# Fertility after deep intra-uterine AI of concentrated low-volume boar semen doses

## Tuempong Wongtawan

# Fertility after
# deep intra-uterine AI
# of concentrated low-volume boar
# semen doses

Tuempong Wongtawan

Division of Comparative Reproduction, Obstetrics and Udder Health
Department of Clinical Sciences
Faculty of Veterinary Medicine and Animal Science

Swedish University of Agricultural Sciences
Uppsala 2004

The present thesis is a partial fulfilment of the requirements for a
Master of Science Degree in Veterinary Medicine for International
Students (MSc), at the Swedish University of Agricultural Sciences
(SLU), in the field of Animal Reproduction

Tuempong Wongtawan
Division of Comparative Reproduction, Obstetrics and Udder health
Department of Clinical Sciences
Faculty of Veterinary Medicine and Animal Science
Swedish University of Agricultural Sciences (SLU)
P.O. Box 7039, SE- 75007 Uppsala, Sweden

แด่ บุพการี.. To my parents

# ABSTRACT

**Wongtawan, T. 2004.** Fertility after deep intra-uterine AI of concentrated low-volume boar semen doses. Master's thesis. ISSN 1403-2201, Report no. 49.

Boar semen can be successfully frozen -highly packed- in small containers (medium-straw, MS or multiple FlatPack, MFP). The use of deep intrauterine artificial insemination (DIU-AI) can make possible the deposition of small volumes of this thawed, non re-extended semen deeply intra-cornual. The fertility achieved after single or double DIU-AI per oestrus was hereby studied, with special attention to the interval between AI and spontaneous ovulation. Semen from two boars of proven fertility was frozen in MS or MFP holding $1x10^9$ total spermatozoa. Multiparous (n=42) crossbred sows were checked for oestrous behaviour after weaning and the occurrence of spontaneous ovulation was checked with transrectal ultrasonography (TUS) to establish the interval between onset of oestrus (OO) and ovulation. Sows were subjected to DIU-AI in the following oestrus using thawed semen (MS=20 or MFP=22), inseminated without further re-extension. Sows were randomly allotted to one of 3 groups: (1) Single DIU-AI 8 h before expected ovulation (Control group, n= 19), (2) Single DIU-AI 4 h before expected ovulation (Treatment group S, n=15) and (3) Double DIU-AI 12 and 4 h before expected ovulation (Treatment group D, n=8). Pregnancy was confirmed by TUS 28 days after OO in those sows not returning to oestrus. These sows were later slaughtered (day 30 to 45 of pregnancy), noting the appearance of the reproductive tract and ovaries, numbers of live foetuses, implantation sites and of CL. Some sows (n= 9) returning to oestrus were re-inseminated (either once [n=4] or twice [n=5]) in the following oestrus with either MFP (n=5) or MS (n=4) and slaughtered 12 to 14 h post-ovulation for recovery of spermatozoa from the utero-tubal junctions (UTJ, sperm reservoir) and of tubal oocytes, to disclose the effectiveness of sperm transport. Post-thaw sperm motility was 44.3±3.21% in MFP and 42.8±0.72 % for MS (LSMean±SEM, n.s.), and did not significantly change from thawing to AI. The DIU-AI could be performed in all sows, but insertion was slow (>5 min) in 5/42 sows of which 4 returned to oestrus. Pregnancy rate averaged 35% (Group D: 25%, Group S: 40%, Control: 36%, n.s.). The interval between DIU-AI and ovulation varied largely (group C: between -13 and -3 h , for S-group: between -11 and +3h , for group D: between -17 and -4 h). Pregnancy rates clearly related to the interval DIU-AI and ovulation, being highest (60%, 12/20) when AI occurred between 8 and 4 h before ovulation. Numbers of apparent implantation sites ranged 6 to 22 and of live foetuses 2 to 11 (n.s. among groups), while fertilization rate (total number of implantations/CL) ranged 48.0 to 69.7%, being highest in group D (P<0.05). The examination of the open sows slaughtered 12 to14 h post ovulation showed low sperm numbers (approx 4,000) in the UTJs. Only 40% of oocytes had spermatozoa bound to the zona pellucida, not more than 2 spermatozoa per oocyte, and only 10 % of recovered oocytes were fertilized, irrespective of using one or two DIU-AI (n.s.). The highest (p<0.05) values for these variables were recorded when DIU-AI (either single or double [second AI]) was done between 4 to 8 h before ovulation, especially when MFP-semen was used (P<0.05). In conclusion; (1) DIU-AI can be easily performed in most sows, (2) pregnancies can be obtained by the DIU-AI of low volumes of highly concentrated frozen-thawed boar semen, once or twice during oestrus, but fertility is still low, probably owing to an incomplete replenishment of the sperm reservoirs, and (3) fertility is mainly related to the interval DIU-AI and ovulation -which should be -8 to -4 h of spontaneous ovulation- and to the package, MFP having shown better results *in vivo*.

