona and attach to the ooplasm membrane in the fertilization medium increases in the same proportion (the linear relationship is shown in Figures 5-3 and 5-5). After sperm have attached to the ooplasm membrane, they may either penetrate into the oocyte, or be stopped at this stage because of the ooplasm membrane block, although this block is weak and incomplete compared to the zona block in the pig (see Parrish and First, 1993). This may explain the quadratic relationship between sperm:oocyte ratio and average number of sperm per penetrated oocyte. For practical purposes, when using these two IVF parameters for discrimination of boars, use of a high sperm:oocyte ratio (e.g. 50,000:1) appears to give a better comparison.

For MPN formation rate, which correlated to sperm:oocyte ratio in a quadratic manner, the boar by quadratic interaction was also significant, suggesting that boars might have different expected trend lines (see Figures 5-2 and 5-6). Therefore, when

using this parameter for comparison of boars, a serial dilution of semen will be needed to characterize the variations in MPN formation rate changes across a large range of sperm:oocyte ratios (e.g. from 3125:1 to 50,000:1 in the current study). The relationship of sperm:oocyte ratio and potential embryo production rate was quadratic and in the main experiment, the boar by quadratic effect was also significant (see Figure 5-7). Since the potential embryo production rate integrated information on penetration rate, monospermy rate and MPN formation rate, the boar by quadratic effect is probably mainly related to the significant boar by MPN interaction. Although the assessment of MPN formation rates and thus potential embryo production rate may be an important predictor of sperm quality, in practice the in vitro methods needed are considerably more complex than for say estimates of penetration rate. Therefore the predictive value of these characteristics will have to be very high to justify such technology in a practical situation.

To find out which IVF parameter(s) could be used for predicting boar fertility, correlations between in vitro fertilization results and in vivo field data were established using stepwise regression analyses. To avoid using confounded parameters in the model, potential embryo production rate instead of penetration, polyspermy and MPN formation rates was analysed together with two other parameters, the number of sperm per penetrated oocyte and the number of sperm attached per oocyte. In the preliminary experiment, at a high sperm:oocyte ratio (50000:1; Dilution 1), no IVF parameters correlated to both farrowing rate and litter size. When a reduced sperm:oocyte ratio (12500:1; Dilution 2) was applied, potential embryo production rate (EmbryoR) correlated positively to farrowing rate. However, when sperm:oocyte ratio was further reduced to 3125:1 (Dilution 3), no IVF parameters correlated to farrowing rate. The combination of two factors, the number of sperm per penetrated oocyte (SpermN; P=0.05) and the number of sperm attached per oocyte (AttachN; P=0.11), contributed to the multiple regression equation describing effects on litter size, of which SpermN was the most important.

In the main experiment, as there was no boar effect on farrowing rate, stepwise analyses were only applied to litter size. For all the three semen dilutions, the potential embryo production rate was a major factor accounting for up to 70% of the predicted litter size obtained from 3 billion sperm dose per AI. The number of sperm attached per oocyte was the major factor accounting for 39% and 36% of the predicted litter size obtained from 2 billion sperm dose per AI, for dilutions 1 and 2, respectively; and for dilution 3, the combination of AttachN and EmbryoR accounted for 53% of the predicted litter size obtained from 2 billion sperm dose per AI. Results from the main experiment showed that the potential embryo production rate, a measurement which probably related to a combination of sperm functions such as penetrability, sperm oocyte interaction and male pronuclear formation, consistently correlated to litter size when a conservative dose of semen (3 billion) was applied for AI. However, when the number of sperm per AI dose was reduced from 3 billion to 2 billion, the number of sperm attached per oocyte (AttachN), a parameter representing a proportion of sperm that have undergone the acrosome reaction and attached to the ooplasma membrane in the whole sperm population used in vitro, became the major factor correlated to litter size. Compared to the results of

the main experiment, in the preliminary experiment relationships between in vivo data and IVF outcomes seemed to depend more on the semen dilutions used in IVF.

In vitro sperm function tests are required to predict the outcomes of numerous processes including efficiency of sperm transport, speed of capacitation, ability to bind to the oocyte surface, undergo the acrosome reaction, penetrate the zona pellucida and fuse with the oolemma. However, different results from in vitro and in vivo fertilization are often observed, since conditions under which sperm are induced to fertilize in vitro clearly differ very greatly from the in vivo situation (Harrison et al., 1996). In vivo, the inseminated sperm must remain viable within the female reproductive tract for a considerable period of time and only a small number of sperm ascend the upper parts of the oviduct shortly before ovulation (Hunter, 1981). The oviductal isthmus acts as a specific sperm reservoir protecting sperm from premature capacitation (Hunter, 1995). As the normal ovulation process takes 2 to 3 hours in the pig, the sperm population response to full capacitating conditions should be diverse rather than rapid (Harrison, 1997). Recently, in vitro evidence has been obtained that within the whole sperm population, individual populations of sperm respond to capacitating conditions at widely different rates. Generally, the sperm population consists of two broad groups, sperm that respond to capacitation effectors rapidly and sperm that respond slowly (Harrison, 1997). It is postulated that the slowly responding sub-population might represent the sperm which would be more likely to fertilize oocytes in vivo (Harrison, 1997). Although there is still no evidence to show whether sperm that penetrated oocytes in vitro are from the rapidly responding sub-population, the strong correlation between the number of sperm attached per oocyte and the litter size obtained using 2 billion sperm per dose for AI suggests that the proportion of sperm attached to oocyte cytoplasm membrane at the time of examination (after insemination for about 14 h) could be the sub-population of sperm responding slowly to the in vitro capacitation condition and might represent the sperm population that would fertilize oocytes in vivo.

