



139
589
THS

This is to certify that the
thesis entitled

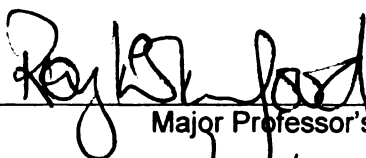
SOW FERTILITY TO INSEMINATION OF
CRYOPRESERVED SPERM

presented by

MIGUEL ABAD-VALCARCE

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Large Animal Clinical Sciences



Major Professor's Signature

7/31/06

Date

MSU is an Affirmative Action/Equal Opportunity Institution

LIBRARY
Michigan State
University

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.
MAY BE RECALLED with earlier due date if requested.

DATE DUE	DATE DUE	DATE DUE

SOW FERTILITY TO INSEMINATION OF CRYOPRESERVED SPERM

By

Miguel Abad-Valcarce

A THESIS

**Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of**

MASTER OF SCIENCE

Department of Large Animal Clinical Sciences

2006

ABSTRACT

SOW FERTILITY TO INSEMINATION OF CRYOPRESERVED SPERM

By

Miguel Abad-Valcarce

This thesis describes studies to assess the effect of the insemination-ovulation interval and the addition of seminal plasma (SP) on the establishment of the sperm reservoir and subsequent sow fertility following artificial insemination (AI) of cryopreserved sperm. In initial experiments, the numbers of sperm located in the reservoir after AI of fresh (FS) or frozen-thawed (FT) semen was studied in gilts. Ovulation was synchronized and gilts inseminated with FS sperm, FT sperm, or FT sperm supplemented with 10% SP. After slaughter, uterotubal junctions (UTJs) were recovered and flushed to recover sperm. The number of sperm cells located in the reservoir was consistently lower in gilts inseminated with FT sperm and results were not affected by the addition of seminal plasma. In subsequent experiments, multiparous sows were inseminated 12 h before ovulation with either FS sperm, FT sperm, or FT sperm with 10% SP. Other sows were inseminated with FT sperm at 2 h before ovulation. Inseminations were either cervical (experiment 2.1) or intrauterine (experiment 2.2). Farrowing rates were lower in sows receiving FT sperm regardless of timing of AI and supplemental SP. Litter size was unaffected. The smaller sperm reservoir and low fertility rates achieved after AI of FT sperm suggests that the injuries produced by the cryopreservation to the sperm cells involves more than just cryocapacitation.

DEDICATION

To the eternal care, to my parents and brother

ACKNOWLEDGMENTS

I am extremely grateful to many people that have contributed widely to the thesis presented here. Risking forgetting many, I would like to mention some.

Firstly Dr Roy Kirkwood, advisor not only limited to that, because of his professional and personal excellence, identifying the time to push me, and the time to push me more. None of these studies would have existed without him.

Dr Dave Sprecher, who repeatedly taught me the way to face the adversity, regardless the size in which it arrives. He did show me the true American character and values.

Dr Bob Friendship, who host me as one more of his privileged students. His guidance and never-ending enthusiasm was my main support during the stay in Canada.

Dr Jose Cibelli, who kindly took interest in this work. His generous character and exceptional leadership have been a permanent referent in my time spent at MSU.

I would also like to acknowledge Arturo, Christine, Jessika, Jose, Pablo and all the graduate students that collaborated to this thesis with their time and comprehension.

Special thanks to Glen Cassar and Missy Vadnais for their help and friendship.

Finally, I would like to thank Faith Peterson and also the staff at MSU and Arkell Swine Farms for their continuous support and availability.

TABLE OF CONTENTS

LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	ix
 Chapter 1	
Literature Review: Reproduction and artificial insemination	1
Swine reproductive anatomy and Physiology	1
History of artificial insemination.....	5
Sow fertility to AI.....	7
Semen backflow	8
Sperm reservoir	9
Insemination of low sperm numbers	10
Timing of insemination relative to ovulation	11
Sperm capacitation	15
Boar semen cryopreservation	17
Freezing protocols	18
Capacitation-like status.....	20
Seminal plasma.....	21
Effect of seminal plasma on sperm capacitation and fertility	22
References	24
 Chapter 2	
Effect of sperm cryopreservation and supplementing semen doses with seminal plasma on the establishment of a sperm reservoir in gilts	32
Contents.....	32
Introduction	33
Material and methods	35
Results	38
Discussion.....	39
Acknowledgements	41
References	42
 Chapter 3	
Effect of insemination-ovulation interval and addition of seminal plasma on sow fertility	44
Contents.....	44
Introduction	45
Material and methods	47
Results	49
Discussion.....	51
Acknowledgements	53
References	54
 Chapter 4	
Summary and Conclusions	57

Summary.....	57
Conclusions	58
Appendices	59
Appendix 1	59
Appendix 2	61

LIST OF TABLES

Table 1.1. Characteristics of a typical boar ejaculate	5
Table 1.2. Volume and number of sperm in the semen backflow during insemination (M1), 0-0.5h after insemination (M2) and 0.5-2.5h after insemination (M3). Semen doses were 80ml with 1, 3 or 6×10^9 sperm	8
Table 1.3. Best time to inseminate relative to the first time detection of standing estrous	12
Table 1.4. hCG based ovulation synchronization protocols.....	14
Table 2.1. Effect of freezing and supplementation with seminal plasma (SP) on number of sperm recovered form the oviduct and uterotubal junction (experiment 1).....	38
Table 2.2. Effect of freezing and supplementation with seminal plasma (SP) on number of sperm recovered from the oviduct and uterotubal junction (experiment 2).....	39
Table 3.1. Farrowing rates and total born litter sizes following artificial insemination of fresh (FS) sperm or cryopreserved (FT) sperm with or without 10% supplemental seminal plasma (SP) inseminated 2 or 12 hours before ovulation (experiment one)...	50
Table 3.2. Farrowing rates and total born litter sizes following artificial insemination of fresh (FS) sperm or cryopreserved (FT) sperm with or without 10% supplemental seminal plasma (SP) inseminated 2 or 12 hours before ovulation (experiment two)...	51

LIST OF FIGURES

Figure 1.1. Reproductive anatomy of the sow	3
Figure 1.2. Fertilization rates from AI with fresh and frozen semen at different times of insemination relative to ovulation	13
Figure 1.3. Model of sperm capacitation.....	16
Figure 3.1. Sow ovulation time (hours post pLH injection).....	49

LIST OF ABBREVIATIONS

AC.....	adenyl cyclase
AI.....	artificial insemination
BSA	bovine serum albumine
cAMP.....	cyclic adenosin monophosphate
CTC	chlortetracycline
ECG	equine chorionic gonadotrophin
FS.....	fresh (sperm cells)
FT	frozen-thawed (sperm cells)
GnRH.....	gonadotrophin releasing hormone
HCG.....	human chorionic gonadotrophin
IM	intramuscular
IU	international unit
LH.....	luteinizing hormone
LSD.....	least significant difference
MSU	Michigan State University
PGF.....	prostaglandin F2a
PKA	protein kinase a
PKC	protein kinase c
PMSG	pregnant mare serum gonadotrophin
PTO.....	predicted time of ovulation
PRRS	porcine respiratory and reproductive syndrome
PSP	porcine seminal plasma protein
ROS	reactive oxygen species

SEM.....standard error of the mean
SP..... seminal plasma
SR sperm reservoir
UTJuterotubal junction
V/Vvolume/volume
ZPzona pellucida

CHAPTER 1

Literature Review: Reproduction and Artificial Insemination

Swine Reproductive Anatomy and Physiology

The reproductive organs of the female pig (*Sus scrofa*) are shown in Figure 1.1. The vulva is the most external and visible portion of the reproductive tract and responds to the cyclic levels of estrogen by showing a red edematous status during the proestrous period. The vulva leads to the vagina, on the floor of which the urethra opens. By directing artificial insemination (AI) catheters dorsally during catheter entry, urethral penetration is prevented. The cervix is located immediately cranial to the vagina, the lumen of which contains multiple interdigitating folds to prevent bacteria and other foreign material from entering the uterus. The cervix is the site of semen deposition during natural breeding and also during conventional AI. In addition, during pregnancy the cervix secretes highly viscous mucus under the influence of progesterone to keep the products of conception isolated from the exposed vaginal tract. The uterus of the sow consists of a small uterine body and two long (approximately 1 m) uterine horns. The oviducts extend from the end of each uterine horn to the ovary. The junction of the uterus and the oviduct is called the uterotubal junction (UTJ). After sperm traverse the UTJ they remain in the first 1 to 2 cm of the oviductal isthmus, which is the site of the functional sperm reservoir (SR). Sperm in the SR are primarily non-capacitated and remain so under the influence of the local microenvironment until near the time of ovulation. The primary function of the ovaries is to produce the female gametes, but they also produce hormones (mainly estrogen and progesterone, but also oxytocin, relaxin, inhibin and activin).

As described by Kirkwood (1999), the 21-day porcine estrous cycle is composed of an approximately 16-day luteal phase and a 5-day follicular phase. During the luteal phase the ovarian corpora lutea produce progesterone, which limits ovarian follicular development to the medium follicle stage and so prevents the onset of estrus. At about 12 to 14 days of the luteal phase endometrial production of prostaglandin $F_{2\alpha}$ (PGF) causes regression of corpora lutea and so terminates progesterone production. This removal of the progesterone block allows resumption of appropriate secretory patterns of the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) which permits the resumption of ovarian follicular development. The developing ovarian follicles secrete estrogen, which ultimately results in behavioral estrus. In weaned sows, a 4 or 5-day wean-to-estrus interval is equivalent to the follicular phase of the estrous cycle. Approximately coincident with the onset of estrus, the increasing circulating levels of estrogen drive a surge release of LH, which causes a cascade of events within the follicle including a switch from estrogen to progesterone production and culminating about 40 hours later with ovulation.

The male reproductive system consists of the testis, epididymis, accessory sex glands, the spermatic cord and the penis. The primary function of the testes is to produce the male gametes (sperm), but they also produce hormones (androgens, estrogens, and progesterone). The epididymides are tubular structures that lie along the testis and they store sperm while they mature. The vas deferens connect each epididymis to the urethra. The boar accessory glands include the seminal vesicles, the prostate and the bulbourethral glands, and these glands secrete the protective non-cellular portion of semen known as seminal plasma.

Testicular function is controlled by pulsatile release of GnRH from the hypothalamus, which stimulates secretion of FSH and LH by the anterior pituitary. The LH stimulates progesterone and testosterone production by the Leydig cells while FSH acts on the Sertoli cells to aromatize androgen to estrogen and to support spermatogenesis. Inhibin, also produced by the Sertoli cells, regulates FSH secretion via a negative feedback loop.

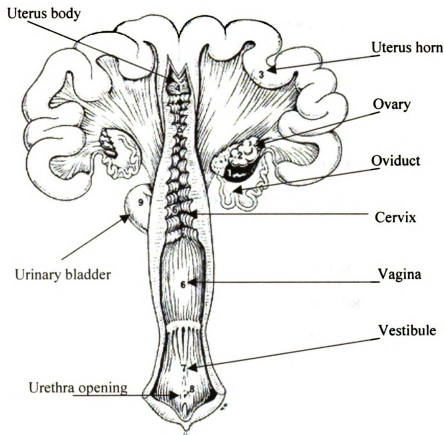


Figure 1.1 Reproductive anatomy of the sow

http://www.wisc.edu/ansci_repro/lec/lec1/female_hist.html

The normal boar ejaculate is composed of several fractions, an initial gel fraction followed by a small volume of clear fluid from the prostate, then the sperm-rich fraction, then more clear prostatic fluid and ending with the majority of the gel fraction. The gel fraction secreted by the bulbourethral glands has a very high estrogen content. Most of the sperm are located in the sperm-rich fraction of the ejaculate. After copulation, the sperm start arriving in the oviduct within 5-30 min (Rodriguez-Martinez et al. 2005). Sperm transport from the uterine body to the UTJ is achieved by coordinated uterine contractions, the result of oxytocin release as well as endometrial PGF released in response to seminal estrogen content (Willemburg et al. 2004).

Currently, techniques for evaluating the quality of boar sperm are similar to those used for bull sperm (Johnson 1998). Differences are found among breeds and seasons, but generally boar ejaculate volumes range from 160 to 266 ml, with concentrations of 374 to 548 x 10⁶ sperm per ml (Ciereszko et al. 2000). Sperm motility immediately after collection ranged from 70-90% (Vyt et al. 2004). For swine AI, the minimum volume of fluid and number of motile sperm necessary at each insemination is suggested to be 50 ml and 3 x 10⁹ motile sperm, respectively. However, insemination of 2 x 10⁹ sperm has been used successfully (Watson and Behan 2002). Currently, typical AI doses have 3 x 10⁹ sperm in 80ml extender.

Table 1.1. Characteristics of a typical boar ejaculate

Criterion	Amount
Total volume, ml	300
Strained volume, ml	210
Gelatinous volume, ml	90
Spermatozoa per ml of strained semen	200×10^6
Total spermatozoa per ejaculate	42×10^9
Motile sperm, %	70
Total motile sperm per ejaculate	29.4×10^9
Number of AI doses possible	10

Straw (<http://www.msu.edu/%7Estraw/>)

History of Artificial Insemination

Artificial insemination involves the collection of semen from the male, its extension (dilution) to produce multiple semen doses and subsequent deposition into the female reproductive tract. In 1779, the Italian Lazzaro Spallanzani performed the first AI in Milan. Using dog sperm to breed a bitch on heat, he obtained a group of normal puppies with the same morphological characteristics as their progenitors. This event, confirmed by Pietro Rossi in 1782, is considered as the beginning of artificial insemination in domestic animals (Abad 2001).