**Key words:** frozen semen, low volume AI, deep intra-uterine AI, boar, pig.

*Author's address:* Tuempong Wongtawan, Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), PO Box 7039, SE-750 07, Uppsala, Sweden.

# Contents

# Background

## Artificial insemination (AI) in pigs

*Historical summary*

Artificial insemination of pigs started in Russia in the beginning of the 20th century, yielding acceptable fertility when cervically inseminated with fresh, extended semen (Rodin & Lipatov, 1936). Since then, use of liquid boar semen for AI, preserved for up to 3 to 5 days at 16-20ºC, constitutes the core of the AI routinely performed by the pig breeding industry, covering more than 99% of the pig AIs done around the world (Wagner & Thibier, 2000). The worldwide tendency foresees an increase in the number of porcine AIs over the next few years (Weitze, 1999) and it is predicted that next year, the level of pig breeding via AI will already reach 80% (Burke, 1999).

*Conventional AI with liquid, not cryopreserved, boar semen*

Conventional AI in domestic pigs is practiced with doses of approximately $3x10^9$ spermatozoa extended to a volume of 80 to 100 mL (Stratman & Self, 1960; Baker *et al.*, 1968). Semen doses are stored at temperatures ranging 16 to 20 °C, usually for up to 3 days in simple extenders, but longer when using other extenders (Levis, 1999; Dubé *et al.*, 2004; Vyt *et al.*, 2004). The semen is deposited into the posterior region of the cervix by using a disposable, often an intra-cervical catheter whose tip simulates the corkscrew shape of the boar penis and engages with the posterior folds of the cervix as it occurs during natural mating. Various catheter shapes are commercially available ranging from the original large moulded rubber "Melrose" catheter (Melrose & O'Hagen, 1961) that mimicks the boar penis, to a simple polypropylene tube with an expanded polyurethane sponge tip (such as the GoldenPig®). In general, the AI process starts 12 h after detection of standing oestrus and it is repeated every 12 to 18 h until standing oestrus is no longer shown. The standing oestrus can vary widely among individual females (20 to 64 h or longer)(Mburu *et al.*, 1995; Soede & Kemp, 1997; Steverink *et al.*, 1999). When proper detection of oestrus is performed, the farrowing rate and litter sizes are comparable with rates/sizes achieved by AI with liquid semen or natural mating, reaching over 90% of farrowing and mean litter sizes of 14 piglets (Nissen *et al.*, 1997).

## Sperm transport in the female pig

After natural mating or conventional AI, billions of spermatozoa pass the cervix (15 to 25 cm) through the uterine body (5 cm) and the uterine horns (90 to 140 cm) to colonize the first segment of the oviduct (the uterotubal junction, UTJ), an area known as the sperm reservoir, from where they reach the site of fertilization (Viring & Einarsson, 1980; Hunter, 1990; Mburu et al., 1996; Rodriguez-Martinez et al., 2001). The main functions of the sperm reservoir are; a) a systematic reduction of spermatozoa before fertilization to prevent polyspermia, b) the

modulation of capacitation and c) the maintenance of sperm viability and fertilizing capacity escaping the female immune system (Rodriguez-Martinez *et al.*, 2001). Only few spermatozoa (less than thousand) reach the site of fertilization at the ampullary-isthmic junction (AIJ) (Hunter, 1990; Mburu *et al.*, 1997).