Among the laboratory semen characteristics measured in the present study, sperm normality (percentage of sperm with normal morphology) was the only measurement that positively correlated to litter size when either 3 billion sperm per dose were used in the preliminary experiment, or 2 billion sperm per dose were used in the main experiment. In the main breeding trial, reduction of sperm numbers from 3 billion to 2 billion per dose did not affect the farrowing rate but reduced the mean litter size from 10.8 to 10.0, which indicates that litter size responds more sensitively to sperm population changes than farrowing rate. This apparent difference in sensitivity is consistent with the observation that whilst one of the semen characteristics contributed to litter size, none of them contributed to differences in farrowing rate. For the adult boars used in the main experiment, 3 billion sperm per AI dose appeared to be conservative and this large number of sperm might 'mask' the real variation in boar fertility. However, using 2 billion sperm per AI dose from adult boars and 3 billion sperm per dose from young boars, allowed discrimination of boar fertility, and sperm normality accounted for 56% and 26%, respectively, of the variation in fertility among boars. As discussed previously, oocyte penetration rates in the preliminary experiment were lower than those in the main

experiment when the same sperm:oocyte ratio was used for IVF; therefore the number of fertilizable sperm in the 3 billion sperm per dose from young boars might be equvalent to that in 2 billion sperm from adult boars. A study in the bull showed that morphology tests of semen quality accounted for 65% of the variation in fertility among bulls (Saacke and White, 1972). The morphology of sperm also plays an important role in fertility in stallions (Jasco et al., 1990; Parlevliet et al., 1994). Our observations support these results and suggest that the percentage of normal sperm may be a useful indicator for predicting boar fertility in vivo. The lack of correlation between in vivo sperm fertilizing potential and some of the semen characteristics such as motility, total number of sperm per ejaculate and volume of ejaculate, is likely a reflection of the very limited variation in these characteristics in fertile males.

In our previous experiments, we observed that sperm motility on Day 7 was a good indicator for boar semen quality tested in vitro (Xu et al., 1996b), however this characteristic did not correlate to the in vivo data in the current studies. Two factors may account for this: firstly, sperm motility evaluation could be rather subjective and in this study it was done by two individuals with different levels of semen evaluation experience. Secondly, boar V709 showed consistently lower sperm motility from Day 0 to Day 8 and motility dropped faster in V709 from Day 0 to Day 8 compared to the other boars in the main experiment; however, this boar showed high in vivo fertility (about 92% farrowing rate and normal litter size). Therefore, a lack of correlation between sperm motility estimates and fertility in vivo was not surprising. Although sperm motility was significantly lower for V709 compared to the other boars, sperm motility at Day 0 for V709 was above 60%, the cut-off line of the boar stud for use of semen for AI during the period of breeding. However, using Day 0 semen from this boar for IVF resulted in a significantly lower oocyte penetration rate (15%) and polyspermy rate (6.7%) at the beginning of breeding trial even when a high sperm:oocyte ratio (50,000:1) was used. These observations suggest that changes in boar fertility seen in vivo appear more slowly in response to changes in semen quality than do changes in the in vitro characteristics. Therefore, use of IVF parameters mentioned above may predict the onset of sub-fertility in boars more precisely and at an earlier stage than routine semen examination. Unfortunately this boar was removed from the AI trial for commercial reasons before we could see the drop in farrowing rate.

In most commercial situations, total numbers of sperm per insemination usually vary between 2 and 5 billion. The commonly accepted minimum number of cells for achieving adequate fertility is 2 billion (see review by Flowers, 1992). The current study showed that reduction of sperm numbers from 3 billion to 2 billion did not change the farrowing rate, and no boar effects was observed on farrowing rate, suggesting that 2 billion sperm per dose is still more than the semen doses that would yield a "linear" fertility response (see Saacke, 1982; Amann and Hammerstedt, 1993; Saacke et al., 1994). Experiments using a further reduction of sperm numbers per dose would give us a better discrimination of variations in semen quality from different boars and clarify the relationships between different in vitro measurements and in vivo fertility data.

In conclusion, this study showed there were variations in results obtained from in vitro and in vivo assessment of semen quality. Some of the in vitro test parameters are positively correlated to the in vivo fertility data and therefore could be used as indicators for the prediction of boar sperm quality in vivo. Relationships between sperm:oocyte ratio used in IVF experiments and different IVF parameters were evident, which provided a better understanding of in vitro sperm oocyte interactions and for a further optimization of IVM-IVF systems.

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6. MEISHAN AND LARGE-WHITE HYBRID PREOVULATORY FOLLICLES DIFFERENTIALLY AFFECT OOCYTE IN VITRO MATURATION AND FERTILIZATION[4]

6.1 INTRODUCTION

The cellular relationship between the oocyte and somatic follicular cells is fundamental to oocyte maturation (Moor et al., 1990). Follicular cells play a critical role in the regulation of oocyte meiotic arrest and the resumption of meiosis, in addition to providing nutrients. Recent studies on the maturation of porcine oocytes in vitro by either co-culture with follicular cells or follicular fluid (Niwa, 1993; Sirard et al., 1993) indicate that follicular cells secrete factors which play a crucial role in supporting oocyte cytoplasmic maturation. Furthermore, Ding and Foxcroft (1992; 1994a) showed that the maturational status of the follicle used for co-culture had a critical influence on cytoplasmic maturation and that this effect was mimicked by the use of conditioned media.

The Chinese Meishan pig (MS) is characterised by its prolificacy, producing an average of 3-4 more piglets per litter than European breeds (Bolet et al., 1986; Haley and Lee, 1993). Substantial differences in the characteristics of follicle maturation between Meishan and Large-White hybrid pigs (LW) have been observed. These include smaller preovulatory follicle diameter, but increased follicular fluid oestradiol concentrations and aromatase activity in both granulosa and theca tissues in Meishan animals (Biggs et al., 1993; Hunter et al., 1994b). Meishan follicles also appear to luteinize more rapidly in response to hCG administration (Downey and Driancourt, 1994) or the endogenous LH surge (Hunter et al., 1996). Importantly however, more oocytes were found to have matured to the metaphase II stage within the estimated 7 hours before ovulation in Meishan than in Large-White hybrid animals, indicating enhanced oocyte nuclear maturation in the Meishan pig (Faillace and Hunter, 1994).

Several studies indicate that the prolificacy of the Meishan pig is due primarily to an enhanced level of early embryonic survival (Bazer et al., 1988; Ford and Youngs, 1993; Haley and Lee, 1993; Hunter et al., 1994a), although the precise mechanisms by which this is achieved remain unclear. It is likely however, that the enhanced maturation of the Meishan follicles is an important component, since the administration of hCG at the onset

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of oestrus (approximately 12 hours before the endogenous LH surge) resulted in a decrease in embryo survival (Hunter and Picton, 1995). The onset of behavioural oestrus occurs earlier relative to the LH surge and ovulation in Meishan than Large-White hybrid animals (Hunter et al., 1993).

Considering all these points, it can be hypothesised that the Meishan follicle provides a 'better' environment for oocyte maturation than Large-White hybrid follicles, resulting in the production of oocytes of improved quality and thus ultimately greater embryo survival. This hypothesis was tested in the current study by two experiments. In Experiment 1, Meishan or Large-White hybrid oocytes were matured in vitro by co-culture with pre-ovulatory follicles of the same breed and in Experiment 2, oocytes recovered from the slaughterhouse were matured in the presence of conditioned media recovered from either Meishan or Large-White hybrid animals, followed by fertilization in vitro.