As reviewed by Abad (2001), food animal AI was introduced by Smith in 1891 and was initially a way to control infertility. However, in 1902 Sand raised the possibility of enhancing domestic animal reproduction by means of AI. During the following 30 years the main advances in AI were made in Russia, where in 1922 Ivanov developed

the first extender for semen and his student, Milanov, designed the first artificial vagina for use in sheep. Ivanov initiated AI of swine in 1907, and the procedure was then introduced into the United States, Japan and Western Europe.

The rapid adoption of artificial insemination in swine originated from a more judicious use of semen and easier accessibility to producers of high-quality genetics. The uptake of AI was rapid in Europe, especially during 1990-2000 (Gerrits et al. 2005), leading to a current AI rate of 90% in most West European countries. The United States was slower to adopt AI (Weitze 2000), having a 15% use rate by 1994 and barely 50% by 1999 (Buhr 2001). Currently, it has been estimated that 70% of sows and gilts in North America are bred by AI (Rodriguez-Martinez et al. 2005).

Many different extenders have been developed to preserve sperm cell viability after collection. The first Russian extenders for boar semen were based on glucose solutions with sodium potassium tartrate or sodium sulfate and peptone, keeping the concentration of electrolytes low (Foote 2002). The recommended storage temperature for semen extended in this manner was from 7 to 12°C. After yolk-phosphate, yolk-citrate, and milk-based extenders were developed for bull semen, they were also used or modified for boar semen (Polge 1956). Ito et al. (1948) were the first group recommending storage temperatures of 15 to 20°C for cooled boar semen.

At temperatures between 15 and 17°C, boar sperm storage times of up to 7 days are achievable (Johnson 1998; Levis 2000). However, the majority of producers use semen within 3-days of collection. The advantages of AI include the widespread use

of superior genetics; increased control of breeding and more uniform pigs; better control of semen quality; decreased risk of injury to boar, sows and personnel handling them; the ability to use heavy boars on light females; elimination of isolation and testing of newly introduced boars; decreased boar housing and feeding costs; reduced risk of disease transmission via boar introductions; reduced breeding work load. The disadvantages of AI include the increased level of management required to implement an AI program and, if collecting boars on farm, the need to train boars for collection and redesign of facilities for semen collection and processing.

In swine, about 99% of AI procedures are performed with fresh-extended (liquid) semen (Johnson et al. 2000). Bull semen extenders and freezing procedures were modified for use with boar semen (for review see Johnson 1998). Initially, the pellet method of freezing developed in Japan (Nagase and Graham 1964) was the most common method used. Today frozen-thawed (FT) boar semen packaged in straws is available. The higher price and the reduced pregnancy rates and litter sizes obtained in comparison to fresh semen limits the use of cryopreserved sperm to specific breeding programs (see “Boar Semen Cryopreservation”, p 23).

Sow Fertility to AI

About 50 years ago several authors, including Wiggins et al. (1951), Polge (1956) and Dziuk and Henshaw (1958), recommended the intracervical deposition of a large volume of liquid (50-200ml) and high numbers of spermatozoa (5 to 6×10^9) in every semen dose to obtain maximum levels of fertility to AI. Few modifications have been made to this practice since, as the current AI procedure in swine involves the

deposition in the sow's cervix of about 3×10^9 sperm cells in a total volume of 70-100ml per dose (for review see Martinez et al. 2001a).

Semen backflow: With intra cervical deposition, approximately 25-30 % of the inseminated sperm cells and up to 70% of the volume are lost by retrograde flow (backflow; Steverink et al. 1998). Although backflow of semen during or shortly after insemination has been a concern, recent evidence suggests its relationship to fertility may be minimal. Steverink et al. (1998) inseminated 1 to 6×10^9 sperm in 80ml and collected semen backflow occurring: during the insemination, for the first 30 min after insemination, and from 0.5 h to 2.5h after insemination. Semen backflow occurred in 63% of the sows during AI and in 98% after AI (Table 1.2). However, a reduction in fertility was only evident after relatively large backflows (>5% number of sperm deposited) during insemination of semen doses with 1×10^9 sperm. Presumably, under these conditions, the backflow reduced the numbers of sperm in the SR.

Table 1.2. Volume and number of sperm in the semen backflow during insemination (M1), 0-0.5h after insemination (M2) and 0.5-2.5h after insemination (M3). Semen doses were 80ml with 1, 3 or 6×10^9 sperm.

Backflow	n % ¹	Volume %		Sperm %	
		Mean $\pm$ se	Range	Mean $\pm$ se	Range
M1	63.3	7 $\pm$ 1.1	1-56	8 $\pm$ 1.3	0.3 – 50
M2	98.2	31 $\pm$ 1.7	3-76	14 $\pm$ 1.0	0.3 – 79
M3	97.5	36 $\pm$ 2.6	1-94	9 $\pm$ 0.8	0.3 - 30

From: Steverink et al. (1998)

¹ Percent of sows exhibiting backflow

The female reproductive tract is very effective at moving sperm out of seminal plasma or extender and up into the oviduct. Consequently, backflow during the 30 min following AI, which was observed after 98% of inseminations, contained less and less sperm per ml fluid lost. With this loss of progressively fewer sperm, the impact of backflow on sow fertility also lessens. Indeed, if the backflow occurred between 0.5 and 2.5h after AI, no effects on fertility were observed, regardless of numbers of sperm originally inseminated. When intra-cervical tampons were used to prevent backflow, sperm numbers in the upper uterus and the oviduct were not increased (Pursel 1982).

Sperm reservoir: For successful fertilization, sperm must be able to reach the oocytes soon after ovulation. With conventional AI, sperm are deposited in the cervix and are then transported through the uterine lumen by myometrial contractions. Sperm reach the UTJ within about 5 min (Rodriguez-Martinez et al. 2005), where a functional sperm reservoir is created (Hunter 1981). This reservoir serves to provide the appropriate number of sperm at the appropriate time and physiological state, therefore ensuring successful fertilization of maximum numbers of oocytes (Suarez 2002).

The oviducts play a key role modulating sperm capacitation (Fazeli et al. 1999; Petrunkina et al. 2001; Hunter 2002) due to the different fluid compartments that can be demonstrated between the isthmus and the ampulla. Non-capacitated sperm are stored in the reservoir by specific carbohydrate-mediated binding to the surface of the mucosal epithelium of the oviduct (Suarez et al. 1998). Low levels of bicarbonate in

the region of the oviductal sperm reservoir act to delay or prevent capacitation prior to sperm release in response to ovulatory signals.

Insemination of low sperm numbers: Currently, swine AI involves the cervical deposition of 3×10^9 sperm diluted to a total volume of 80-90ml with extender. However, backflow rapidly reduces the liquid portion of inseminates. Within approximately 75 min after AI almost no seminal fluid/extender can be recovered. However, from personal experience, semen backflow is minimized with intra-uterine insemination. Therefore, the bypassing of the cervix may increase viable sperm numbers available to reach the sperm reservoir, permitting an original insemination of lower sperm numbers. Indeed, it has been shown that for optimal fertility, progressively fewer sperm are required the closer they are deposited to the uterotubal junction. When deposited near the uterotubal junction using either surgical (Krueger and Rath 2000) or endoscopic techniques (Vazquez et al. 2000; Martinez et al. 2001a), very low numbers of sperm (10 to 150×10^6) are required. Such deep intrauterine AI techniques would only be justified when dealing with sperm of extremely high genetic value, or for sex pre-selected sperm. However, the effect of site of sperm deposition has received less attention than other fertility-related parameters, such as the addition of different components to seminal doses, the effect of the seminal plasma on sperm transport, or the timing of insemination relative to ovulation (Martinez et al. 2001b).

Recently, several commercial companies have developed AI catheters that are capable of being passed via manual manipulation through the cervix of the sow to allow deposition of the semen dose into the uterine body. Using this technique allows the

insemination dose to be reduced to 1×10^9 per insemination (Watson and Behan 2002). Field trials have confirmed that farrowing rates statistically comparable to standard AI can be maintained using intrauterine insemination, although there may be a reduction in litter size (Watson and Behan 2002; Rozeboom et al. 2004). Further, it is likely that the timing of insemination relative to ovulation will become progressively more important as the number of sperm deposited is reduced.

Timing of insemination relative to ovulation: The time of insemination relative to ovulation is important for sow fertility (Kemp and Soede 1996). Inseminations performed outside a window of time from 24h before to 4h after ovulation resulted in a reduced fertilization rate and a consequent reduction in farrowing rates and litter sizes (Waberski et al. 1994a,b; Nissen et al. 1997). Currently, there are no practical methods for the detection of ovulation in large groups of sows. Consequently, the timing of insemination is based on detection of estrus, although the time from detection of estrus to ovulation is known to vary considerably.

Group weaning serves as an effective tool for synchronizing estrus and approximately 95% of sows are likely to express estrus between 3 and 8 days after weaning (Knox and Rodriguez-Zas 2001). The duration of estrus averaged 40 hours for gilts and 48 hours for sows (Steverink et al. 1997), but ranged widely (12-88 h) depending on parity, season, weaning-to-estrus interval and farm management (Soede et al. 2000). Ovulation has been shown to occur approximately 70% of the way through the estrous period independent of duration of estrus, although the exact timing relative to ovulation was variable (10 to 85 hours; Weitze et al. 1994; Nissen et al. 1997).

Although the onset of estrus is the parameter used in commercial practice to time swine AI, it is evidently not a good predictor of the time of ovulation.

As a consequence of variable estrus to ovulation intervals, multiple inseminations are performed in order to ensure sperm deposition at a time associated with optimal fertility. However, since studies have shown that AI causes an inflammatory reaction in the uterus that is detrimental to sow fertility (Rozeboom et al. 1998; Kaeoket et al. 2003), insemination too frequently is not recommended. Common commercial practices of timing AI relative to heat detection are described in Table 1.3. Although not recommended by these authors, my personal recommendation is that females standing on day 3 of estrus should also be bred.

Table 1.3. Best time to inseminate relative to the first detection of standing estrous

Frequency of estrus detection	Best time to inseminate	
	Gilts	Sows
Once daily	0 and 24 hours	0 and 24 hours
Twice daily (12 hours apart)	12 and 24 hours	24 and 36 hours

From Estienne et al. (2005).

Currently, optimal fertility is achieved when inseminations with fresh-extended semen are performed during the 24 hours prior to ovulation, with the peak fertility obtained from insemination about 12 hours before ovulation (Waberski et al. 1994a; Kemp and Soede 1996). When employing frozen semen, fertility rates are considerably lower. However, fertilization rates similar to those following insemination of fresh semen

have been reported when insemination of frozen sperm occurred in the 4 hours before ovulation (Figure 1.2; Waberski et al. 1994b).

If we knew when ovulation was to occur, we could use a single AI, or possibly increase the use of frozen semen. However, to obtain this degree of predictability of ovulation, control of follicular development and ovulation is required, which is achievable via the administration of exogenous hormones.

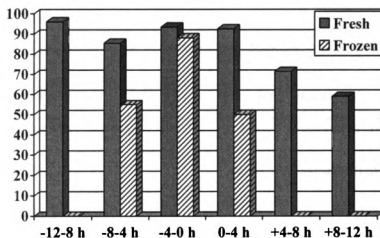


Figure 1.2. Fertilization rates from AI with fresh and frozen semen at different times of insemination relative to ovulation (0h). From Waberski et al. (1994b).

Several hormones, such as human chorionic gonadotrophin (hCG), gonadotrophin releasing hormone (GnRH) or porcine luteinizing hormone (pLH) have been used to control ovulation in sows (De Rensis et al. 2003; Cassar et al. 2004; 2005). Although equally efficient for induction of ovulation, the difference between protocols is the duration of the interval from injection to ovulation, and possibly the synchrony of the

ovulation in the responding animals. Classically hCG was believed to cause a very synchronized ovulation at around 40–42h post injection (Table 1.4). However, recent studies have revealed a relatively wide variation in the interval to ovulation (Soede et al. 1998; Nissen et al. 2000). When ovulation was induced using GnRH or pLH, the interval from injection to ovulation was shorter (36 to 38h) and the degree of synchrony appeared greater (Cassar et al. 2005). Sow fertility to AI at a controlled estrus and ovulation was improved (Candini et al. 2001; De Rensis et al. 2005; Cassar et al. 2004, 2005) even when single inseminations were employed (Cassar et al. 2005).

Table 1.4. hCG based ovulation synchronization protocols.

