ELSEVIER

# EFFECTS OF SEMEN PLASMA FROM DIFFERENT FRACTIONS OF INDIVIDUAL EJACULATES ON IVF IN PIGS

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## ABSTRACT

We applied IVM/IVF techniques to investigate effects of preincubation of sperm with different fractions of semen plasma harvested from fresh ejaculates on in vitro penetration and fertilization of in vitro matured oocytes. Three fractions of semen plasma were separated from the complete ejaculate of three Landrace boars and used to coincubate sperm obtained from the first sperm-rich fraction of the same ejaculates. After 14 to16 h coincubation at room temperature, sperm were preincubated in capacitation medium and then inseminated into fertilization medium containing porcine oocytes matured in vitro. The semen plasma used for coincubation affected penetration rate ($P<0.001$); Sperm coincubated with Fraction 1 semen plasma had a higher penetration rate compared with sperm coincubated with Fraction 2 ($P<0.05$), but not with Fraction 3. Boar affected male pronucleus formation rates after insemination ($P<0.05$), but no difference among boars was found in monospermy rate, average number of sperm penetrating into each fertilized oocyte, or the average number of sperm attached. No boar by fraction interaction was found for any parameters studied.



Key words: boar, semen plasma, ejaculate fraction, IVF

## INTRODUCTION

In vitro fertilization (IVF) in pigs has been studied since sperm capacitation was first described in 1951 (1, 4). This technique has been successfully used in evaluating boar sperm fertility (9, 12, 20) and, in our laboratory, more consistent IVF results were obtained using spermatozoa from a standardized sperm-rich fraction (21). Penetration rate, male pronucleus formation rate, polyspermy rate and average number of spermatozoa penetrating the oocyte were all higher when using spermatozoa from the first sperm-rich fraction (Fraction 1) f or IVF

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compared to use of spermatozoa from the sperm-depleted fraction (Fraction 2) or the second sperm-rich fraction (Fraction 3). Whether this variability in function capacity comes from the semen plasma or from the sperm themselves has not been clarified.

This study, in which three standardized fractions of semen plasma were separately incubated with sperm pellets from the first sperm-rich fraction of the same ejaculate, was designed to compare possible influences of semen plasma on sperm function using IVM/IVF techniques.

## MATERIALS AND METHODS

### Preparation and Maturation of Oocytes

Ovaries with large antral follicles were obtained from freshly slaughtered prepubertal gilts at a local abattoir (Gainers Co., Edmonton, AB). They were held in a plastic jar and transferred to our laboratory in a polystyrene box within 40 min after recovery to avoid major changes in temperature. After washing with saline, cumulus oocyte complexes (COCs) were aspirated together with follicular fluid from healthy-looking 3 to 6 mm follicles using a 10-mL syringe attached to an 18½ gauge needle. After removal of supernatant, spontaneously precipitated COCs were washed three times with PBS supplemented with 1% (v/v) porcine follicular fluid (pFF), which was prepared from 3 to 6 mm, transparent and grape-like follicles during our preliminary study. Only COCs containing oocytes with evenly-distributed cytoplasm and multilayered compact cumulus cells were harvested for maturation culture using a dissecting microscope at x 16 magnification.

The oocyte maturation medium contained 85% (v/v) TCM-199 medium (12340-30, Gibco Life Technologies, Grand Island, NY, USA), 10% (v/v) pFF and 5% (v/v) additive mixture containing 2 mg/mL glutamine (BDH, Toronto), 1.4 mg/mL L-ascorbic acid (BDH, Toronto, Ont., Canada), 0.7 mg/mL insulin (I-1882, Sigma, St. Louis, MO, USA) and 1 mg/mL PVA in TCM-199 medium. Two mL of this solution was added to individual 35×15 mm petri-dishes and 0.1 mL multi-gonadotropin stock containing 5 mg/mL FSH (USDA-pFSH-B-1, AFP-5600), 0.05 mg/mL LH (NIADDK-oLH-26, AFP-5551b), 0.4 μg $mL^{-1}$ prolactin (USDA-pprl-B-1, AFP-5000) and 1 mg/mL PVA in TCM-199 medium, was then added to each dish. All details of this methodology were previously described by Xu et al. (21), except that in the present study the pFF supernatant used in the media was directly filtered with a 0.2 μm syringe filter (Millipore Corp., Bedford, Mass., USA) without heat treatment.