6.2 MATERIALS AND METHODS

6.2.1 Experiment 1

6.2.1.1 Animals

Six Meishan gilts (Cotswold Pig Development Co. UK) and seven Large-White hybrid gilts (University of Nottingham herd) were group penned according to breed and checked daily for oestrous behaviour with mature vasectomized boars of the appropriate breed. Previous comparisons between animals from the same sources had confirmed the higher embryonic survival level in the Meishan animals (Hunter et al., 1994a). Ovaries were recovered at slaughter on the equivalent of day 20 of the oestrous cycle (oestrus minus 1 day). The date of slaughter was determined using mean cycle length and previous oestrous date in combination with observing the gilts for the physical signs of approaching oestrus as described previously (Biggs et al., 1993).

6.2.1.2 Follicle and Oocyte Collection

The handling and dissection of ovarian tissue, the measurement of follicles and the collection of follicular fluid were as described by Foxcroft et al. (1987), Biggs et al. (1993) and Faillace and Hunter (1994). All follicles 3-4 mm were dissected, incised and their cumulus oocyte complexes (COCs) located using a dissection microscope. The ten largest follicles from each gilt which were incised had the follicular fluid aspirated separately for subsequent radioimmunoassay of individual follicle oestradiol, and they were then everted.

6.2.1.3 Oocyte Culture and Preparation of Conditioned Media

All COCs of normal, healthy appearance (defined as those with dark uniform oocyte cytoplasm and layers of compact cumulus and ranged from 12-27 recovered per animal)

were randomly divided into two groups and placed in a dish containing either of the two largest everted follicles and 2.5 ml culture medium. The culture medium was TCM 199 supplemented with 10% fetal calf serum, 100 mg ml^{-1} glutamine, 70 mg ml^{-1} L-ascorbic acid, 35 mg ml^{-1} insulin and 50 mg ml^{-1} gentamycin sulphate (all from Sigma Chemical Co. Poole, UK). Gonadotrophin supplementation was 50 ng ml^{-1} each of LH (USDA-pLH-B1) and FSH (USDA-pFSH-I-2). Culture was carried out in 5% CO_2 in humidified air at 39°C. One of the two dishes containing oocytes in addition to a follicle shell was removed from culture after 27 h and the other after 34 h of culture. Oocytes were recovered, denuded of cumulus cells by repeated pipetting with a fine-bore pipette and fixed in acetic acid:ethanol (1:3) for at least 48 h. The nuclear status of each oocyte was identified after staining with 1 % lacmoid according to the criteria of Hunter and Polge (1966).

The remaining 8 everted follicles per animal were cultured individually for 34 h without oocytes in 2.5 ml culture media for generation of conditioned medium (CM). At the end of culture, 1 ml of medium from each dish was harvested, filtered using a 0.22 mm filter and pooled and stored at -20°C for later use in Experiment 2. The remaining medium from each dish was recovered, stored at -20°C until pooled before determination of progesterone, oestradiol and insulin-like growth factor-1 (IGF-1) concentrations.

6.2.1.4 Hormone Radioimmunoassays

The concentration of oestradiol in follicular fluid was measured directly by the method described by Grant et al. (1989). The limit of sensitivity was 4.9 pg per tube and the inter-and intra-assay coefficients of variation were 14.1 and 6.6% respectively. The concentrations of oestradiol and progesterone in the conditioned media were each measured directly in a single assay as described by Grant et al. (1989) and Hunter et al., (1988). The limits of sensitivity were 4.4 and 11.6 pg per tube and the intra-assay coefficients of variation were 8.7 and 4.2% respectively.

IGF-1 concentrations in conditioned media were determined using the heterologous double antibody radioimmunoassay previously described by Cosgrove et al. (1992). The assay was further validated for use with conditioned medium. Serial dilution of media showed parallelism to the standard curve. Cold recovery of standard from pooled conditioned medium was 51.5 ±2.9 %, and potencies were corrected for recovery. The assay procedure was modified by taking 0.5 ml of the conditioned medium into a total volume of 3.8 ml acid ethanol for extraction and 0.3 ml of this neutralized sample was assayed. All the samples were measured in a single assay, with a limit of sensitivity of 8 pg per tube and an intra-assay coefficient of variation of 9.7%.

6.2.2 Experiment 2

6.2.2.1 Oocyte Collection and Culture

Ovaries were collected from a minimum of 150 prepubertal gilts on each occasion, largely representing Large-White/Landrace type commercial genotype, at a local abattoir and transported to the laboratory as described previously (Xu et al., 1996a). Oocyte cumulus complexes were collected by aspiration through an 18-gauge needle into a disposable 10-ml syringe from healthy follicles with a diameter of 3-6 mm. The COCs were washed three times using warm D-PBS containing 1% pig follicular fluid and then assessed and selected for use in IVM. The collection of follicular fluid and COC selection procedures were as described by Funahashi and Day (1993). Normal and healthy COCs were pooled and randomly divided into three groups. For treatment comparisons, group 1 COCs were cultured in the conditioned media produced from MS gilts (MS-CM) and group 2 COCs were cultured in the conditioned media from LW gilts (LW-CM). To confirm the effectiveness of this IVM/IVF system for evaluation of effects of different conditioned media, group 3 COCs were co-cultured with fresh follicle shells (FS) with a diameter of 5-6 mm obtained from the same slaughterhouse ovaries and following the procedure described by Ding and Foxcroft (1992). In each dish 10 to 15 COCs were cultured in 2 ml culture medium (MS-CM or LW-CM) or in 2ml modified TCM199 culture medium with 1 everted follicle shell for 46 h, with 2 or 3 replicate dishes for each sample of culture medium on each occasion. Effects of treatment were evaluated on at least three occasions.

6.2.2.2 In Vitro Fertilization

Fresh ejaculates were collected regularly from one adult boar by the gloved-hand method at Alberta Swine Genetics Corporation. A 15 ml semen sample from the first sperm-rich fraction was selected for use in IVF. Semen handling and pre-insemination treatments followed the procedures as described by Xu et al., (1996a). The in vitro matured oocytes cultured with each sample of CM or a follicle shell were pooled and washed twice with fertilization medium (modified medium TCM 199 supplemented with 10 mM caffeine sodium benzoate and 10% FCS; All from Sigma, St Louis, MO, USA). Then 10 to 15 oocytes were randomly transferred to a 4-well culture dish containing 1 ml fertilization medium per well, with at least 2 wells per medium sample. The final sperm concentration for fertilization was 5×10^5 cells ml^{-1}. Oocytes were co-cultured with spermatozoa for 6 h at 39°C in an atmosphere of 5% CO_2 in air and then transferred to a sperm-free medium for a further 6 h of culture. The sperm-free oocyte culture medium was modified Whitten's medium (Whitten and Biggers, 1968) which was modified by a change in the concentration of bovine serum albumin (BSA; Fraction V, Sigma) to 1.5 % (w/v) as reported by Beckmann and Day (1991).