Synchronization protocol	Ovulation (h)	Source
750 IU hCG 68h after weaning	43	Soede et al. 1998
1250 IU eCG 24h after weaning + 750 IU hCG 72h later	40-42	Martinez et al. 2001a,b
750 IU hCG 96h after weaning	38-48	Nissen et al. 2000
750 IU hCG 76h after weaning	40	Nissen et al. 1995
PG600 + 500IU hCG 72h later	42	Bertani et al. 1997

Sperm Capacitation

Mammalian sperm are not able to fertilize eggs immediately after ejaculation. They acquire fertilizing capacity after residing in the female tract for a certain period of time during which several biological changes occur to render the sperm cells capable of binding with the oocyte and fertilizing it (Visconti et al. 2002). The physiological changes that sperm undergo are collectively called 'capacitation' (Yanagimachi 1994). Capacitated sperm cells show a number of properties including an inability to bind oviductal epithelium in the sperm reservoir, penetration of the cumulus layers surrounding the ova, and binding to the zona pellucida (ZP) that permits the acrosome reaction (Fazeli et al. 1999; Rodriguez-Martinez et al. 2005). During capacitation, several proteins derived from the cauda epididymidis and from seminal plasma are removed from the sperm surface, which then becomes accessible to lipid-binding components of oviductal luminal fluids that extract cholesterol from the sperm plasma membrane. In this state, membrane fluidity is enhanced, causing lipid scrambling and initiating further capacitation changes such as the uptake of extracellular Ca^{2+} , tyrosine phosphorylation (Tardif et al. 2001; 2003), and reorganization of the sperm membrane (Topfer-Petersen et al. 2002). In the pig, bicarbonate appears to be the effector molecule that initiates membrane disorder, which is considered one of the earliest signs of capacitation (Harrison et al. 1996; Gadella and Harrison 2000). Bicarbonate is virtually absent from epididymal fluids [0.5 mM] whereas high concentrations [20 mM] are detected in the oviduct, except in the region of the sperm reservoir (Suarez et al. 1998). These modifications of membrane fluidity precede specific changes in Ca^{2+} movement and of motility patterns (e.g. hyperactivated motility) that predispose to the acrosome reaction and penetration of the ZP (Rodriguez-Martinez et al. 2005).

In vitro, capacitation has been accomplished using sperm cells incubated under a variety of conditions in defined media (Breitbart et al. 1997; Visconti and Kopf 1998; Baldi et al. 2000; 2002). It has been suggested that during capacitation intracellular pH and Ca^{2+} rise, adenylate cyclase is activated resulting in a rise in cAMP levels, and specific proteins (including extracellular signal regulated cyclases) are then tyrosine phosphorylated (Tardif et al. 2003; De Lamirande 1997). Although the molecular basis of capacitation is still not well understood, the following model (Figure 1.3) proposed by Baldi et al. (1996) is currently among the most accepted:

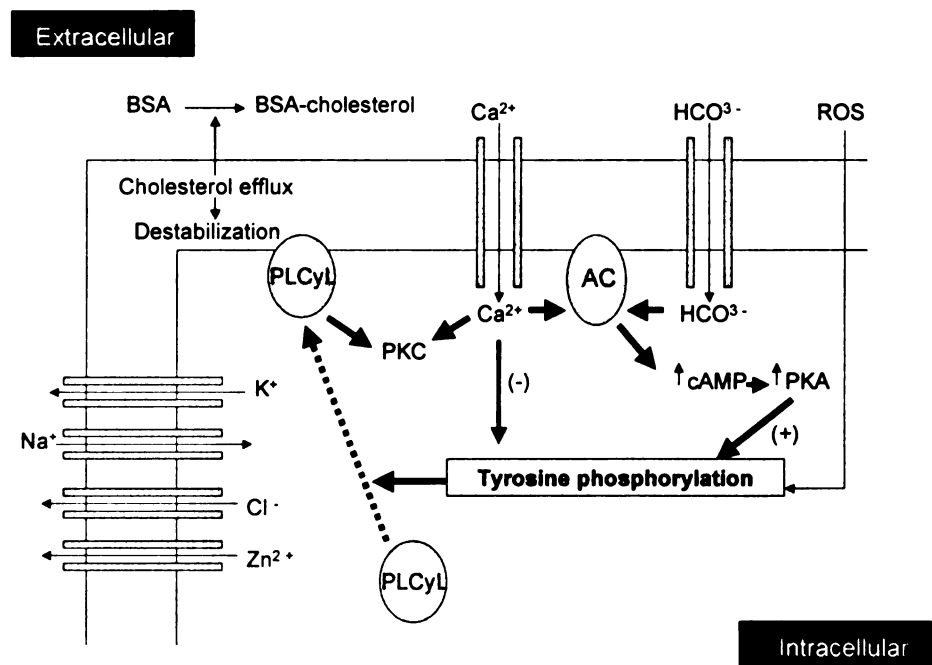


Figure 1.3. Model of sperm capacitation. Cholesterol removal from the cell membrane (accelerated by the presence of bovine serum albumin (BSA) in the medium) increases the fluidity to ions, including bicarbonate (HCO_3^-) and calcium (Ca^{2+}). Both ions activate adenyl cyclase (AC) inside the cell, raising the levels of cyclic AMP (cAMP) and subsequently activating the protein kinase A (PKA). PKA and the incorporated reactive oxygen species (ROS) increase the tyrosine phosphorylation, activating the phospholipases (PLCyL), which destabilize the cell structure, remodeling the sperm membrane phospholipids. A possible activation of the protein kinase C (PKC) has also been reported (Baldi et al. 1996).

Boar Semen Cryopreservation

The first attempts at freezing boar sperm were described by Polge (1956). After repeated failures with cervical and deep intrauterine inseminations, Polge et al. (1970) became the first group obtaining progeny from frozen-thawed (FT) boar sperm by surgical deposition of the FT sperm directly into the oviduct. One year later, using the same cryopreservation protocol, three other groups (Crabo and Einarsson 1971; Graham et al. 1971; Pursel and Johnson 1971) obtained similar success (for review see Pelaez 2004). Following these initial studies, efforts were directed towards improving the procedures as the potential benefits of FT sperm to the swine industry were recognized. These benefits, reviewed by Eriksson (2000), include an ability to store semen until completion of any health test, allowing the semen to be quarantined before use in the herd. Also, specific genetic lines may be preserved and used in future breeding programs. Similarly, improved genetics could be introduced from worldwide sources, eliminating the current geographic limitations. Frozen semen would allow planned matings, which are essential at the top of the breeding pyramid for optimal progress in every gestation.

The utilization of FT boar semen is estimated to be less than 1% of all inseminations worldwide (Johnson et al. 2000). The limitations for a wider use have remained invariable for years, being associated with lower fertility (farrowing rates of 40 to 70% and litter sizes of 7 to 10) compared with those achieved with fresh semen (farrowing rates of 75 to 90% and litter sizes of 9 to 12). A large variation exists between individual boars in freezing success measured as both in-vitro sperm viability and in-vivo fertility post-thaw (Holt 2000a; Eriksson 2000). This, added to the lack of reliable laboratory tests for assessment of sperm quality becomes an even larger

problem when trying to relate in vitro viability with fertility outcomes in-vivo (Selles 2001). The time of AI relative to ovulation must be accurate, as the optimal fertility “window” for FT boar sperm appears to be substantially shorter than that for fresh semen (Waberski et al. 1994b). In addition, costs are higher when using FT semen, due to the more expensive laboratory procedures and higher number of sperm cells needed per insemination dose (5×10^9) compared with fresh semen (3×10^9).

Although FT semen has been commercially available since 1975 (Johnson and Larksson 1985), it has been used primarily for export and transfer of specific genetics within the domestic market. Some usage of frozen semen for market hog production has been reported (Hofmo and Grevle 2000) although it is not widely used in the commercial production of swine because it is not economical in comparison to the use of fresh semen (Wagner and Thibier 2002).

Freezing protocols: The susceptibility of sperm to low temperatures was first described by Milanov in 1934 (see Parks 1997), who observed the irreversible loss of motility of bull sperm after cooling quickly to temperatures near 0°C , an event that he termed “cold shock”. Boar semen differs from the semen of other domestic species in that it is produced in large volumes and the sperm cells are particularly sensitive to cold shock. The severity of the shock depends on cooling rate and final temperature obtained, being maximal at high cooling rates and final temperatures of 12 to 2°C and minimum at cooling rates below $10^{\circ}\text{C}/\text{min}$ and temperatures above 4°C (Pelaez 2004).

Success in the freezing of boar sperm depends on an understanding of how several factors influence the capacity of sperm to survive freezing and thawing while

retaining their ability to fertilize. These factors are either internal, such as the innate differences among boars and ejaculates, or external such as the composition of extenders, type and concentration of the cryoprotective agent applied, rates of extension and cooling or equilibration, and the method of freezing and thawing of the semen (Johnson et al. 2000). Only external factors can be modified to optimize freezing protocols.

Semen cryopreservation involves dilution, cryoprotection, cooling, freezing, storage and thawing, steps which may cause a detrimental effect on cell structure and function (Hammerstedt et al. 1990). Immediately after collection, the semen is diluted in an appropriate medium, often based on egg yolk or milk, at temperatures close to body temperature (30 to 39°C; Bailey et al. 2003). The usual extension rate is 1:1 v/v because high dilution rates reduce sperm function, likely due to the reduced contact between sperm and components of seminal plasma (Garner et al. 2001), the so-called “dilution effect” (Mann and Lutwak-Mann 1981; cited in Maxwell and Johnson 1999).

For most domestic species, cooling of sperm from body temperature to near freezing represents a major stress to the sperm plasma membrane resulting in the rearrangement and destabilization of membrane components and calcium influx (Bailey et al. 2003). The addition of a cryoprotective agent increases both the viability and the motility of the sperm cells at thawing. Glycerol is the cryoprotectant of choice for most mammalian sperm, and is usually added at rates below 3% after cooling of sperm to 5°C (Holt 2000b). The efficacy of glycerol is partially due to its osmotic effect that dramatically increases the osmolality of the medium, retarding intracellular

ice formation (Hammerstedt et al. 1990). The osmotic effects of glycerol are marked, but transitory, and a rapid change in cell volume does not appear to be detrimental to sperm function (Liu and Foote 1998). Furthermore, glycerol acts directly on the sperm plasma membrane, possibly reducing some of the phase transitions and increasing membrane fluidity during cooling (Noiles et al. 1995). The most reliable boar sperm cryopreservation protocols, described in 1975, are freezing in straws (Westendorf et al. 1975) and pellets (Pursel and Johnson 1975), or modifications of these.

Capacitation-like status: The cooling of sperm to 4°C, a step inherent in standard freezing protocols, causes molecular changes to the sperm that effectively mimic capacitation (Bailey et al. 2003). This capacitation-like reaction, or “cryo-capacitation”, does not occur in the entire population of sperm. Cryopreserved boar sperm supplemented with heparin, an established capacitating agent for bull sperm (Parrish et al. 1988), penetrated nearly twice as many oocytes as the heparin-free cryopreserved sperm (Bailey et al. 2000). Further, these authors provided evidence that both cooling and full cryopreservation induced precocious capacitation of bull sperm. Similar findings have been reported for boar sperm (Gillan et al. 1997). The manifestation of the capacitation-like effect in a larger proportion of the sperm population after cooling between 22°C and 5°C indicates that membrane lipid phase transitions occur between these temperatures in boar spermatozoa (Green and Watson 2001).

Capacitation is known to involve a destabilized sperm membrane (Harrison 1997), and the same is true for cryocapacitation. In this state the sperm membrane is fragile and susceptible to deterioration or to undergoing spontaneous acrosome reactions and

death if fertilization has not occurred. Therefore, the in-vivo fertile lifespan of FT sperm is reduced and the fertility of the insemination dose is compromised, based on the proportion of cryocapacitated sperm present (Bailey et al. 2000).

Seminal Plasma

Seminal plasma is the major portion of a boar's ejaculate and plays an important role in carrying the sperm through the female reproductive tract. Produced by the accessory glands of the male reproductive system, seminal plasma nourishes the sperm cells and also influences the capacitation and fertilization processes (Zhu 2000).

The composition of seminal plasma varies with species, the interval between ejaculations, and the health of the animal (Maxwell and Johnson, 1999). Seminal plasma is especially rich in proteins, each of which may play different roles at different stages in the fertilization process. Spermadhesins are the predominant family of boar seminal plasma proteins, accounting for over 90% of total proteins in seminal plasma (Calvete et al. 1997). Several studies by this latter author have reported that Porcine Seminal Plasma Protein I (PSP-I) and II (PSP-II) are the two major members of the spermadhesin family, representing well over 50% of total proteins in seminal plasma (Rodriguez-Martinez et al. 2005). They are usually found combined in heterodimers that show a marked zona pellucida glycoprotein-binding activity, suggesting an important role during gamete interaction (Zhu 2000).

Seminal plasma hormones are potentially associated with sperm function. The Leydig cells secrete large amounts of estrogens, which modulate protein synthesis and

secretion as well as possibly affecting the timing of ovulation (Claus 1990). However, the role of seminal plasma in the timing of ovulation remains unclear, as its addition pre-insemination advanced ovulation time in some studies (Waberski et al. 1997) but not others (Soede et al. 1998).