Fifty to sixty selected COCs were transferred to individual petri dishes and incubated for 44 h under 5% (v/v) $CO_2$ at 39°C . Oocytes with many evenly-expanded cumulus cells attached were regarded as matured oocytes and randomly transferred to individual 4-well dishes, with 10 COCs in each well containing 0.9 mL preincubated fertilization medium.

Collection and Treatment of Semen

Three young, fertile Landrace boars (coded as Boar A, B and C) housed at the Alberta Swine Genetics Corperation boar stud (Leduc, Alta., Canada) were selected for experimental use and were regularly collected twice a week with a gloved-hand method. On experimental days, the whole ejaculate from all three boars was collected into a series of 15-mL sterile tubes and the sperm concentration in each tube was measured at 540 nm with a Spectronic 301 spectrophotometer (Milton Roy Co., Rochester, NY, USA) immediately following collection. The most concentrated tube of the first sperm-rich fraction of the ejaculate was defined as Fraction 1, the least concentrated tube after the first sperm-rich fraction as Fraction 2 and the most concentrated tube of the second sperm-rich fraction as Fraction 3. Only ejaculates showing 80% or higher sperm motility as measured immediately after collection in Fraction 1 spermatozoa, were used in IVF; they were delivered to our laboratory before 1800 on the day of collection.

The semen was centrifuged at 550×g for 10 min at 25°C and 0.5 mL precipitated sperm from Fraction 1 was then resuspended in 2.0 mL of Fraction 1, Fraction 2 and Fraction 3 semen plasma. The recombined semen was gently mixed and then kept in a polystyrene box for 14 to16 h at 25°C.

Capacitation and Insemination of Semen

After co-incubation, semen was washed twice with sperm washing medium (9 mg/mL NaCl, 1 mg/mL BSA (Fraction V) (Sigma, St Louis, MO, USA), 0.1 mg/mL Kanamycin (Sigma, St Louis, MO) and a third time with sperm preincubation medium (0.55 mg/mL D-Glucose, 0.9 mg/mL Calcium lactate, 0.1 mg/mL Sodium pyruvate, and 0.1 mg/mL Kanamycin in M-199 medium, with 4 mg/mL BSA added just before use), using centrifugation at 550×g for 5 min for the three washes. Sperm concentration was then adjusted to $4\times10^8$ spermatozoa mL with preincubation medium before incubation at 39°C under 5% (v/v) $CO_2$.

After 1.5 h of preincubation, sperm agglutination was scored on a 5-point scale for 20 aggregations of spermatozoa. Grades 1, 2, 3, 4 and 5 represent 1 to 3, 4 to 6, 7 to 9, 10 to 12 and 13 to 15 spermatozoa in each aggregate, respectively. Semen was then further diluted to a concentration of $2\times10^7$ spermatozoa/mL in fertilization medium (sperm preincubation medium containing 0.3884 mg/mL caffeine-sodium benzoate (50:50, w/w, mixture) (Sigma, St. Louis, MO, USA) and 100 µl was subsequently inseminated into each culture well containing expanded COCs. The final sperm concentration was $2\times10^6$ sperm mL and the sperm to oocyte ratio was $2\times10^5$:1 during fertilization.

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

Evaluation of Fertilized Oocytes

After completion of fertilization, oocytes were denuded of cumulus cells, mounted on glass slides with 10 to 20 oocytes on each slide, and fixed in acetic acid-ethanol (1:3, v/v) solution. After 48 h, oocytes were stained with 0.75% (w/v) lacmoid in 45% (v/v) acetic acid and

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

Stained oocytes were observed at x 200 or x 400 magnification under a phase-contrast microscope. Oocytes containing one or more unswollen or swollen sperm head(s), or male pronuclei with one or more detached sperm tails were considered as penetrated. Monospermic fertilized oocytes were defined as oocytes containing only one sperm or male pronucleus. Oocytes classified as polyspermic possessed two or more sperm or male pronuclei. The number of sperm penetrating each fertilized oocyte included the total number of sperm and male pronuclei. The above observation criteria have been previously described by Ding and Foxcroft (8) and Xu et al. (21). In the present study, only spermatozoa with their heads attached to the oocyte membrane were counted as "attached". Any unfertilized or recently penetrated oocyte without a visible polar body was regarded as immature (MI or earlier oocyte) and was not included in the total number of scored oocytes used for analysis. The experiment was repeated three times.