At the end of culture, oocytes were denuded of cumulus cells, mounted on a slide and fixed in acetic acid:ethanol solution (1:3) for at least 48 h. Oocytes then were stained with 1% lacmoid and washed with 45% acetic acid to clear the background. The incidence of

sperm penetration and male pronuclear development was examined under a phase-contrast microscope and determined as described by Ding and Foxcroft (1992). Those oocytes with unswollen or slightly swollen sperm head(s) (SH1) and/or further swollen sperm head(s) (SH2) and/or male pronuclei (MPN) with detached sperm tail(s) were classified as being penetrated. Those penetrated oocytes with full size MPN were considered to have undergone normal MPN development. Oocytes with more than one sperm head or MPN were considered to be polyspermic. The average number of spermatozoa penetrating each oocyte (total number of spermatozoa penetrating / total number of oocytes inseminated) was recorded.

6.2.3 Statistical analyses

6.2.3.1 Experiment 1

Breed effects on follicular diameter, volume of follicular fluid, concentration of oestradiol in follicular fluid (FFE) and steroid and IGF-1 concentration in the conditioned media were analysed using one-way ANOVA with gilts within breed as the error term.

Data for oocyte maturation were analysed using split-plot analysis of variance with breed as the main plot and gilts within breed as the error term, and time as the split plot with breed × time as the error term. Although in preliminary analyses FFE was used as a covariate in this analysis no significant contributions were observed. Also, in preliminary analyses data were transformed using the arcsin square root transformation (Steel and Torrie, 1980), but as the results for transformed and untransformed variables were the same, only the original data are presented.

6.2.3.2 Experiment 2

This was a nested factor design. Data were analysed by ANOVA fitting type of media as the main effect and using gilts within type of media as the error term. For measurements with significant differences among type of media, multiple comparisons were made using the Student-Newman-Keuls (SNK) test. Data shown in the Tables and Figures are expressed as least squares means (LSM), and standard errors of LSM are given (SAS 6.11, SAS Institute, Cary, NC).

6.3 RESULTS

6.3.1 Experiment 1

6.3.1.1 Characteristics of the 10 Largest Follicles from Both Breeds

Mean values of follicle diameter, follicular fluid volume and follicular fluid oestradiol concentration of the 10 largest follicles from animals within each breed are summarized in

Table 6-1. The follicles from Meishan gilts were significantly smaller (P<0.001) and contained less follicular fluid (P<0.001) than did the follicles from Large-White hybrid gilts. There was a tendency for the follicular fluid oestradiol concentration to be higher in the Meishan animals than in the Large-White hybrid animals, however, the difference was not significant (P=0.095).

Table 6-1. Characteristics of the ten largest follicles recovered from six Meishan (MS) and seven Large-White hybrid (LW) gilts*

Follicle characteristic	LW	MS	P value
Diameter (mm)	7.8±0.11	6.1±0.12	0.0001
Fluid volume (ml)	155.9±6.13	75.5±6.53	0.0001
Oestradiol (ng ml^{-1})	323.7±28.92	394.7±30.79	0.0955

*Values presented are least squares means (LSM) and standard errors derived from the mean values of the 10 follicles for each individual animal.

6.3.1.2 In Vitro Maturation of Oocytes Recovered from Both Breeds

For the 6 Meishan gilts, 62 COCs were cultured and 56 oocytes were positively identified in the 27 h culture group; 64 COCs were cultured and 61 oocytes were positively identified in the 34 h culture group. For the 7 Large-White hybrid gilts, the number of COCs cultured and the number of oocytes positively identified were 56 and 54 in the 27 h culture group, and 56 and 56 in the 34 h culture group.

The meiotic stages of oocyte development for the two breeds (LW and MS) at the two culture times (27 h and 34 h) are shown in Figure 6-1. There was no difference between the breeds across all the seven meiotic maturation stages after 27 h culture (all P>0.05), but there was a significant difference between the breeds in the proportion of oocytes that developed to Metaphase II stage after 34 h culture (P<0.01). For both LW and MS, over 50% of the oocytes underwent germinal vesicle breakdown (GVBD) and most of those oocytes reached Metaphase I stage (48.5% and 54.7% for LW and MS, respectively), however only a small percentage of oocytes had developed beyond the Metaphase I stage and none had developed to Metaphase II at the end of 27 h in vitro culture. With a further 7 h of culture, more oocytes from MS gilts (56.0%) reached nuclear maturation stage Metaphase II than those from LW (3.2%).