Effect of seminal plasma on sperm capacitation and fertility: Incubation of boar sperm in the presence of seminal plasma induces resistance to cold shock compared with sperm incubated in the absence of seminal plasma (Pursel et al. 1973). In contrast, at freezing temperatures the presence of seminal plasma caused a detrimental effect on post-thaw sperm motility (Memon et al. 1985). Thus, boar sperm cryopreservation protocols usually include an incubation phase of several hours at room temperature in the presence of seminal plasma before centrifugation for its removal prior to cooling and freezing. In both fresh and frozen-thawed boar sperm, chlortetracycline staining indicated that incubation in the presence of seminal plasma can both prevent and reverse capacitation (Vadnais et al. 2005a,b). Although the addition of seminal plasma to the thawing extender resulted in a decrease in the percent of capacitated spermatozoa, the effects of seminal plasma addition on cryocapacitation are dependent on the temperature of the sperm suspension as well as the presence or absence of egg yolk from the thawing extender (Vadnais et al. 2005b).

Rath and Nieman (1997) showed that the in vitro fertilizing capacity of frozen-thawed boar epididymal sperm was better than that of frozen-thawed ejaculated sperm, reflecting the absence of decapacitation factors that would have been added to the sperm in seminal plasma during ejaculation. Furthermore, the motility of frozen-thawed ram sperm was better after the addition of seminal plasma, and there were

fewer capacitated and acrosome reacted cells than in controls without seminal plasma (Maxwell et al. 1999). Although boar sperm cells are less susceptible than are rams' to the dilution effect (Ashworth et al. 1994), the optimal level of seminal plasma in the extender to improve sperm motility is currently considered to be 10% (Maxwell and Johnson 1999). Lower (5%) and higher (20%) concentrations of seminal plasma appeared to be detrimental to the survival and membrane integrity of boar sperm (Maxwell et al. 1997).

Various authors have studied sperm fertility after the addition of seminal plasma to AI doses, reporting inconsistent results (Maxwell and Johnson 1999; Bellin et al. 1998; Graham 1994). The variation obtained among species and individuals could be associated with the presence, absence or critical concentration of certain components, probably proteins, in some seminal plasma's but not in others (Maxwell and Johnson 1999). Indeed, in bulls, heparin-binding proteins are secreted in a testosterone dose-dependent manner. Further, some seminal plasma proteins have been related to higher conception rates in cattle (Bellin et al. 1998) and concentrations of these seminal plasma proteins vary among males.

Boar differences were noted by Rozeboom et al. (2000) in the immunological response of the uterus after seminal plasma addition during AI. Seminal plasma from some boars may have more of the component(s) that stimulate the uterus to protect the new embryos from the dam's immune system. These various studies all support the idea that seminal plasma has valuable components, which might benefit establishment of pregnancy. However, it appears clear that both the components and their actions must be much better understood before commercial application is possible.

References

- Abad-Gavin M, 2001: History of Animal Reproduction. III Iberian Congress of Animal Reproduction, Porto (Portugal) pp 11-22.
- Ashworth PJC, Harrison RAP, Miller NG, Plummer JM, Watson PJ, 1994: Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. *Reprod Fertil Develop* **6**, 173-180.
- Baldi E, Luconi M, Bonaccorsi L, Krausz C, Forti G, 1996: Human sperm activation during capacitation and acrosome reaction: Role of calcium, protein phosphorylation and lipid remodelling pathways. *Front Biosci* **1**, d189-205.
- Baldi E, Luconi M, Bonaccorsi L, Muratori M, Forti G, 2000: Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. *Front Biosci* **5**, E110-E123.
- Baldi E, Luconi M, Bonaccorsi L, Forti G, 2002: Signal transduction pathways in human spermatozoa. *J Reprod Immunol* **53**, 121-131.
- Bailey JL, Bilodeau JF, Cormier N, 2000: Semen cryopreservation in domestic animals; a damaging and capaciting phenomenon. *J Androl* **21**, 1-7.
- Bailey J, Morrier A, Cormier N, 2003: Semen cryopreservation: Successes and persistent problems in farm species. *Can J Anim Sci* **83**, 393-401.
- Bellin ME, Oyarzo JN, Hawkins HE, Zhang H, Smith RG, Forrest DW, Sprott LR, Ax RL, 1998: Fertility-Associated antigen on bull sperm indicates fertility potential. *J Anim Sci* **76**, 2032-2039
- Bertani GR, Scheid IR, Rubin MIB, Wentz I, Goncalves PBD, 1997: Effect of the time of artificial insemination with frozen-thawed or fresh semen on embryo viability and early pregnancy rate in gilts. *Theriogenology* **48**, 933-945
- Breitbart H, Rubinstein S, Lax Y, 1997: Regulatory mechanisms in acrosomal exocytosis. *Rev Reprod* **2**, 165-174.
- Buhr M, 2001: Emerging tools in artificial insemination. London Swine Conference - The Pork Industry and Public Issues 5-6 April 2001
- Calvete JJ, Ensslin M, Mburu J, Iborra A, Martinez P, Adermann K, Waberski D, Sam L, Topfer-Petersen E, Weitze KF, Einarsson S, Rodriguez-Martinez H, 1997: Monoclonal antibodies against boar sperm zona pellucida-binding protein AWN- 1. Characterization of a continuous antigenic determinant and immunolocalization of AWN epitopes in inseminated sows. *Biol Reprod* **57**, 735-742.
- Candini PH, Moretti AS, Silveira PRS, Zanella E, Viana CHC, Santos ICC, 2001: Utilization of a protocol of synchronization of ovulation, induced by porcine LH, to perform insemination programs in fixed time, with one or two inseminations per sow. *Proc 6th Int Conf Pig Reproduction*, 2001.

- Cassar G, Kirkwood RN, Poljak Z, Friendship RM, 2004: Effect of estrogen formulation and site of deposition on fertility of artificially inseminated sows treated with human chorionic gonadotrophin to induce ovulation. *J Swine Health Prod* **12**, 285-287.
- Cassar G, Kirkwood RN, Poljak Z, Bennett-Steward K, Friendship RM, 2005: Effect of single or double insemination on fertility of sows bred at an induced estrus and ovulation. *J Swine Health Prod* **13**, 254-258.
- Ciereszko A, Ottobre JS, Glogowski J, 2000: Effects of season and breed on sperm acrosin activity and semen quality of boars. *Anim Reprod Sci* **64**, 89-96.
- Claus R, 1990: Physiological role of seminal components in the reproductive tract of the female pig. *J Reprod Fertil (Suppl)* **40**, 117-31.
- Crabo B, Einarsson S, 1971: Fertility of deep frozen spermatozoa. *Acta Vet Scand* **12**, 125-127.
- De Lamirande E, Leclerc P, Gagnon C, 1997: Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod* **3**, 175-94.
- De Rensis F, Benedetti S, Silva P, Kirkwood RN, 2003: Fertility of sows following artificial insemination at a gonadotrophin-induced estrus coincident with weaning. *Anim Reprod Sci* **76**, 245-250.
- Dziuk PJ, Henshaw G, 1958: Fertility of boar semen artificially inseminated following in vitro storage. *J Anim Sci* **17**, 554-558.
- Eriksson B, 2000: Cryopreservation of boar semen. Studies on sperm viability in vitro and fertility. PhD Thesis. Uppsala: Swedish University of Agriculture Sciences.
- Estienne MJ, Allen FH, 2005: Using artificial insemination in swine production: Detecting and synchronizing estrous and using proper insemination technique. Virginia Cooperative Extension Publication 414-038.
- Fazeli A, Duncan AE, Watson PF, Holt WV, 1999: Sperm-oviduct interaction: induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol Reprod* **60**, 879-86.
- Foote RH, 2002: The history of artificial insemination: Selected notes and notables. *J Anim Sci*. 80. Electronic Supplement 2. Peer-reviewed papers from the 2001 ASAS National Meeting.
- Gadella BM, Harrison RAP, 2000: The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behaviour in the sperm plasma membrane. *Development* **127**, 2407-2420.

- Garner DL, Thomas CA, Gravance CG, Marshall CE, DeJarnette JM, Allen CH, 2001: Seminal plasma addition attenuates the dilution effect in bovine sperm. *Theriogenology* **56**, 31–40.
- Gerrits RJ, Lunney JK, Johnson LA, Pursel VG, Kraeling RR, Rohrer G, Dobrinsky JR, 2005: Perspectives for artificial insemination and genomics to improve swine populations. *Theriogenology* **63**, 283-299.
- Gillan L, Evans G, Maxwell WMC, 1997: Capacitation status and fertility of frozen-thawed ram spermatozoa. *Reprod Fertil Dev* **9**, 481–487.
- Graham JK, 1994: Effect of seminal plasma on the motility of epididymal and ejaculated spermatozoa of the ram and bull during the cryopreservation process. *Theriogenology* **41**, 1151-1162.
- Graham EF, Rajamannan AHJ, Schmehl MKL, Maki-Laurila M, Bower RE, 1971: Fertility studies with frozen boar spermatozoa. *AI Digest* **19**:61.
- Green CE, Watson PF, 2001: Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation. *Reproduction* **122**, 889-898.
- Hammerstedt RH, Graham JK, Nolan P, 1990: Cryopreservation of mammalian sperm: What we ask them to survive. *J Androl* **11**, 73–88.
- Harrison RAP, Ashworth PJC, Miller NGA, 1996: Bicarbonate/CO₂, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Mol Reprod Dev* **45**, 378–91.
- Harrison RAP, 1997: Sperm plasma membrane characteristics and boar semen fertility. *J Reprod Fertil (Suppl)* **52**, 195–211.
- Hofmo PO, Grevle IS, 2000: Development and commercial use of frozen boar semen in Norway. In: Johnson LA, Guthrie HD, (eds). *Boar semen preservation IV*. Lawrence KS: Allen Press; pp. 71-77.
- Holt WV, 2000a: Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* **53**, 47-58.
- Holt WV, 2000b: Basic aspects of frozen storage of semen. *Anim Reprod Sci* **62**, 3-22.
- Hunter RHF, 1981: Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J Reprod Fertil* **63**, 109–117.
- Hunter RHF, 2002: Vital aspects of fallopian tube physiology in pigs. *Reprod Dom Anim* **37**, 186-190.
- Ito T, Niwa T, Kudo A, 1948: Studies on artificial insemination in swine. *Zootech Exp Sta Res Bull* **55**, 1-74.

Johnson LA, Larksson K, 1985: eds. Deep freezing of Boar semen. Uppsala: Swedish University of Agriculture Sciences Press; 1985. 310 p.

Johnson LA, 1998: Current developments in swine semen: preservations, artificial insemination and sperm sexing. *Proc IPVS*, **1**, 225–229.

Johnson LA, Witze KF, Fiser P, Maxwell WMC, 2000: Storage of boar semen. *Anim Reprod Sci* **62**, 143-172.

Kaeoket K, Persson E, Dalin AM, 2003: Influence of pre-ovulatory insemination and early pregnancy on the infiltration by cells of the immune system in the sow endometrium. *Anim Reprod Sci* **75**, 55–71.

Kemp B, Soede NM, 1996: Relationship of weaning-to-estrus interval to timing of ovulation and fertilization in sows. *J Anim Sci* **74**, 944-949.

Kirkwood RN, 1999: Pharmacological intervention in swine reproduction. *Swine Health Prod* **7**, 29-35.

Knox RV, Rodriguez-Zas SL, 2001: Factors influencing estrous and ovulation in weaned sows as determined by transrectal ultrasound. *J Anim Sci* **79**, 2957–2963.

Krueger C, Rath D, 2000: Intrauterine insemination in sows with reduced sperm number. *Reprod Fertil Dev* **12**, 113-117.

Levis DG, 2000: Liquid boar semen production: current extender technology and where we go from here. In: Johnson LA, Guthrie HD, (eds). *Boar semen preservation IV*. Lawrence, KS: Allen Press pp 121-128.

Liu Z, Foote RH, 1998: Osmotic effects on volume and motility of bull sperm exposed to membrane permeable and non-permeable agents. *Cryobiology* **37**, 207-218.

Martinez EA, Vazquez JM, Roca J, Lucas X, Gil MA, Parrilla I, Vazquez JL, Day BN, 2001a: Successful non-surgical deep intrauterine insemination with small numbers of spermatozoa in sows. *Reproduction* **122**, 289-296.

Martinez EA, Vazquez JM, Roca J, Lucas X, Gil MA, Parrilla I, Vazquez JL, 2001b: Deep intrauterine insemination in sows with a low number of spermatozoa: A new and simple procedure. *Theriogenology* **55**, 248 (abstr).

Maxwell WMC, Welch GR, Johnson LA, 1997: Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. *Reprod Fertil Develop* **8**, 1165-1178.

Maxwell WMC, Evans G, Mortimer ST, Gillan L, Gellatly ES, Mcphie CA, 1999: Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. *Reprod Fertil Develop* **11**, 123-126.