Statistical Analysis of Data

The experiment was analyzed as a split-plot (29) design using the General Linear Model procedures of the Statistical Analysis System (version 6.08, SAS Institute Inc., Cary, NC). Boar was the main plot and the effects of boar were tested against the replicate (block) by boar (main plot) interaction. The effects of semen plasma (sub-plot) were tested against the boar by fraction interaction, and the interaction between boar and fraction was tested against the replicate by boar by fraction interaction. Where appropriate, multiple comparisons were carried out among individual means using the Student-Newman-Keul test. All of the differences were evaluated at the level of $P<0.05$.

## RESULTS

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

Table 1. Physiological characteristics of the three ejaculates from each of three boars used for IVF (Mean±SEM)

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

[ab]Within columns, different superscripts indicate significant differences ($P<0.05$).

Post-capacitation sperm motility and agglutination are presented in Table 2. In relation to the main factor under investigation, the fraction of semen plasma used for in vitro fertilization affected penetration rate ($P<0.001$) but no other measure of oocyte penetration or development. Fraction 1 spermatozoa co-incubated with Fraction 1 semen plasma had a higher penetration rate than Fraction 1 spermatozoa co-incubated with Fraction 2 semen plasma (Figure 1). Male pronuclear formation rate was also affected by boar ($P<0.05$; Figure 2). There was no significant interaction between boar and semen plasma fraction for any of the in vitro characteristics measured.

Table 2. Sperm motility and agglutination estimated after in vitro capacitation (Mean±SEM)

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

## DISCUSSION

The use of Fraction 1 semen plasma for co-incubation with Fraction 1 sperm resulted in a higher penetration rate than the use of Fraction 2 semen plasma. No significant interaction was found between boar and semen plasma fraction in our study. As the different batches of co-incubated semen was kept overnight under the same circumstances and then handled in the same IVF procedure, it is assumed that the variability in penetration rate observed was related to the characteristics of the different boars and fractions of semen plasma used. Previously we have reported more extensive differences of ejaculate fraction on IVF results when spermatozoa and semen plasma came from the same fraction (21). This suggests that a considerable part of the fraction effect seen in the earlier study came from differences in spermatozoa quality per se, with a lesser part of the difference due to semen plasma effects. Nevertheless, semen plasma is clearly able to affect the function of spermatozoa obtained from the first sperm-rich fraction of the ejaculate and the basis for this effect is of practical significance.

Like other plasma, semen plasma consists of many components, some of which are thought to influence sperm motility, capacitation/decapacitation and fertilization. High concentrations of estrone, estradiol-17β and estrone-sulfate are present in boar semen with considerable differences in mean concentrations and total amounts among individual ejaculates (5, 7, 8). Claus and colleagues further demonstrated that the addition of a physiological amount of amount of estrogens to semen extender used for artificial insemination could increase both conception rate and litter size born (6).