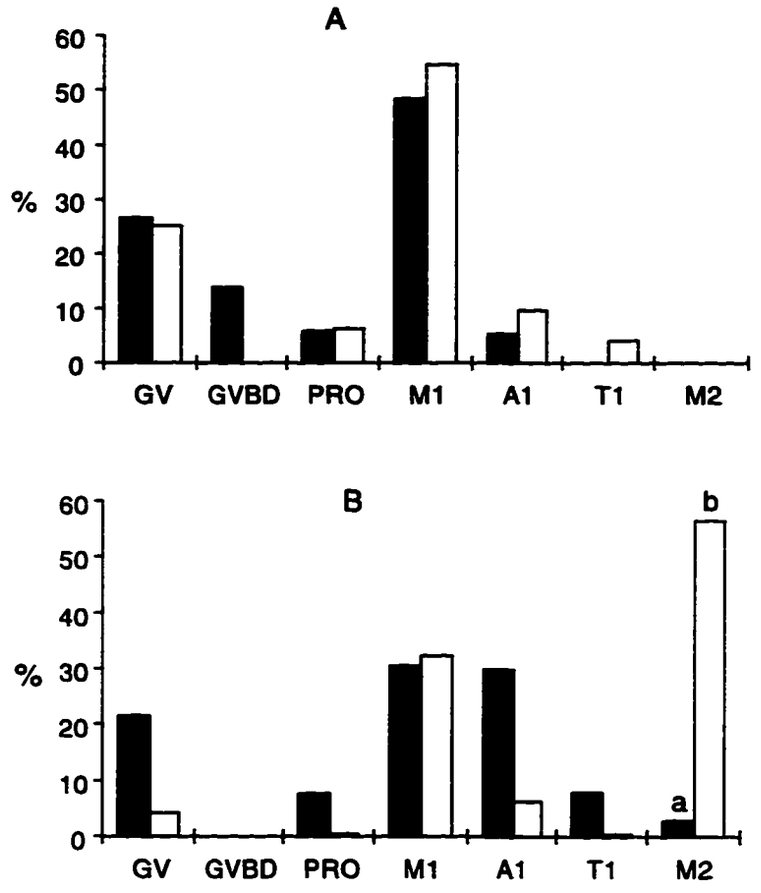

Figure 6-1. Meiotic development in LW (■) and MS (□) oocytes cultured in vitro for 27 (A) and 34 h (B), expressed as the percentage of oocytes reaching each stage of development. The total number of oocytes examined after 27h and 34h were 54 and 56, and 56 and 61 for LW and MS, respectively. GV: Germinal Vesicle; GVBD: Germinal Vesicle Breakdown; PRO: Pro-metaphase; M1: Metaphase I: A1: Anaphase I; T1: Telophase I; M2: Metaphase II. a-b: Bars with different letters are significantly different (P<0.01).

6.3.2 Experiment 2

6.3.2.1 Steroid and IGF-1 Concentrations in Conditioned Media

The concentrations of both progesterone and oestradiol and their ratio were not different in the CM between the two pig breeds (P>0.1). IGF-1 concentrations in the LW conditioned medium were higher than those in the MS conditioned medium (P<0.01) (see Table 6-2).

Table 6-2. Concentrations of steroids and IGF-1 in Conditioned Media from six Meishan (MS) and seven Large-White hybrid (LW) gilts*

Concentration ($ng\ ml^{-1}$)	LW	MS	P value
Progesterone (P_4)	276.3±40.9	180.2±48.4	0.160
Oestradiol (E_2)	38.5±7.1	46.5±8.4	0.485
P_4/E_2	7.8±1.3	4.2±1.5	0.105
IGF-1	6.5±0.24	5.2±0.27	0.008

*Values presented are least squares means (LSM) and standard errors.

6.3.2.2 In Vitro Maturation and Fertilization of Slaughterhouse Oocytes

The total number of oocytes cultured in LW-CM, in MS-CM and in FS were 731, 612 and 117, respectively. The percentages of oocytes penetrated by spermatozoa, the percentages of penetrated oocytes with a male pronucleus (MPN) and the percentages of un-penetrated oocytes at different maturation stages are illustrated in Figure 6-2. The penetration rate in LW-CM (33.7 ± 5.3 %) was significantly lower ($P<0.05$) than that in MS-CM (72.0 ± 5.7 %) and FS (80.8 ± 14.5 %), both of which were similar ($P>0.10$). Male pronuclear formation rates were significantly different among the three culture conditions (all $P<0.05$). Monospermy rates for LW-CM, MS-CM and FS were 79.3 ± 4.3, 66.1 ± 4.6 and 58.3 ± 11.9, respectively and did not differ ($P>0.05$). The average number of spermatozoa per penetrated oocyte was similar ($P>0.05$) for LW-CM (1.31 ± 0.08) and MS-CM (1.58 ± 0.09), but both were less ($P<0.05$) than that for FS (2.05 ± 0.22). The average number of swollen or decondensed spermatozoa (SH2) per penetrated oocyte showed the same trend as the average number of spermatozoa per oocyte. More penetrating spermatozoa underwent decondensation in FS (0.59 ± 0.07) ($P<0.05$) than that in LW-CM (0.08 ± 0.03) and in MS-CM (0.17 ± 0.03).

Among the population of unpenetrated oocytes, there was no difference in the maturational stages reached by these oocytes in the three different culture conditions (all $P>0.05$) (Figure 6-2).

6.4 DISCUSSION

Both of these experiments are consistent with the hypothesis that the Meishan preovulatory follicle environment is more favourable for oocyte maturation than that of the Large-White hybrid follicle. Oocyte maturation was enhanced in terms of both nuclear and cytoplasmic maturation when oocytes were cultured in the presence of a follicle shell or conditioned media from a Meishan compared to a Large-White hybrid pig.

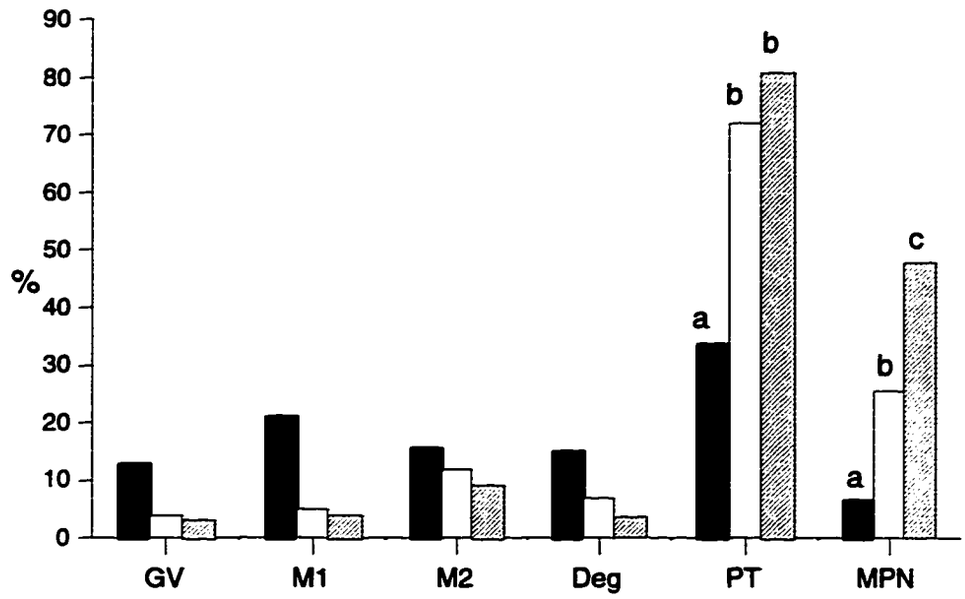

Figure 6-2. Effects of conditioned media on oocyte nuclear and cytoplasmic maturation. LW-CM (■): Large White hybrid gilt follicle shell conditioned media; MS-CM (□): Meishan gilt follicle shell conditioned media; FS (▨): Culture medium with one follicle shell. The total number of oocytes cultured in LW-CM, in MS-CM and in FS were 731, 612 and 117, respectively. GV: Germinal Vesicle stage; M1: Metaphase I stage; M2: Metaphase II stage; Deg: degenerated. PT: penetration rate; MPN: male pronuclear formation rate. a-c: Bars with different letters are significantly different ($P<0.05$).