- Maxwell WMC, Johnson LA, 1999: Membrane status of boar spermatozoa after cooling or cryopreservation. *Theriogenology* **48**, 209-219.
- Memon MA, Bretzlaff KN, Ott RS, 1985: Effect of washing on motility and acrosome morphology of frozen-thawed goat spermatozoa. *Am J Vet Res* **46**, 473-475.
- Nagase H, Graham EF, 1964: Pelleted semen: Comparison of different extenders and processes on fertility of bovine spermatozoa. In: *Proc 5th Int. Congr Anim Reprod AI*, Trento, Italy. **4**, 387-391.
- Nissen AK, Schmidt M Hyttel P, Greve T, 1995: Ultrasonography of ovulation in relation to first cleavage in hCG treated sows. *Theriogenology* **43**, 287.
- Nissen AK, Soede NM, Hyttel P, Schmidt M, D'Hoore L, 1997: The influence of time of insemination relative to time of ovulation on farrowing frequency and litter size in sows, as investigated by ultrasonography. *Theriogenology* **47**, 1571-1582.
- Nissen AK, Schmidt M, Hyttel P, Greve T, 2000: Ovulation and embryonic developmental rate following hCG-stimulation in sows. *Acta Vet Scand* **41**, 321-328.
- Noiles EE, Bailey JL, Storey BT, 1995: Temperature dependence of the water permeability, L_p , of murine sperm shows a discontinuity between 4° and 0°C. *Cryobiology* **32**, 220-238.
- Parks JE, 1997: Hypothermia and mammalian gametes. In: Karow AM, Critser J K (eds) *Reproductive tissue banking*. San Diego: Academic Press 1997, pp 229-261.
- Parrish JJ, Susko-Parrish J, Winer MA, First NL, 1988: Capacitation of bovine sperm by heparin. *Biol Reprod* **38**, 1171-1180.
- Pelaez J, 2004: Congelacion de semen porcino. Aportaciones a la congelabilidad y fertilidad. PhD Thesis. University of Leon (ES).
- Petrunkina AM, Friedrich J, Drommer W, Bicker G, Waberski D, Topfer-Petersen E, 2001: Kinetic characterization of the changes in protein tyrosine phosphorylation of membranes, cytosolic Ca^{2+} concentration and viability in boar sperm populations selected by binding to oviductal epithelial cells. *Reproduction* **122**, 469-480.
- Polge C, 1956: Artificial insemination in pigs. *Vet Rec* **68**, 62-76.
- Polge C, Salamon S, Wilmut I, 1970: Fertilizing capacity of frozen boar semen following surgical insemination. *Vet Rec* **87**, 424-429.
- Pursel VG, 1982: Effect of uterine ligation and cervical plugs on retention of frozen-thawed boar sperm. *J Anim Sci* **54**, 137-141.
- Pursel VG, Johnson LA, 1971: Procedure for the preservation of boar spermatozoa by freezing. *USDA ARS, Bull No 44-227*, 1-5.

- Pursel VG, Johnson LA, Schulman LL, 1973: Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. *J Anim Sci* **37**, 528-31.
- Pursel VG, Johnson LA, 1975: Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J Anim Sci* **40**, 99-102.
- Rath D, Niemann H, 1997: In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. *Theriogenology* **47**, 785-793.
- Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vazquez JM, Martinez E, Roca J, Sanz L, Calvete JJ, 2005: Boar spermatozoa in the oviduct. *Theriogenology* **63**, 514-535.
- Rozeboom KJ, Troedsson MHT, Crabo BG, 1998: Characterization of uterine leukocyte infiltration in gilts after artificial insemination. *J Reprod Fertil* **114**, 195-199.
- Rozeboom KJ, Troedsson MH, Hodson HH, Shurson GC, Crabo BG, 2000: The importance of seminal plasma on the fertility of subsequent artificial inseminations in swine. *J Anim Sci* **78**, 443-448.
- Rozeboom KJ, Reicks DL, Wilson ME, 2004: The reproductive performance and factors affecting on-farm application of low-dose intrauterine deposit of semen in sows. *J Anim Sci* **82**, 2164-2168.
- Selles E, 2001: Estudio de diferentes factores que afectan a la calidad y capacidad fecundante de los espermatozoides crioconservados en la especie porcina. PhD Thesis. University of Murcia (ES).
- Soede NM, Bowman EG, Kemp B, 1998: Seminal plasma does not advance ovulation in hCG-treated sows. *Anim Reprod Sci* **54**, 23-29.
- Soede NM, Steverink DW, Langendijk P, Kemp BJ, 2000: Optimized insemination strategies in swine AI. In: LA Johnson, HD Guthrie (eds): *Boar Semen Preservation IV*. Allen Press Inc. Lawrence, Kansas. pp. 185-190.
- Steverink DW, Soede NM, Bouwman EG, Kemp BJ, 1997: Influence of insemination-ovulation interval and sperm cell dose on fertilization in sows. *J Reprod Fertil* **111**, 165-171.
- Steverink DWB, Soede NM, Bouwman EG, Kemp B, 1998: Semen backflow after insemination and its effect on fertilization results in sows. *Anim Reprod Sci* **54**, 109-119.
- Straw B. Swine Health Resource. Michigan State University Extension Program. <http://www.msu.edu/%7Estraw/>

- Suarez SS, Revah I, Lo M, Koelle S, 1998: Bull sperm binding to oviductal epithelium is mediated by a Ca²⁺-dependent lectin on sperm that recognizes Lewis trisaccharide. *Biol Reprod* **59**, 39–44.
- Suarez SS, 2002: Formation of a reservoir of sperm in the oviduct. *Reprod Dom Anim* **37**, 140-143.
- Tardif S, Dube C, Chevalier S, Bailey JL, 2001: Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. *Biol Reprod* **65**, 784–92.
- Tardif S, Dube C, Bailey JL, 2003: Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein phosphorylation is only associated with calcium. *Biol Reprod* **68**, 207–13.
- Topfer-Petersen E, Wagner A, Friedrich J, Petrunkina A, Ekhlas-Hundriesen M, Waberski D, 2002: Function of the mammalian oviductal sperm reservoir. *J Exp Zool* **292**, 210–5.
- Vadnais ML, Kirkwood RN, Tempelman RJ, Sprecher DJ, Chou K, 2005a: Effect of cooling and seminal plasma on the capacitation status of fresh boar sperm as determined using chlortetracycline assay. *Anim Reprod Sci* **87**, 121-132.
- Vadnais ML, Kirkwood RN, Sprecher DJ, Chou K, 2005b: Effects of extender, incubation temperature, and added seminal plasmas on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Anim Reprod Sci* **90**, 347-354.
- Vazquez JM, Martinez EA, Parrilla I, Cuello C, Carvajal G, Gil MA, Lucas X, Centurion F, Vazquez JL, Roca J, 2000: Deep uterine insemination in sows with flow cytometric sorted sperm. *Theriogenology* **53**, 389 (abstr).
- Visconti PE, Kopf GS, 1998: Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod* **59**, 1–6.
- Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB, 2002: Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol* **53**, 133-150.
- Vyt P, Maes D, Rijsselaere T, Dejonckheere E, Castryck F, Van Soom A, 2004: Motility assessment of porcine spermatozoa: a comparison of methods. *Reprod Dom Anim* **39**, 447-453.
- Waberski D, Weitze KF, Lietmann C, Lubbert zur Lage W, Bortolozzo FP, Willmen T, Petzoldt R, 1994a: The initial fertilizing capacity of long term-stored liquid boar semen following pre- and postovulatory insemination. *Theriogenology* **41**, 1367-1377.

Waberski D, Weitze KF, Gleumes T, Schwartz M, Willmen T, Petzold R, 1994b: Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology* **42**, 831-840.

Waberski D, Claassen R, Hahn T, Jungblut PW, Parvizi N, Kallweit E, Weitze KF, 1997: LH profile and advancement of ovulation after transcervical infusion of seminal plasma at different oestrous stages in gilts. *J Reprod Fertil* **109**, 29–34.

Wagner HG, Thibier M, 2002: World Statistics for Artificial Insemination in small ruminants and swine. In: Proceedings of the 14th International congress on Animal Reproduction **2**, 77.

Watson PF, Behan JR, 2002: Intrauterine insemination of sows with reduced sperm numbers: results of a commercially based field trial. *Theriogenology* **57**, 1683-1693.

Weitze KF, Wagner-Rietschel H, Waberski D, Richter L, Krieter J, 1994: The onset of estrus after weaning, estrus duration and ovulation as major factors in AI timing in sows. *Reprod Dom Anim* **29**, 433-443.

Weitze KF, 2000: Update on the worldwide application of swine AI. In: LA Johnson, HD Guthrie (eds). *Boar semen preservation IV*. Lawrence, KS: Allen Press; 2000. pp. 141-145.

Westendorf P, Richter L, Treu H, 1975: Deep freezing of boar sperm. Laboratory and insemination results using the Hulsenberger paillete method. *Dtsch Tierarztl Wochenschr* **82**, 261-267.

Wiggins EL, Grummer RH, Casida LE, 1951: Minimal volume of semen and number of sperm for fertility in artificial insemination of swine. *J Anim Sci* **1**, 138-143.

Willenburg KL, Knox RV, Kirkwood RN, 2004: Effect of estrogen formulation and its site of deposition on serum PGFM concentrations, uterine contractility, and time of ovulation in artificially inseminated sows. *Anim Reprod Sci* **80**, 147-156.

Yanagimachi R, 1994: Mammalian fertilization. In: Knobil A, Neill JD (eds). *The Physiology of Reproduction*. New York: Raven Press. 189–317.

Zhu, J, 2000: Boar seminal plasma components and fertilization. MSc Thesis, University of Alberta, Canada.

CHAPTER 2

Effect of sperm cryopreservation and supplementing semen doses with seminal plasma on the establishment of a sperm reservoir in gilts

M Abad¹, DJ Sprecher¹, P Ross¹, RM Friendship², RN Kirkwood¹

¹Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, USA. ²Department of Population Medicine, University of Guelph, Canada

Contents

Frozen-thawed (FT) boar sperm have a reduced fertile life, due in part to a capacitation-like status induced by cooling. Reversal of this cryocapacitation in-vitro by exposure to boar seminal plasma (SP) has been demonstrated. The objective of these studies was to determine the effect of SP on the ability of FT sperm to create an oviductal sperm reservoir following artificial insemination (AI). In Experiment One, 35 prepubertal gilts were injected (IM) with 400 IU equine chorionic gonadotropin (eCG) plus 200 IU human chorionic gonadotropin (hCG) to induce estrus. At detection of estrus, gilts were inseminated with 3×10^9 live sperm, either fresh (FS; n=13), FT (n=10), or FT supplemented with 10% v/v SP (n=12). Gilts were sacrificed 8 h later, their reproductive tracts recovered and the uterotubal junctions (UTJs) flushed to recover sperm. Fewer ($P < 0.01$) sperm were recovered following FT compared to FS inseminations, and there was no evident effect of SP. In Experiment Two, 30 prepubertal gilts received IM injections of 1,000 IU eCG followed by 5 mg porcine luteinizing hormone (pLH) 80 h later to control time of ovulation. Gilts were inseminated with 3×10^9 live FS sperm (n=6), FT sperm (n=15) or FT sperm plus 10% SP (n=9) at 12 h before ovulation and then sacrificed 8 h later. The UTJs were dissected and flushed for sperm recovery. Fewest ($P < 0.001$) sperm were recovered following FT insemination and there was no evident effect of SP. These data

demonstrate that the size of the sperm reservoir is markedly reduced in gilts inseminated with FT sperm. However, the lack of effect of SP suggested that either it did not reverse cryocapacitation or that such a reversal does not impact the in-vivo ability to create a sperm reservoir.

Introduction

After deposition into the female reproductive tract, boar sperm are passively transported by gravity and uterine contractions to the tubal end of the uterine horns. Thereafter, the sperm are passively and actively (sperm motility) transported through the uterotubal junction (UTJ) into the oviduct (Langendijk et al. 2002). Only a small percentage of the inseminated cells get to the UTJ (Rodriguez-Martinez et al. 2005), where due to the narrower lumen of the region (Fazeli et al. 1999), binding of non-capacitated sperm cells to oviductal epithelial cells (Suarez 1998; Petrunkina et al. 2001), and the local oviductal fluid environment (Rodriguez-Martinez et al. 2005), a functional sperm reservoir is formed in the distal 1 to 2 cm of the isthmus (Hunter 2002). The sperm remain in this region until immediately prior to ovulation, at which time they are released in waves to redistribute towards the ampulla-isthmus junction for fertilization. Presumably, a sufficient number of viable sperm cells are needed in the sperm reservoir in the peri-ovulatory period to maximize fertility rates.

In pigs, artificial insemination (AI) with frozen-thawed semen results in reduced farrowing rates and litter sizes (Johnson et al. 2000) due to cryoinjury to sperm including the induction of a capacitation-like status of the sperm cells, so-called cryocapacitation (Bailey et al. 2003). Since capacitated cells are not stored in the sperm reservoir, to be able to fertilize ova cryopreserved sperm must presumably

encounter oocytes soon after insemination. Therefore, greater accuracy in timing of the insemination relative to time of ovulation is likely needed when using frozen-thawed semen (Larsson 1976). Hormones such as equine chorionic gonadotropin (eCG), gonadotropin-releasing hormone (GnRH), human chorionic gonadotropin (hCG) or porcine luteinizing hormone (pLH) and their combination can be used effectively to control the time of ovulation in pigs, which may be monitored by real time ultrasonography (Soede et al. 1994).