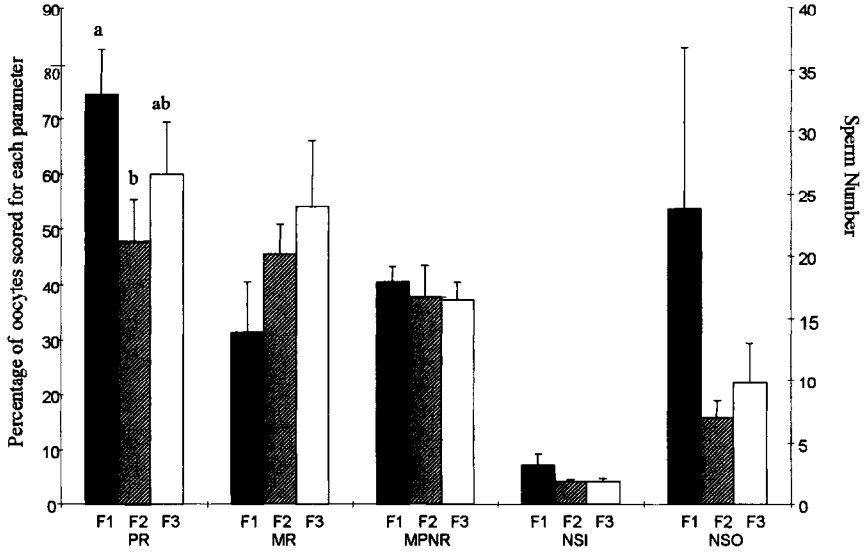


Figure 1. Effects of three fractions of semen plasma (F1, F2, F3) on penetration rate (PR), monospermy rate (MR), male pronuclear formation rate (MPNR), number of sperm penetrating each fertilized oocyte (NSI), and number of sperm attached to each matured oocyte (NSO). Fractions from three boars were tested simultaneously in each of three replicates. Between 69 and 116 matured oocytes were scored for each semen plasma sample. [abc] different letters on histograms indicate effects of fraction ($P>0.05$).

Proteins are also major components of semen plasma. In addition to maintenance of semen osmolarity and nourishment for spermatozoa, they are involved in sperm capacitation and gamete recognition and binding (3, 16, 20). Porcine Semen Plasma Protein I and II (PSP-I and PSP-II) were identified as glycoproteins with 50% amino acid sequence homology with a family of zona pellucida-binding proteins (11, 14) and could prevent premature acrosome reaction and immunosuppression (11). Fertilization-promoting peptide was shown to be present in the mammalian prostate at a high concentration and enhanced fertilization potential of sperm (17). At least two kinds of sperm motility inhibiting factors were purified and identified from boar

semen (10, 16), and sperm motility inhibiting factors were able to inhibit sperm motility in a non species-specific manner (16). A group of zona pellucida binding proteins (ZPBP, 16-23 kDa) has also been described in boar ejaculates. They were derived from semen plasma, as shown by their absence on epididymal spermatozoa and their presence in semen plasma as detected by N-terminal amino acid sequence analysis, and could regulate the rate of sperm capacitation and survival in the female genital tract (16). More than one decapacitation factor was reported in boar semen plasma by Bonilla et al. (2) and these factors were greater than 100 kDa in molecular weight.

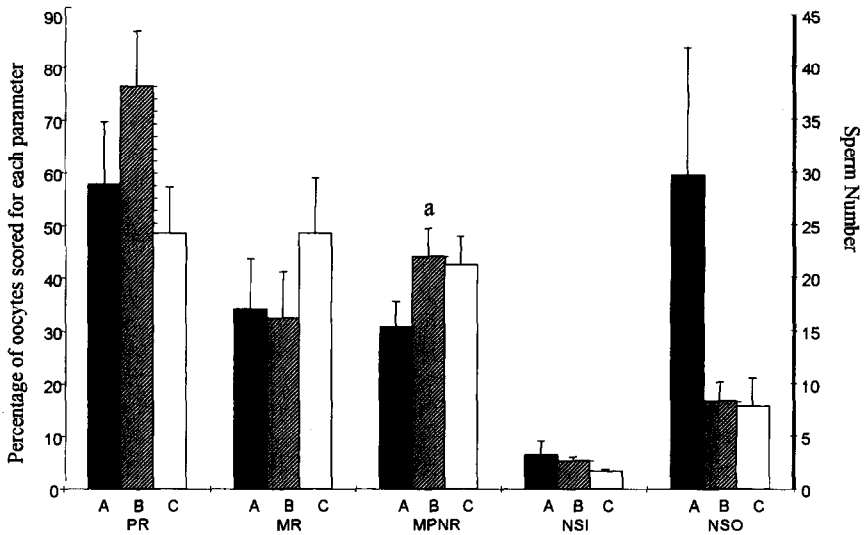


Figure 2. Effects of semen plasma from three boars (A,B,C) on IVF results. The parameters evaluated include penetration rate (PR), monospermy rate (MR), male pronucleus formation rate (MPNR), number of spermatozoa penetrating (NSI) and attached to each matured oocyte (NSO). Means represent average values of three fractions for Boars A and B, and two fractions for Boar C, with three replicates per boar. A total of 69 to 116 matured oocytes were scored for each semen plasma sample. [a] Overall effect of boars on MPN formation rates ($P<0.05$).