It is now well established that follicle cells play a major role in supporting oocyte maturation (Niwa, 1993; Sirard et al., 1993) and that this is further dependent on the maturational status of the follicle (Ding and Foxcroft, 1992). We have reported previously that the maturational characteristics of the Meishan preovulatory follicle are different to those of the Large-White hybrid (see Introduction) and this is supported by the results in the current study. We have suggested furthermore, that the MS preovulatory follicle is in a more advanced state of maturation than that of the LW (Hunter et al., 1993). This is based on higher follicular fluid oestradiol concentrations (Biggs et al., 1993), aromatase activity (Hunter et al., 1994b), the cyclic AMP response to LH (Ranson et al., 1994) and a more rapid nuclear maturation of oocytes in vivo in response to the endogenous LH surge (Faillace and Hunter, 1994). This more rapid progression to Metaphase II in vivo was also found in vitro in Experiment 1, with more Meishan oocytes at the Metaphase II stage after 34 h of culture than LW. The culture times of 27 and 34 h used in Experiment 1 are shorter than the 44-46 h approximately which is usually selected for porcine oocyte maturation in vitro (Niwa, 1993; Sirard et al., 1993) but these times were selected based on the timing of Meishan oocyte maturation in vivo (Faillace and Hunter, 1994). The precise maturational stage(s) which occur more rapidly in the MS oocyte are interesting and require further

study. Regardless of the precise stage however, the result would be the ovulation of more mature oocytes which are reported to be more rapidly fertilised (Terqui et al., 1992). Since only 22% of LW oocytes in Experiment 1 had not undergone germinal vesicle breakdown by 34 h of culture, then it may be that some of other 78% would have progressed through to Metaphase II with further time in culture. In Experiment 1, oocytes were cultured with a follicle shell from the same breed. Although the results of Experiment 2 clearly indicate that the follicle itself has an influence, it is possible nevertheless in Experiment 1 that the increased progression to Metaphase II at 34 h by MS oocytes was due not only to the follicle shell but to a combination of both follicle and oocyte.

Another point which needs to be considered is the time of follicle collection in both breeds. This was based on the time prior to the predicted onset of oestrus as has been used previously in several studies (Biggs et al., 1993; Hunter et al., 1993; Faillace et al., 1994). It is known however, that behavioural oestrus in the MS occurs approximately some 12 h earlier relative to the LH surge and ovulation than in the Large-White hybrid (Wilmut et al. 1992; Hunter et al., 1993; Faillace et al., 1994). This means therefore that if anything follicles and oocytes were recovered at a slightly earlier time from MS than from LW gilts. The smaller follicle diameter and volume of follicular fluid is consistent with previous studies, as is the trend for higher follicular fluid oestradiol concentrations (Biggs et al., 1993). Even when follicles were followed through to ovulation in another study (Faillace and Hunter, 1994), they remained smaller in diameter and corpora lutea are also smaller (Biggs et al., 1993; Downey and Driancourt, 1994). Thus the positive effect of MS follicles cannot be explained by time of follicle collection or follicle size.

The results of Experiment 2 clearly show that compared to LW conditioned media, MS conditioned media affected oocyte status, as evidenced by more penetrated oocytes, and enhanced cytoplasmic maturation of oocytes, as evidenced by the higher rate of male pronuclear formation.

However, the results with both MS and LW conditioned media were lower than those reported by Ding and Foxcroft (1994a) who used follicle shell conditioned media to achieve oocyte maturation in vitro. The higher rates in Ding and Foxcroft's study may be due to a longer culture time (48 h) and the use of strictly selected follicles from a slaughterhouse pool for making conditioned medium (2ml per follicle shell), compared to one follicle cultured for 34 h in 2.5 ml culture media for producing MS-CM and LW-CM in the present study. The supportive effects of follicular secretions on oocyte maturation vary with the different maturity of follicle shells. More mature follicles provide a better environment for oocytes when they are used for co-culture or for production of conditioned medium (Ding and Foxcroft, 1992). However, despite the lower rates of in vitro maturation, any influence of the oocyte on the treatment difference observed can be discounted, since the same pool of oocytes was used for co-culture with both MS and LW conditioned media. The observed treatment effect must be due therefore to follicle secretions into the conditioned media. Progesterone and oestrodial concentrations in the conditioned media were not different between the breeds and therefore although the precise role of steroids secreted by the follicle shell in oocyte maturation is still unclear, differences in the secretion of these

steroids into conditioned media did not mediate the treatment effects observed. Data from the current study support Ding and Foxcroft's (1992) results and indicate that steroids in the coditioned media are not the main regulatory factors for cytoplasmic maturation. Although IGF has been reported to enhance mammalian oocyte maturation (Reed et al., 1993; Lorenzo et al., 1994; Xia et al., 1994), concentrations were actually higher in the LW than MS and thus IGF is unlikely to be responsible for the improved maturation. Various other factors have been reported to enhance oocyte maturation and fertilization such as Epidermal Growth Factor (Reed et al., 1993; Ding and Foxcroft, 1994b; Lorenzo et al., 1994) and this may be worthy of further study. Interestingly, it has recently been shown that the presence of growth hormone during the maturation of bovine oocytes in vitro significantly accelerated nuclear maturation and subsequent cleavage and embryonic development (Izadyar et al. 1996). Furthermore, other unidentified factors have been found in porcine follicular fluid that promote oocyte maturation (Yoshida et al., 1992b, factor Mr 10-20,000; Daen et al., 1992, factor <6.5 kD) although the precise structure of these substances requires further elucidation. Indeed, in additional studies we observed that the percentage of oocytes recovered from slaughterhouse ovaries and achieving nuclear maturation was higher when cultured in the presence of follicular fluid recovered from MS follicles than from LW (V. Brankin and M.G. Hunter, unpublished observations).