If the primary cryoinjury associated with reduced fertility is cryocapacitation, then reversal of cryocapacitation should enhance sperm fertility. It has recently been demonstrated that based on chlortetracycline staining patterns, exposure to seminal plasma will reverse cryocapacitation of boar sperm in-vitro (Vadnais et al. 2005a,b). Further, it has been documented that addition of seminal plasma to cryopreserved ram semen improved subsequent ewe fertility (Maxwell et al. 1999). A beneficial effect of thawing cryopreserved boar sperm in seminal plasma on in-vivo sperm longevity (Einarsson and Viring 1973) and gilt and sow fertility (Crabo and Einarsson 1971; Larsson 1976) has also been documented. However, we are not aware of any recent studies examining the effect of a relatively low (10%) content of seminal plasma in thawing diluents on in-vivo fertility in pigs. The present experiments were designed to test the hypothesis that cryopreserved boar sperm are less able to form a sperm reservoir in the oviduct and that this relative inability would be reversed by prior exposure to seminal plasma.

Materials and Methods

These studies were performed at Michigan State University Swine Facility and University of Guelph (Arnell) Research Station and were approved by the institutional Animal Care committees.

In Experiment One, 60 prepubertal Landrace x Yorkshire gilts (124.3 ± 1.8 kg) were housed in groups of approximately 10 and were allowed ad libitum access to a standard finisher ration. Gilts received an IM injection of 400 IU equine chorionic gonadotrophin (eCG) plus 200 IU human chorionic gonadotrophin (hCG) (PG600, Intervet) to induce a synchronous onset of estrus. From Day 1 post-injection, gilts were exposed to a mature boar for 15 min daily to facilitate detection of estrus. Only those gilts exhibiting standing estrus on Day 5 were selected for the study (35/60). Selected gilts were assigned by weight to receive a single insemination either of fresh semen (FS; n=13), frozen-thawed semen (FT; n=10), or FT semen supplemented with 10% v/v seminal plasma (SP; n=12). The seminal plasma was pre-mixed in the thawing extender, thus allowing the thawed sperm immediate contact with seminal plasma constituents. Commercial semen from 2 boars was used in this study and both fresh and frozen doses contained 3×10^9 live sperm with boars equally represented within treatment. All inseminations were cervical and were performed by the same personnel using conventional foam-tip catheters in the presence of a mature boar.

In Experiment Two, 30 prepubertal Yorkshire gilts (127.5 ± 2.4 kg) without previous boar contact were housed as groups of approximately 10 and fed ad libitum a standard dry sow ration. Following formation of groups (Day 0), ovulation was induced by IM injection of 1,000 IU eCG (Pregnenol, Bioniche Animal Health, Bellville, Ontario,

Canada) followed by 5 mg pLH (Lutropin, Bioniche Animal Health) 80 h later. Ovulation was predicted to occur 38 h after pLH injection. Gilts were allowed 15 min exposure to a mature boar at 12 h intervals to facilitate detection of estrus.

At 26 h after pLH injection (12 h before the predicted time of ovulation) gilts were assigned by weight to receive a single insemination of FS semen (n=6), FT semen (n=15), or FT semen + 10% v/v seminal plasma (n=9). The FT semen group was over-represented because of an expectation of lower sperm recovery. The same personnel using foam-tip catheters in the presence of a mature boar performed all of the inseminations.

The boars supplying semen and seminal plasma for Experiment Two were housed in the Ponsonby Research Facility, University of Guelph. All boars were 8 to 12 months of age and were routinely collected weekly using the gloved-hand method.

Immediately after collection, entire filtered ejaculates were transported to the laboratory at 37°C and analyzed for volume, motility and sperm concentration. The same pool of 3 boars was used to obtain both fresh and FT semen doses. Sperm cryopreservation employed the method of Pursel and Johnson (1975), as modified by Buhr et al. (2001). The vitality of cryopreserved sperm at thawing was determined using Eosin-Nigrosin staining. All doses (FS and FT) were extended in Beltsville Thawing Solution to a total volume of 80 ml and 3×10^9 live sperm.

In Experiments One and Two, seminal plasma (SP) was obtained after centrifugation (800 x g for 10 min at 25°C) of a pool of semen derived from 5 and 10 Yorkshire boars, respectively, and was stored at -80°C until needed. Thawing of SP was done in

a 37°C water bath and semen doses that included the 10% v/v SP were incubated for 20-30 min at 39°C before insemination. This interval was chosen based on previous data showing that reversal of cryocapacitation in vitro required at least 15 min (Vadnais et al. 2005b).

At 8 h after insemination, gilts were either subjected to commercial slaughter procedures (Experiment One) or euthanized by IV injection of 100 mg/kg pentobarbital (Experiment Two), and their reproductive tracts recovered. Both UTJs, defined as the portion of the tract comprising the last 1 cm of the uterine horn and first 3 cm of oviduct, were dissected and clamped. For flushing of sperm from the UTJs, a blunted 18 g 12 mm needle was introduced into the oviduct and the UTJ's filled with 0.5 ml of Dulbecco's PBS. A gentle massage was performed for 1 min to detach sperm cells from the oviductal epithelium and the volume emptied into a 15 ml tube. This was repeated once and thereafter, two more flushings were performed by forcing 5 ml DPBS to pass through the UTJ lumen. An average total volume of 11 ml was recovered for each UTJ.

The recovered fluid was centrifuged at 800g for 10 min (25°C), and the supernatant discarded. The pellet was resuspended to 0.5 ml and then frozen overnight (-20°C) to destroy any maternal cells. After thawing by submersion in a 37°C water bath, the tubes were gently agitated to re-suspend sperm and the number of cells counted using a hemacytometer. The average of two readings was recorded for each oviduct and the average from both oviducts was recorded. If sperm were evident on the slide but absent from the designated counting area, the gilts were given a nominal total sperm count of 500.

Statistical analysis

Data were analyzed using INFOSTAT/E v2.0 (University of Cordoba, Argentina).

Differences in mean numbers of recovered spermatozoa were tested using analysis of variance with LSD Fisher approximation. Body weight was used as a blocker in all analyses. Results are presented as mean $\pm$ SEM and $P < 0.05$ was considered statistically significant.

Results

In both experiments, examination of gilt ovaries post mortem revealed that none had ovulated. The effect of semen type and supplemental SP in Experiment One are shown in Table 2.1. The insemination of FT sperm resulted in a significantly smaller sperm reservoir ($P < 0.01$). There was no effect of SP on numbers of sperm recovered.

The effect of semen type and of supplemental SP in Experiment Two are shown in Table 2.2. The results were very similar to those of Experiment One, with the insemination of FT sperm resulting in a significantly smaller sperm reservoir ($P < 0.001$) and with sperm numbers unaffected by supplemental SP.

Table 2.1. Effect of freezing and supplementation with seminal plasma (SP) on number of sperm recovered from the oviduct and uterotubal junction (Experiment 1)

	AI group		
	Fresh	Frozen	Frozen+SP
N	13	10	12
Body weight (kg)	125.2 $\pm$ 2.7	123.2 $\pm$ 2.9	124.2 $\pm$ 3.9
Sperm recovered ($\times 10^5$)*	211.8 $\pm$ 84.9	9.1 $\pm$ 2.4	8.6 $\pm$ 2.2

* $P < 0.01$

Table 2.2. Effect of freezing and supplementation with seminal plasma (SP) on number of sperm recovered from the oviduct and uterotubal junction (Experiment 2)

	AI group		
	Fresh	Frozen	Frozen+SP
N	6	15	9
Body weight (kg)	119.2±5.3	126.1±3.3	135.6±3.9
Sperm recovered (x10 ³)*	141.6±22.9	19.3±1.3	9.4±1.0

* P<0.001

Discussion

The absence of evidence of ovulation in the gilts was taken to indicate that we had achieved our objective, i.e., induction of estrus followed by insemination and establishment of sperm reservoirs in the preovulatory period. Regarding the establishment of the sperm reservoir, the numbers of sperm recovered following the fresh semen inseminations were of the same order of magnitude as that reported previously (Langendijk et al. 2002; Willenburg et al. 2003). Therefore, the numbers of sperm observed are likely sufficient for normal fertility, although the minimum number of sperm within the sperm reservoir at the time of ovulation necessary to maximize oocyte fertilization rates remains unknown. We are not aware of any recent studies examining establishment of a sperm reservoir subsequent to AI with cryopreserved semen. However, the present results clearly indicate that cryopreservation markedly reduces the ability of sperm to associate with the oviductal epithelium of the sperm reservoir. It is now accepted that only non-capacitated sperm reside in the sperm reservoir (Fazeli et al. 1999; Tienthai et al. 2004). Therefore, the capacitation-like reaction induced by cooling (Vadnais et al. 2005a,b) may explain the inability of cryopreserved sperm to enter the sperm reservoir. If it is accepted that a

small sperm reservoir will compromise fertility, then this inability to form an adequate sperm reservoir may partly explain fertility depressions associated with insemination of cryopreserved sperm.

Interestingly, using chlortetracycline (CTC) staining, seminal plasma has been shown to prevent and/or reverse capacitation in-vitro (Maxwell et al. 1999; Vadnais et al. 2005a,b). Others have demonstrated improved gilt and sow fertility when frozen sperm were thawed in seminal plasma (Crabo and Einarsson 1971; Einarsson et al. 1973; Larsson and Einarsson 1975). Also, thawing in seminal plasma increased sperm survival time in-vivo (Einarsson et al. 1973; Einarsson and Viring 1973). Clearly, some component(s) of seminal plasma can affect the fertility of cryopreserved boar sperm. However, in the present study the addition of 10% v/v seminal plasma to cryopreserved semen at thawing had no effect on the establishment of the sperm reservoir in-vivo. This may indicate that the effect of 10% seminal plasma observed with CTC staining in vitro is not biologically meaningful. However, we have shown by flow cytometry of sperm stained with merocyanine and yo-pro that seminal plasma did prevent further capacitation when cryopreserved sperm were incubated in a capacitation-supporting medium (unpublished data). Therefore, if it is accepted that seminal plasma will affect the capacitation status of cryopreserved sperm, the implication is that the failure of cryopreserved sperm to form an effective sperm reservoir involves aspects of cryoinjury in addition to cryocapacitation. In support of this suggestion, it has been shown that approximately 60% of cryopreserved sperm exhibit cryocapacitation on thawing, with about 40% appearing “normal”. If these normal appearing sperm are indeed normal, a markedly larger number of sperm would be anticipated in the sperm reservoir.

From our data, we conclude that cryopreserved boar sperm did not form an effective sperm reservoir subsequent to insemination. Further, the apparent lack of effect of seminal plasma on the establishment of a sperm reservoir suggests that aspects of cryoinjury in addition to the cryocapacitation effect are involved. Whether a greater seminal plasma inclusion level, as used by earlier workers, would alter the observed effect remains unknown.

Acknowledgements

We gratefully acknowledge Ontario Pork, Michigan State University Foundation, and the University of Guelph-Ontario Ministry of Agriculture and Food Animal Research Program for financial support of these studies. We are indebted to Dr. Jose Cibelli for allowing access to his laboratory (Experiment One) and to Glen Cassar, Christine Pelland and Arturo Ruiz for invaluable assistance in the performance of Experiment Two.

References

- Bailey J, Morrier A, Cormier N, 2003: Semen cryopreservation: Successes and persistent problems in farm species. *Can J Anim Sci* **83**, 393–401.
- Buhr M, 2001: Emerging tools in artificial insemination. *Proc London Swine Conference 2001*, pp 123-135.
- Crabo B, Einarsson S, 1971: Fertility of deep frozen boar spermatozoa. *Acta vet scand* **12**, 125-127.
- Einarsson S, Swensson T, Viring S, 1973: A field trial on the fertility of deep-frozen boar spermatozoa. *Nord Vet –Med* **25**, 372-376.
- Einarsson S, Viring S, 1973: Distribution of frozen-thawed spermatozoa in the reproductive tract of gilts at different time intervals after insemination. *J Reprod Fert* **32**, 117-120.
- Fazeli A, Duncan AE, Watson PF, Holt WV, 1999: Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviductal epithelial cells in porcine species. *Biol Reprod* **60**, 879-886.
- Hunter RHF, 2002: Vital aspects of fallopian tube physiology in pigs. *Reprod Dom Anim* **37**, 186-190.
- Johnson LA, Witze KF, Fiser P, Maxwell WMC, 2000: Storage of boar semen. *Anim Reprod Sci* **62**, 143-172
- Langendijk P, Bouwman EG, Kidson A, Kirkwood RN, Soede NM and Kemp B, 2002: Role of myometrial activity in sperm transport through the genital tract and in fertilization in sows. *Reproduction* **123**, 683-690.
- Larsson K, 1976: Fertility of deep frozen boar spermatozoa at various intervals between insemination and induced ovulation. Influence of boars and thawing diluents. *Acta vet scand* **17**, 63-73.
- Larsson K, Einarsson S, 1975: Fertility and post-thawing characteristics of deep frozen boar spermatozoa. *Andrologia* **7**, 25-30.
- Maxwell WMC, Evans G, Mortimer ST, Gillian L, Gellatly ES, McPhie CA, 1999: Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. *Reprod Fertil Dev* **11**, 123-126.
- Petrunkina AM, Gehlhaar R, Drommer W, Waberski D, Topfer-Petersen E, 2001: Selective sperm binding to pig oviductal epithelium in vitro. *Reproduction* **121**, 889-896.
- Pursel VG, Johnson LA, 1975: Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J Anim Sci* **40**, 99-102.