The above observations indicate that many proteins and hormones in semen plasma are related to sperm function. Masuda et al. (13) observed differences in the concentrations of protein, as well as citric acid and zinc, which were positively correlated with the agglutinating activity in semen plasma, either among different fractions of semen plasma or among different seasons. It can be reasonably inferred that during the 14 to 16 h pre-capacitation sperm culture with substituted semen plasma, certain qualitative and quantitative changes took place in the

proteins and hormones coating the sperm surface, and that this eventually induced functional alterations in the capacitated sperm. Interestingly, about half of the total amount of semen estrogens are reportedly bound to the sperm membrane (7). Whichever hormones or proteins might be involved, the net effect of exposure of standard aliquots of sperm from Fraction 1 semen with Fraction 1 semen plasma was more beneficial than co-incubation with Fraction 2 semen plasma during capacitation and fertilization. Whether this is due to the binding of beneficial molecules or hormones, or the removal of factors inhibitory to sperm capacitation is unknown.

At the same sperm:oocyte ratios used for IVF in this study, there was no difference in penetration rate among the three boars tested, when results from all three fractions were combined for analysis. Again, these results contrast to earlier data using homologous spermatozoa and semen plasma (21), suggesting that the a large part of among boar differences in spermatozoa function in IVF systems relates to differences in the spermatozoa themselves.

There was also no obvious correlation between penetration rate and post-ejaculation sperm motility, or post-centrifugation sperm motility (Tables 1 and 2). Although high and equivalent sperm motility (≥80%) was recorded for each replicate among the three boars when semen was evaluated immediately after collection of the ejaculates, a dramatic post-centrifugation decrease (53%), and then an obvious post-capacitation rebound (70, 63 and 63% respectively for Fractions 1, 2 and 3) was observed in Boar B, while Boar C exhibited a gradual drop in sperm motility, along with the poorest post-capacitation sperm agglutination (Tables 1 and 2). These coincubation-induced effects likely contributed to sperm penetration rates. Although there was no significant difference in sperm motility among the three boars, the sperm co-incubated with Fraction 1 semen plasma possessed a higher motility and more obvious post-capacitation agglutination than sperm co-incubated with Fraction 2 or Fraction 3 semen plasma after capacitation, as shown in Tables 1 and 2.

A high penetration rate was always accompanied by a relatively low monospermy rate (80.47% vs. 27.43%, 89.23% vs. 17.37%, respectively, for Boar A and B in Fraction 1), consistent with previous results from our laboratory using similar sperm:oocyte ratios (21). Although lower sperm:oocyte ratios could have been used in the present study to better describe the effects of Fraction 1 semen plasma, preliminary results indicated that a further reduction in sperm:oocyte ratio led to a dramatic drop in penetration rates with Fraction 2 semen plasma, making it difficult to make comparisons across fractions at the lower sperm:oocyte ratio.

In conclusion, three fractions of boar semen plasma within one ejaculate had different effects on in vitro penetration rate of in vitro matured porcine oocytes when they were separately used for co-incubation with the same Fraction 1 spermatozoa. Although only Fraction 1 spermatozoa were used for IVF in the present study, boars were still different in their effects on in vitro developmental of oocytes, as measured by male pronuclear formation rates. These results suggest that semen plasma components such as proteins and steroids contribute to semen plasma effects on the fertilization process. From a practical perspective, the observation that Fraction 2 semen plasma has a negative impact on the high quality spermatozoa in Fraction 1 of the ejaculate, suggests that collection of this part of the ejaculate should be avoided if possible when semen is being collected for use in IVF systems. The implications for AI use are less clear, as

lower penetration rates in vitro may indicate delayed capacitation of spermatozoa, which may be an advantage in preserving the fertilizing capacity of sperm in vivo after insemination.

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