In Experiment 2, the penetration and MPN formation rates of oocytes co-cultured with fresh follicle shells (the FS control group) were similar to those reported previously by Xu et al., (1996a), indicating that our in vitro maturation system was working effectively. However, cytoplasmic maturation as determined by MPN formation rate in penetrated oocytes of the FS group in (48.1%) was significantly higher than that of the LW-CM group and MS-CM group. The differences could be explained by the conditioned medium preparation procedure in which one follicle shell was cultured in 2.5 ml oocyte maturation medium for 34 h in the absence of oocytes. The length that follicle shells were cultured for producing CM was therefore 12 h less than the duration of follicle shell co-culture with oocytes in the control group (1 follicle shell cultured in 2ml medium for 46 h in Experiment 2). It is likely therefore that oocytes were less exposed to oocyte maturation factors in the conditioned media treatments (groups LW-CM and MS-CM) than in the situations that involve direct co-culture with follicle shells (the FS control group). Also, the frozen-thawed condition media may not work as efficiently as the fresh follicle shell co-culture medium on oocyte maturation. It has been reported that sufficient glutathione (GSH) is an important cytoplasmic factor for sperm chromatin decondensation and hence for male pronuclear formation following sperm penetration in pig oocytes (Yoshida et al., 1992a). GSH synthesis occurs throughout the in vitro maturation of pig oocytes, and the composition of the maturation medium has a significant impact on the ability of oocytes to form a male pronucleus (Yoshida et al., 1992a).

Previous studies indicate clearly that pig oocytes matured in vitro in the absence of any follicle cells or in the presence of cumulus cells alone fail to induce normal male pronuclear development (Moor et al., 1990). These differences can be related to studies that established that oocytes require follicle cell stimulation for at least the first 32 h of maturation (Moor et al., 1990) and the role of the cumulus cells is to maintain functional

intercellular coupling between the oocyte and the follicular compartment (Mattioli et al., 1988b). Even when cumulus cells are present, the addition of follicle shells to cultures of cumulus-enclosed oocytes confers normal condensation competence (Irritani et al., 1978; Mattioli et al., 1988a; Moor et al., 1990). Therefore the fresh follicle shell co-culture condition in Experiment 2 probably provided better support for complete oocyte maturation than did the LW-CM and MS-CM. Oocytes have been demonstrated to secrete soluble factors that promote granulosa cell proliferation and differentiation, enable the cumulus cells to synthesize hyaluronic acid and to undergo cumulus expansion in vitro, and regulate granulosa cell steroidogenic activity (Vanderhyden et al., 1993; Vanderhyden and Tonary 1995; Nagyová et al., 1997). Therefore, the absence of oocytes in the culture media during conditioned medium preparation probably resulted in a lack of such oocyte-derived factor(s), which could affect the quality of the conditioned media and therefore oocyte maturation in vitro. The nature of oocyte-derived factors and their possible regulatory mechanism on follicular cells are unknown. Ding and Foxcroft (1994a) also suggested that the maturational status of follicles might affect their secretory activity during in vitro culture. Collectively, these studies suggest that the lower MPN formation rate of penetrated oocytes cultured in the conditioned media could be caused by a number of factors.

In contrast to the effect on penetration rate there was no effect on monospermy rate, although the trend towards an inverse relationship between these characteristics is consistent with data from our previous experiments (Xu et al., 1996a,b). Although Ding and Foxcroft (1994b) showed a positive relationship between penetration rate and the number of penetrated spermatozoa per oocyte, no such relationship was seen in this study, and the number of spermatozoa that underwent decondensation was not affected by the source of conditioned medium. Thus, although penetration rate and MPN rate effectively discriminated the breed effects under the conditions described in these experiments, modification of the in vitro culture system (e.g. a change in the sperm:oocyte ratio) might place greater emphasis on other measures of oocyte maturation (Xu et al., 1996b).

In conclusion, the results from these two experiments support the hypothesis that differences in follicle maturation in the prolific Meishan compared to the Large-White hybrid pig result in an improved ability of the follicles to support oocyte maturation and fertilization in vitro.

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7. GENERAL DISCUSSION

When IVM-IVF systems are used to assess oocyte maturation and sperm function, they need to be effective, stable, relatively easy to set up and most importantly, should identify characteristics that are essential for in vivo oocyte maturation and fertilization.

In vitro maturation of follicular oocytes from slaughterhouse ovaries has been described as an effective means of obtaining large numbers of uniform and fertilizable oocytes (Ivanova and Mollova, 1993). As reviewed in Chapter 2, follicular cells, hormones, growth factors and other factors are involved in the regulation of both oocyte nuclear and cytoplasmic maturation. In vivo, oocytes are matured inside follicles. The intimate relationship between the oocyte and the surrounding follicular somatic cells establishes a functional basis for oocyte-follicular cell interactions during oocyte maturation. In vitro experiments demonstrated that follicular cells not only control nuclear maturation, but also facilitate cytoplasmic maturation in mammalian oocytes (Thibault et al., 1987; Moor et al., 1990). However, the cumulus alone can not support full oocyte cytoplasmic maturation in vitro in some domestic species (pig: Motlik and Fulka, 1974; Cattle: Thibault et al., 1975; Sheep: Moor and Trounson, 1977) and co-culture of cumulus-enclosed oocytes with granulosa cells or follicular shells greatly improved cytoplasmic maturation and development potential (Mattioli et al., 1989; Lu et al., 1989; Staigmiller and Moor, 1984). In our IVM system, co-culture pig cumulus-enclosed oocytes with 1 or 2 fresh follicular shell(s) proved to be very effective and gave an oocyte maturation rate as high as 92% (see Chapter 3), which is consistent with the results of Ding et al. (1992) when the same system was used. However, the use of follicle dissection as a means of oocyte collection is very time-consuming compared with follicle aspiration. In the studies described in Chapters 4 and 5, the large number of semen treatment groups required a more efficient method for recovery of a large number of oocytes. Therefore, an aspiration method was adopted, which produced a 4- to 5-fold increase in the number of culturable oocytes compared to the follicle dissection method. In addition, 10% (v/v) pig follicular fluid (pFF) was added in the oocyte maturation medium in these studies replace of follicle shell co-culture and fetal calf serum, since follicular fluid and follicular cell conditioned medium have supportive effects on oocyte maturation (Niwa, 1993) and the use of pFF from a standard batch reduced experimental costs and produced stable and satisfactory IVF results (Funahashi and Day, 1993).

It is generally believed that the LH surge triggers the preovulatory oocyte to resume meiosis in vivo (Tsafriri, 1978; Moor et al., 1981). In vitro observations showed that using either follicle shell co-culture (Ding et al., 1992) or follicular fluid (Naito et al., 1988), both nuclear and cytoplasmic maturation can only be properly achieved in the presence of gonadotropins. Acceleration of nuclear maturation by prolactin has also been observed during IVM of oocytes (Hoshino, 1988). Insulin, a growth factor structurally related to IGF-1, improves fertilization and cleavage rate of in vitro matured pig oocytes (Zhang et al., 1991). Therefore, gonadotropins (LH and FSH), prolactin and insulin were

added to a basic tissue culture medium 199 (M199) in a constant concentration in the IVM systems for all studies described in this thesis.