Rodriguez-Martinez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vazquez JM, Martinez E, Roca J, Sanz L, Calvete JJ, 2005: Boar spermatozoa in the oviduct. *Theriogenology* **63**, 514-535.

Suarez SS, 1998: The oviductal sperm reservoir in mammals: mechanisms of formation. *Biol Reprod* **58**, 1105–1107.

Soede NM, Helmond FA, Kemp B, 1994: Periovulatory profiles of oestradiol, LH and progesterone in relation to oestrus and embryo mortality in multiparous sows using transrectal ultrasonography to detect ovulation. *J Reprod Fertil* **101**, 633-641.

Tienthai P, Johannisson A, Rodriguez-Martinez H, 2004: Sperm capacitation in the porcine oviduct. *Anim Reprod Sci* **80**, 131-146.

Vadnais ML, Kirkwood RN, Sprecher DJ, Chou K, 2005b: Effects of extender, incubation temperature, and added seminal plasmas on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Anim Reprod Sci* **90**, 347-354.

Vadnais M, Kirkwood RN, Tempelman RJ, Sprecher DJ, Chou K, 2005a: Effect of cooling and seminal plasma on the capacitation status of fresh boar sperm as determined using chlortetracycline assay. *Anim Reprod Sci* **87**, 121-32.

Willenburg KL, Miller GM, Rodriguez-Zas SL, Knox RV, 2003: Influence of hormone supplementation to extended semen on artificial insemination, uterine contractions, establishment of a sperm reservoir, and fertility in swine. *J Anim Sci* **81**, 821-829.

CHAPTER 3

Effect of insemination-ovulation interval and addition of seminal plasma on sow fertility to insemination of cryopreserved sperm

M Abad¹, JC Garcia¹, DJ Sprecher¹, G Cassar², RM Friendship², RN Kirkwood¹

¹Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, USA.

²Department of Population Medicine, University of Guelph, Canada.

Contents

In swine, the use of frozen-thawed (FT) sperm for artificial insemination (AI) is limited due to poor sow fertility, possibly associated with a post-thaw capacitation-like status resulting in fewer fully viable sperm. Sow fertility to AI with FT sperm may improve with deeper deposition of sperm within the female tract, insemination very close to ovulation, or reversal of cryocapacitation by seminal plasma (SP). We performed two experiments to examine these suggestions. In Experiment One, 122 multiparous Yorkshire sows received 600 IU eCG at weaning and 5mg pLH 80 h later to control time of ovulation. The predicted time of ovulation (PTO) was 38 h after pLH injection. Thereafter, sows were assigned on the basis of parity to a single AI of FT sperm at 2 h before PTO, or at 12 h before PTO, or FT sperm supplemented with 10% SP at 12 h before PTO. Control sows received fresh semen at 12 h before PTO. All semen doses were adjusted to 3×10^9 live cells and deposited into the cervix. Experiment Two employed 99 multiparous sows and was a repeat of Experiment One except that all FT inseminations were intrauterine.

In both experiments, farrowing rates were lower ($P < 0.01$) following FT inseminations with no effect of time of insemination or of supplemental SP. In Experiment One, litter size was smaller following FT insemination, but no effect on litter size was

evident in Experiment Two. Supplemental SP had no effect on litter size in either experiment. The lack of effect of either SP or timing of FT insemination on sow fertility suggests that the non-lethal sperm cryoinjury affecting fertility involves more than just cryocapacitation.

Introduction

Artificial insemination (AI) is the technique of choice in the breeding of domestic animals (Verberckmoes et al. 2004). However, in contrast to AI in cattle, swine AI involves almost exclusively liquid semen collected within the previous 3 to 7 days. This use of semen within a short interval from collection limits the time available to test for pathogenic contamination and, as such, increases the risk of disease transmission. Indeed, liquid semen is known to be a vehicle for dissemination of pathogens including the Porcine Reproductive and Respiratory Syndrome (PRRS) virus and porcine circovirus. In addition to improved flexibility in timing of matings and access to worldwide genetics (reviewed by Eriksson, 2000), the cryopreservation of boar sperm would allow sufficient time to ensure absence of pathogens and so improve herd biosecurity.

The limited use of frozen-thawed (FT) sperm in swine is due primarily to relatively poor farrowing rates and litter sizes associated with FT inseminations (Johnson et al. 2000). This poor performance results from cryoinjury to sperm including the induction of a capacitation-like status of the sperm cells, so-called cryocapacitation (Bailey et al. 2003).

Since cryocapacitated sperm cells are not stored in the oviductal sperm reservoir (Abad et al. 2006), their fertile lifespan will be very short. Therefore, for acceptable fertilization rates cryopreserved sperm must presumably encounter ova soon after insemination. Indeed, when inseminated very close to the time of ovulation (eg. ≤ 4 h), fertility to FT inseminations was greatly improved (Roca et al. 2003; Bolarin et al. 2006), with fertilization rates being comparable to those from inseminations of fresh semen (Waberski et al. 1994).

Potentially, a further way to improve fertility of FT sperm is to reverse cryocapacitation. Using chlortetracycline staining, it has been demonstrated that incubation of fresh or FT sperm in media supplemented with seminal plasma (SP) will reverse (cryo)capacitation (Suzuki et al. 2002; Vadnais et al. 2005a,b). Further, supplementing semen doses with SP has been shown to improve in-vivo fertility of ewes (Maxwell et al. 1999) and mares (Alghamdi et al. 2005). A beneficial effect of thawing cryopreserved boar sperm in seminal plasma on in-vivo sperm longevity (Einarsson and Viring 1973) and on gilt and sow fertility has also been documented (e.g. Crabo and Einarsson 1971; Einarsson et al. 1973). However, to our knowledge, sow fertility to insemination of FT sperm in a commercial extender supplemented with SP has not been examined. The present experiments were undertaken to test the hypothesis that supplementing FT sperm with SP will extend in-vivo sperm longevity and so allow maintenance of sow fertility when insemination occurs 12 hours before ovulation.

Material and methods

These studies were performed at the University of Guelph, Arken Research Station (Experiment One) and Michigan State University Swine Facility (Experiment Two). The respective institutional Animal Care committees approved the studies.

Prior to conducting the insemination studies, a pilot study was performed to verify the efficacy of a protocol for hormonally controlling the time of ovulation (Cassar et al. 2005). At weaning, 37 multiparous sows received an injection (IM) of 600 IU equine chorionic gonadotrophin (eCG; Pregnenol®, Bioniche Animal Health, Bellville, Ontario, Canada) to stimulate ovarian follicular development. At 80 h after the eCG, sows received an injection (IM) of 5 mg porcine luteinizing hormone (pLH; Lutropin®, Bioniche) to induce ovulation. Sows were subjected to transrectal real-time ultrasound examinations of the ovaries using an Aloka 500 with a 7.5 MHz linear array transducer every 2 h from 34 h after pLH injection until ovulation was complete. Ovulation time was defined as the time midway between an observed absence of ovulatory follicles (>6mm) and the previous examination (Weitze et al. 1989).

For Experiment One, 122 multiparous sows were assigned at weaning to estrus and ovulation control as described above. The predicted time of ovulation (PTO) was 38h after pLH injection. Immediately before breeding, the sows were assigned by parity to insemination with: 1) fresh sperm 12h before PTO, 2) FT sperm 12h before PTO, 3) FT sperm supplemented with 10% (v/v) SP 12h before PTO, 4) FT sperm 2h before PTO. The SP was obtained after centrifugation (800 x g for 10 min at 25°C) of a pool of semen derived from 10 Yorkshire boars, and was stored at -20°C until needed.

Thawing of SP was done in a 37°C water bath and the SP was added to extender prior to thawing sperm to allow immediate contact between the sperm and SP. Both fresh and FT sperm were obtained from the same pool of boars housed at a commercial boar stud (Ontario Swine Improvement, Innerkip, ON). Sperm were cryopreserved by the method of Pursel and Johnson (1975) as modified by Buhr et al. (2001), and thawed by submersion for 5 sec in a 60°C water bath. The post-thaw vitality of sperm was determined using Eosin-Nigrosin staining. All doses (fresh and FT) were extended in Beltsville Thawing Solution to a total volume of 80 ml and 3×10^9 live sperm and were deposited intracervically in the presence of a boar. Sows were allowed to go to term and farrowing rates and subsequent litter sizes were recorded.

For Experiment Two, 99 multiparous sows were assigned at weaning to treatments as described for Experiment One, except that all FT inseminations were intrauterine. The SP was obtained after centrifugation ($800 \times g$ for 10 min at 25°C) of a pool of semen derived from 5 Yorkshire boars, and was stored at -20°C until needed. Thawing of SP was done in a 37°C water bath and the SP was added to extender prior to thawing sperm to allow immediate contact between the sperm and SP. The fresh and FT semen was obtained commercially (International Boar Semen, IA) with boars equally represented among treatments. Semen thawing was performed by exposure to room temperature for 7 sec followed by submersion in 42°C water bath for 43 sec. The post-thaw vitality of sperm was determined using Eosin-Nigrosin staining and doses (fresh and FT) were extended in X-cell® extender (IMV, Maple Grove, MN) to a total volume of 80 ml and 3×10^9 live sperm. Doses of FT were deposited using an intrauterine catheter (IMV) while fresh semen was deposited intracervically, all in the

presence of a boar. Sows were allowed to go to term and farrowing rates and subsequent litter sizes were recorded.

Statistical analysis

Data were analyzed using INFOSTAT/E v2.0 (University of Cordoba, Argentina).

Differences in farrowing rates and litter sizes (total born) were tested using analysis of variance with LSD Fisher approximation, and with parity as a covariable. Results are presented as mean $\pm$ SEM and $P < 0.05$ was considered significant.

Results

The distribution of ovulation times in response to injections of eCG and pLH is shown in Figure 3.1. All ovulations occurred between 35 and 42 hours, with 75.7% of the animals ovulating between 37 and 39 hours. The time of ovulation (mean $\pm$ SEM) was 38.4 $\pm$ 0.3 hours after pLH injection.

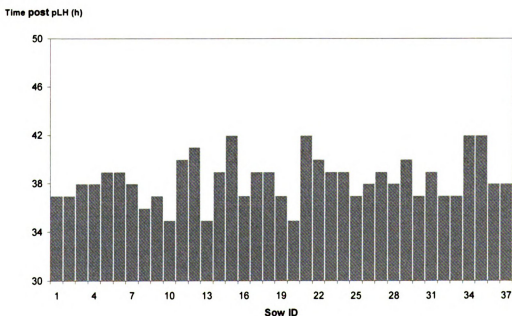


Figure 3.1. Sows ovulation time (hours post-pLH injection)

In Experiment One, a mean of 34.1% of the thawed sperm cells were observed to be alive in randomly selected semen samples. The effects of semen type, supplemental SP, and time of AI relative to ovulation are shown in Table 3.1. The insemination of FT sperm resulted in significantly reduced farrowing rates and smaller litter sizes ($p<0.01$). There was no effect of supplemental SP on farrowing rate or litter size (Table 3.1).

In Experiment Two, a mean of 65.4% of the thawed sperm cells were observed to be alive in randomly selected semen samples. The effects of semen type, supplemental SP, and time of AI relative to ovulation are shown in Table 3.2. The insemination of FT sperm resulted in significantly reduced farrowing rates ($p<0.01$) but semen type did not affect litter size. There was no effect of supplemental SP on farrowing rate or litter size (Table 3.2).

Table 3.1. Farrowing rates and total born litter sizes following artificial insemination of fresh (FS) sperm or cryopreserved (FT) sperm with or without 10% supplemental seminal plasma (SP) inseminated 2 or 12 hours before ovulation (Experiment one).¹

	FS-12h	FT-12h	FT-12h+SP	FT-2h
Number of sows	32	26	30	34
Parity ²	3.2±0.3	3.3±0.3	3.7±0.4	3.0±0.2
Farrowing rate, %	68.8a	7.7b	10.0b	14.7b
Total born litter size ²	12.1±0.8a	5.0±1.0b	3.3±0.9b	8.0±1.8ab

¹ All inseminations were intracervical

²Means ± SEM

ab, means followed by different letters differ, $P<0.01$

Table 3.2. Farrowing rates and total born litter sizes following artificial insemination of fresh (FS) sperm or cryopreserved (FT) sperm with or without 10% supplemental seminal plasma (SP) inseminated 2 or 12 hours before ovulation (Experiment two).¹

	FS-12h	FT-12h	FT-12h+SP	FT-2h
Number of sows	25	24	24	26
Parity ²	2.8±0.3	2.7±0.3	2.7±0.3	3.0±0.4
Farrowing rate, %	92.0a	37.5b	41.7b	38.5b
Total born litter size ²	10.3±0.9	8.1±1.3	9.4±1.6	9.6±1.4

¹FS sperm were inseminated intracervically but all FT sperm were inseminated transcervically into the uterine body

²Means ± SEM

ab, means followed by different letters differ, P<0.01

Discussion

Our data confirms that of others (Cassar et al. 2005), in that the described ovulation control protocol resulted in ovulation consistently at about 38 hours after the pLH injection. Therefore, we are confident that the targeted times of insemination relative to time of ovulation were accurate. This is further supported by the 92% farrowing rate observed in Experiment Two following a single insemination of fresh semen on a farm with a historical average of about 70% farrowing rate.