When the IVM-IVF systems are used for assessment of oocyte maturation and sperm fertilizability, experimental conditions must be controlled carefully in order to minimize the variability among experiment replicates and remove all possible artifacts. In our studies, standardized procedures of follicle selection, oocyte collection and maturation, evaluation of cumulus expansion, selection and randomization of IVM oocytes for IVF, use of chemicals, hormones and pFF from the same sources, and use of duplicate or triplicate fertilization dishes for each semen sample overcame problems of oocyte variability and therefore minimized the female effects and other factors on the IVF results.

Other limiting factors in porcine IVM-IVF may come from the male, since large variations in semen quality among individual boars and even variations between ejaculates from the same boar have been observed (Sirard et al., 1993). Standardization of semen collection methods seems a necessary step to obtain stable IVF results. Differences of semen characteristics in different fractions of an ejaculate have been observed by Einarsson (1971). Studies in Chapter 3 further showed that sperm fertilizing ability could be different among ejaculate fractions. Factors that modified sperm function in vitro are presumably present in the sperm or the seminal plasma before or during ejaculation. Seminal plasma has been shown to play an essential role in the fertilization capacity of sperm and all mammalian species studied so far have seminal decapacitation factors which could be glycoproteins, peptide proteinase inhibitors, sterols or lipids (see Chapter 2). Porcine spermadhesins have been characterized and thought to serve as decapacitation factors by coating the boar sperm head membrane. The decapacitated (ejaculated) sperm must then be recapacitated before they become capable of fertilizing oocytes (Yanagimachi, 1994). It has been found that sperm from some males can be capacitated more easily than those from others of the same species (Shalgi et al., 1981). Taking these observations and results in Chapter 3 together, we suggest that the variable fertilizing ability in vitro among sperm from different fractions within an ejaculate could be due to decapacitation factors in the seminal plasma. Therefore, a standardized use of specific sperm-rich fractions in the IVF system would reduce the variability among ejaculates from the same male and make IVM-IVF outcome more stable and comparisons of boar fertility more precise.

The IVM-IVF systems adopted in the studies of this thesis proved to be effective and reliable. As research in pig IVM/IVF proceeds and more unknown phenomena are clarified, use of frozen-thawed boar semen and a simplified IVM-IVF procedure will make the evaluation of gamete quality more efficient and applicable in the swine industry.

The above-described IVM-IVF systems were successfully used for assessment of oocyte maturation in Meishan and Large-White hybrid pig breeds (see Chapter 6). Using

these systems for evaluation of sperm fertilizing ability and prediction of boar fertility, however, appears to be more complicated.

Among in vitro tests for evaluation of sperm quality, IVF of homologous porcine oocytes is potentially more suitable for assessing overall sperm fertilizing function. Studies in this thesis validated such in vitro sperm quality tests and provided comparable information of laboratory semen characteristics, comprehensive IVF results, breeding field data and their correlation in boars. When semen from different boars were used in different studies, IVF criteria used allowed for discrimination between boars in terms of apparent semen quality, especially when serially diluted semen was used for IVF. Results showed that use of IVF parameters, such as oocyte penetration rate, polyspermy rate and average number of sperm per penetrated oocyte, may predict the onset of sub-fertility in boars more precisely and earlier than routine semen examination. However, these parameters could not detect differences between two boars with a similar high fertility when a relative high sperm:oocyte ratio was used for IVF. This suggests that further semen dilutions are needed to give lower sperm:oocyte ratios in IVF for a better discrimination between boars in vitro.

Semen dilution effects on IVF results have been shown previously (Rath, 1992). Results in this thesis were consistent with the observation of Rath (1992) and provided more information about the relationships between sperm:oocyte ratio (semen dilution) and different IVF parameters. The establishment of the quadratic or linear relationship between each IVF parameter and serial semen dilutions has significance in determining the optimal range of sperm:oocyte ratios used in IVF for the best outcome. Also it offers possibilities for the application of a certain IVF parameter in the AI industry for comparison of semen quality among boars in vitro using a simplified IVM-IVF procedure. It would be necessary, therefore, to characterize boars with different 'best-fit' lines by establishing the relationships between sperm:oocyte ratio and IVF parameters in a preliminary experiment before using semen of these boars in an IVF experiment for the purposes of embryo production or evaluation of gamete quality.

To find out which IVF parameters could be used for predicting boar fertility, two breeding trials (one using semen from young boars and another using semen from adult boars of the same group) were carried out and correlations between IVF results and field data were revealed (see Chapter 5). In vivo and in vitro data showed that semen from young boars might produce a lower number of sperm with fertilizing ability compared to adult boars; therefore, age of boars should be taken into account when selecting a sperm:oocyte ratio for IVF or applying a dose of extended semen for AI. For the adult boars, the reduction of sperm numbers from 3 billion to 2 billion per AI dose reduced the litter size from 10.8 to 10.0, but did not change the farrowing rate. This suggests that 2 billion sperm per dose is still more than the semen doses that would yield a 'linear' fertility response. Using less than 2 billion per AI dose in a future breeding trial would therefore allow a clearer relationship between in vitro and in vivo data to be established,

although the economics of conducting such trials is a big challenge for the pig production industry.

As shown previously in other laboratories (Harrison et al., 1996), different results from in vitro and in vivo fertilization were again observed in our studies. Since in vitro conditions under which sperm are induced to fertilize oocytes differ greatly from the in vivo situation, a sub-group of sperm that fertilized oocytes in vivo may not be the same group of sperm fertilized oocytes in vitro (Harrison et al., 1996). Our data suggest that under the right conditions the proportion of sperm attached to oocyte plasma membrane at the time of microscopic examination might represent the sperm population that would fertilize oocytes in vivo. If this can be confirmed, the development of a simplified sperm oocyte-attachment assay may become of practical significance.

In conclusion, results from the two sets of studies in this thesis suggest that: (1) IVF criteria used in these studies allowed for discrimination between boars in terms of apparent semen quality; (2) Use of a standardized sperm-rich fraction from an ejaculate reduced variation between ejaculates and provided the best semen for used in pig IVF; (3) The relationship revealed between sperm:oocyte ratio and different IVF parameters provided information for a better understanding of sperm oocyte interactions in vitro and for a further optimization of IVM-IVF systems; (4) The IVF parameters, potential embryo production rate and sperm number attached per oocyte were positively correlated to boar in vivo fertility data. These parameters, together with the laboratory semen characteristic, percentage sperm normality, could be used as an efficient indicator of boar sperm quality in vivo; (5) The IVM-IVF systems can be adopted to evaluate porcine oocyte maturation in vitro.

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