In the present studies, insemination of FT sperm was associated with reduced farrowing rates regardless of site of sperm deposition or time of sperm deposition relative to ovulation. Others have previously observed that fertilization rates following FT inseminations were comparable to those following insemination of fresh sperm, if the FT inseminations occurred with 4-hours of spontaneous ovulation (Waberski et al. 1994). However, comparison with the present work is problematic because sow fertility beyond determination of fertilization rates was not measured.

More recently, Wongtawan et al. (2006) observed that pregnancy rates in sows following deep intrauterine insemination of non-extended FT sperm were improved with closer timing of insemination relative to spontaneous ovulation. However, using a model of spontaneous ovulation resulted in very few sows within any specified time period relative to ovulation. Indeed, using a model of spontaneous ovulation, sow fertility to FT insemination was less than that from fresh semen inseminations, but were statistically comparable when FT inseminations occurred at 2 to 4 hours before hormonally controlled ovulation (Roca et al. 2003). However, the latter authors employed only 150×10^6 fresh sperm and a greater distinction between fresh and FT insemination results may have been evident if a larger number of fresh sperm had been used. Alternatively, we may have observed an effect of timing of insemination if sperm deposition had been deeper. Although speculative, if this were the case it would implicate a problem with transport of FT sperm into the oviduct, as suggested by Holt (2000).

It has been suggested that the poor fertility associated with insemination of FT sperm involves the cooling-associated induction of a capacitation-like state (cryocapacitation), which would render the FT sperm incapable of forming an enduring oviductal sperm reservoir. That FT sperm do not form an effective sperm reservoir has been documented (Wongtawan et al. 2006; Abad et al. 2006).

Interestingly, although seminal plasma has been shown to reverse cryocapacitation, such exposure did not affect the ability of FT sperm to form a sperm reservoir (Abad et al. 2006). In addition to reversing cryocapacitation, seminal plasma has also been shown to modulate uterine immune responses to sperm (Rozeboom et al. 1998) and to

be involved in the regulation of early embryo development (O'Leary et al. 2004).

However, there were no effects of seminal plasma on litter size.

The lack of effect of seminal plasma observed in our studies suggests that either the supplemental seminal plasma did not affect the cryocapacitation, or more likely that cryopreservation caused sperm injuries in addition to cryocapacitation that adversely impacted fertility. Indeed, others have observed effects of cryopreservation on integrity of sperm membranes and nuclear DNA (Rybar et al. 2004, Maldjian et al. 2005). Alternatively, it is possible that the specific seminal plasma used in these studies was not effective. Others have shown that seminal plasma from different boars can have different effects (Caballero et al. 2004). However, we used different pools of seminal plasma in each experiment and neither indicated a positive effect. What remains to be resolved is whether a higher concentration of seminal plasma would yield a different response.

From the data presented, we conclude that sow fertility to insemination of cryopreserved sperm is not improved by insemination close to the time of ovulation. Further, there was no benefit associated with supplementing semen doses with seminal plasma, suggesting that the reduced fertility results from sperm cryoinjury in addition to cryocapacitation.

Acknowledgments

We gratefully acknowledge Ontario Pork, Michigan State University Animal Industry Coalition, and the University of Guelph-Ontario Ministry of Agriculture and Food Animal Research Program for financial support of these studies.

References

- Abad M, Sprecher DJ, Ross P, Friendship RM, Kirkwood RN, 2006: Effect of sperm cryopreservation and supplementing semen doses with seminal plasma on the establishment of a sperm reservoir in gilts. *Reprod Dom Anim* (in press).
- Alghamdi AS, Madill S, Foster DN, 2005: Seminal plasma improves fertility of frozen equine semen. *Anim Reprod Sci* **89**, 242-245.
- Bailey J, Morrier A, Cormier N, 2003: Semen cryopreservation: Successes and persistent problems in farm species. *Can J Anim Sci* **83**, 393–401.
- Bolarin A, Roca J, Rodriguez-Martinez H, Hernandez M, Vazquez JM, Martinez EA, 2006: Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility between farms when frozen-thawed semen is used. *Theriogenology* **65**, 669-680.
- Buhr M, 2001: Emerging tools in artificial insemination. *Proc London Swine Conference 2001*, pp 123-135.
- Caballero I, Vazquez JM, Centurion F, Rodriguez-Martinez H, Parrilla I, Roca J, Cuello C, Martinez EA, 2004: Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. *Reprod Dom Anim* **39**, 370-375.
- Cassar G, Kirkwood RN, Poljak Z, Bennett-Steward K, Friendship RM, 2005: Effect of single or double insemination on fertility of sows bred at an induced estrus and ovulation. *J Swine Health Prod* **13**, 254-258.
- Crabo B, Einarsson S, 1971: Fertility of deep frozen boar spermatozoa. *Acta vet scand* **12**, 125-127.
- Einarsson S, Swensson T, Viring S, 1973: A field trial on the fertility of deep-frozen boar spermatozoa. *Nord Vet –Med* **25**, 372-376.
- Einarsson S, Viring S, 1973: Distribution of frozen-thawed spermatozoa in the reproductive tract of gilts at different time intervals after insemination. *J Reprod Fert* **32**, 117-120.
- Eriksson B, 2000: Cryopreservation of boar semen. Studies on sperm viability in vitro and fertility. PhD Thesis. Swedish University of Agriculture Sciences.
- Holt WV, 2000. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology* **53**:47-58.
- Johnson LA, Witze KF, Fiser P, Maxwell WMC, 2000: Storage of boar semen. *Anim Reprod Sci* **62**, 143-172.
- Maldjian A, Pizzi F, Gliozzi T, Cerolini S, Penny P, Noble R, 2005: Changes in sperm quality and lipid composition during crypreservation of boar semen. *Theriogenology* **63**, 411-421.

- Maxwell WMC, Evans G, Mortimer ST, Gillan L, Gellatly ES, McPhie CA, 1999: Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. *Reprod Fertil Dev* **11**, 123-126.
- O'Leary S, Jasper MJ, Warnes GM, Armstrong DT, Robertson SA, 2004: Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* **128**, 237-247.
- Pursel VG, Johnson LA, 1975: Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J Anim Sci* **40**, 99-102.
- Roca J, Carvajal G, Lucas X, Vazquez JM, Martinez EA, 2003: Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. *Theriogenology* **60**, 77-87.
- Rozeboom KJ, Troedsson MHT, Molitor TW, Crabo BG, 1998: The effect of spermatozoa and seminal plasma on leukocyte migration into the uterus of gilts. *J Anim Sci* **77**, 2201-2206.
- Rybar R, Faldikova L, Faldyna M, Machatkova M, Rubes J, 2004: Bull and boar sperm DNA integrity evaluated by sperm chromatin structure assay in the Czech Republic. *Vet Med* **49**, 1-8.
- Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodriguez-Martinez H, 2002: Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int j andrology* **25**, 84-93.
- Vadnais M, Kirkwood RN, Tempelman RJ, Sprecher DJ, Chou K, 2005a: Effect of cooling and seminal plasma on the capacitation status of fresh boar sperm as determined using chlortetracycline assay. *Anim Reprod Sci* **87**, 121-32.
- Vadnais ML, Kirkwood RN, Sprecher DJ, Chou K, 2005b: Effects of extender, incubation temperature, and added seminal plasmas on capacitation of cryopreserved, thawed boar sperm as determined by chlortetracycline staining. *Anim Reprod Sci* **90**, 347-354.
- Verbeckmoes S, Van Soom A, de Kruif A, 2004: Intra-uterine insemination in farm animals and humans. *Reprod Dom Anim* **39**, 195-204.
- Waberski D, Weitze KF, Gleumes T, Schwartz M, Willmen T, Petzold R, 1994: Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology* **42**, 831-840.
- Weitze KF, Habeck O, Willmen T, Rath D, 1989: Detection of ovulation in sow using transcutaneous sonography. *Zucht-hygiene* **24**, 40-42.

Wongtawan T, Saravia F, Wallgren M, Caballero I, Rodriguez-Martinez H, 2006. Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. *Theriogenology* **65**, 773-787.

CHAPTER 4

Summary and Conclusions

Summary

The insemination-ovulation interval and the addition of seminal plasma on the establishment of the sperm reservoir and the subsequent sow fertility following artificial insemination (AI) of cryopreserved sperm were studied.

Firstly, gilts were injected 5mg of PG600 (n=35, experiment 1.1) or a combination of eCG plus pLH (n=30, experiment 1.2) to induce ovulation. At breeding animals were assigned by weight to receive single AI of either FS sperm, FT sperm or FT sperm plus 10% v/v seminal plasma. Semen was obtained from the same commercial boars equally represented among treatments. Gilts were sacrificed 8h post AI and their reproductive tracts recovered. All uterotubal junctions (UTJs) were dissected and flushed with PBS to recover sperm, which were counted using a hemacytometer. The total number of sperm recovered per gilt was analyzed.

Secondly, multiparous crossbred sows were randomly selected to follow an ovulation synchronization protocol (eCG at weaning plus pLH 80 hours later). This protocol was verified previously by monitoring the ovulation of 37 animals by transrectal real time ultrasonography. The predicted time of ovulation (PTO) was 38 hours post pLH injection. Immediately before breeding, sows were assigned by parity to insemination with either: 1) FS sperm 12h before PTO; 2) FT sperm 12h before PTO; 3) FT sperm supplemented with 10% (v/v) SP 12h before PTO; 4) FT sperm 2h before PTO. The FT sperm was deposited cervically in the Experiment 2.1 and intrauterine in

Experiment 2.2. Sows were allowed to go to term in both cases and farrowing rates and litter sizes recorded.

Conclusions

The number of sperm located at the sperm reservoir was significantly reduced after AI with FT sperm in comparison with AI with FS sperm. These data clearly indicated that FT sperm are not capable of forming an adequate sperm reservoir. This inability was assumed to be a result of a cooling-induced capacitation-like reaction (cryocapacitation). However, exposure of FT sperm to seminal plasma had no effect suggesting that the adverse fertility to AI of FT sperm involves more than cryocapacitation.

Neither the addition of seminal plasma to FT sperm nor the timing of insemination of FT sperm affected sow fertility. These data support the earlier conclusion that the occurrence of cryoinjuries in addition to cryocapacitation either at membrane and/or at nuclear levels, negatively affect fertility. Our studies indicate that the direction for further studies should be to determine the specific damage the cryopreservation process produces to the integrity of the sperm membrane and DNA.

APPENDIX 1

Sperm cryopreservation protocol

- 1- The rich sperm fraction of the boar ejaculate is regularly collected by the gloved-hand method in a 37C prewarmed thermos.
- 2- Sperm motility is evaluated subjectively, the total volume by weight and the sperm concentration by spectrophotometry (Spectronic 20D+) within 20 minutes from collection.
- 3- Ejaculate is divided into fractions of 15ml and allowed to cool to room temperature at the rate of 0.1C/min.
- 4- Samples are centrifuged at 800g for 10 min at 25C (Jouan CT 422) to remove seminal plasma. The obtained sperm pellets are re-suspended in extender Beltsville F5-A to 1.2×10^9 spz/ml and cool to 5C over 3 hours.
- 5- Extender Beltsville F5-B is added to a final concentration of 6×10^8 spz/ml and 0.5ml straws are filled and sealed (Filling Frigory, IMV).
- 6- Straws are frozen by contact with nitrogen vapor about 3cm above the liquid nitrogen level for 20 min in an expandable polystyrene box, plunged into the nitrogen tank and stored until use.

APPENDIX 2

Sperm Vitality – Eosin-Nigrosin

Principles

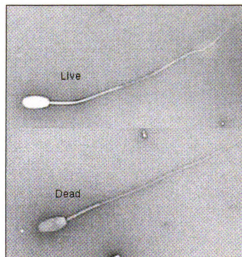
A cell with an intact cell membrane does not take up the stain eosin Y, while a dead cell (i.e. one with damaged cell membrane) takes up the red stain. Nigrosin is used as a background stain to provide contrast for the unstained (white) live cells.

Procedure

- One drop (50ul) of undiluted, well-mixed liquefied semen is mixed with one drop (50ul) of eosin-nigrosin solution (eg in a porcelain spotting plate) and incubated for 30sec.
- A drop of the solution (12-15ul per microscope slide) is placed on a clean microscope slide, and a smear made. The drop is smeared by sliding a cover slip in front of the drop. If two smears are made simultaneously by spreading between two microscope slides without feathering, one drop of 20-30 ul is used.
- The smear is allowed to air dry and examined directly, or mounted permanently the same day and examined after the mount has dried (eg overnight). Mounted smears are stored at room temperature.
- At least 200 sperm are assessed at 1000x (or 1250x) magnification under oil immersion with a high-resolution 100x objective (not phase contrast) with correct adjustment of the bright field optics. Sperm that are white (unstained) are classified as 'live' and those that show any pink or red coloration are considered as 'dead'.
- WHO does not specify duplicate counting for vitality assessment.

Reagents

0.67g of Eosin Y and 0.9g sodium chloride were dissolve in 100ml distilled water under gentle heating to afterwards add 10g Nigrosin. The solution was brought to boil and allowed to cool to room temperature. Paper filter was used to filter the solution before stored in a sealed glass bottle until needed, always at room temperature.



MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 02845 2690