Establishment of the sperm reservoir occurs rapidly after insemination (5-15 min) (First *et al.*, 1968; Baker & Degen, 1972). The sperm population is maintained and increased by migration from the uterine horn during the first 24 h after insemination (Rigby, 1966; Pursel *et al.*, 1978). During the same time, the number of spermatozoa in the uterus is dramatically decreased (First *et al.*, 1968; Pursel *et al.*, 1978; Viring & Einarsson, 1980).

Backflow of the inseminate and the phagocytosis of spermatozoa by polymorphonuclear leukocytes (PMNs) are the major mechanisms of sperm elimination after AI with extended semen. Between 25 to 40% of the spermatozoa (Viring & Einarsson, 1981; Steverink *et al.*, 1998; Woelders & Matthijs, 2001; Matthijs *et al.*, 2003) and up to 70% of the volume (Steverink *et al.*, 1998; Matthijs *et al.*, 2003) are retrogradely expelled after AI. Prevention of the backflow with an artificial tamponade of the cervix failed to increase the number of spermatozoa in the uterus (Pursel, 1982). Approximately 50 % of those inseminated spermatozoa not lost with the backflow are ingested by incoming PMNs few hours after AI (First *et al.*, 1968; Viring & Einarsson, 1981; Matthijs *et al.*, 2003).


## Restricted use of frozen semen in porcine AI

Contrary to what occurs in cattle, where frozen-thawed (FT) semen is routinely used for AI (Curry, 2000), cryopreserved boar semen is used in less than 1% of the AIs performed around the world (Johnson *et al.*, 2000; Wagner & Thibier, 2000). Among the reasons behind this restricted use of FT-boar semen are the low survivability of spermatozoa after the freezing-thawing process and the shorter lifespan of the surviving spermatozoa. This results in lower farrowing rates and smaller litter sizes compared with AI using semen preserved in liquid form (Johnson, 1985; Johnson *et al.*, 2000). Furthermore, owing to the restricted life-span of the FT-boar spermatozoa, excessive sperm numbers are used, often 5-6 billion spermatozoa per dose. Moreover, two AIs are usually performed per oestrus (Johnson, 1985) in order to reach acceptable fertility rates in the field (Eriksson *et al.*, 2002). Altogether, few doses can be obtained from a single ejaculate and too many spermatozoa are used to ensure fertilization, thus contributing to the low efficacy of a sub-optimal cryopreservation technique.

A decrease in the number of spermatozoa per dose is therefore required to improve the use of ejaculates, so that the production will be cheaper and the use of genetically superior sires more effective. Recently, Saravia et al (2004) proved that boar spermatozoa can be packed in concentrated form (2 billion per mL) in small packages, such as 0.5 mL medium plastic straws (MS) or a 0.7 mL variant of the FlatPack (Eriksson & Rodriguez-Martinez, 2000), the so-called multiple-FlatPack (MFP), which keeps its cryobiological advantage of a large surface-to-volume ratio. The post-thaw spermatozoa showed comparable *in vitro* viability, in terms of sperm membrane integrity and functionality, to semen frozen at lower

concentrations in a higher volume of recognized cryogenic suitability. Although the results of the small dosages were satisfactory *in vitro*, there are no comprehensive *in vivo* study results available, using non re-extended, frozen-thawed semen under these conditions.

## Intra-uterine artificial insemination

Although few spermatozoa are required for fertilization within the oviduct, this reduced number is the product of a sequential and very effective reduction along the process of sperm transport in the female reproductive tract. The problem to be overcome during AI is to get an adequate number of spermatozoa to the UTJ that could ensure the establishment of the functional sperm reservoir with enough viable, potentially-fertile spermatozoa to ensure maximal fertilization. One strategy proposed to accomplish this is to decrease the number of spermatozoa per AI-dose and, by depositing the semen directly in the uterus, get sufficient spermatozoa into the UTJ. Such intra-uterine AI with reduced sperm numbers is a relatively new reproductive practice that has attracted the attention of the swine industry. Such a method could be also advantageous for the spreading of AI with FT-semen. Usually, FT-boar semen is re-extended to volumes of 80 to 100 mL, since such volumes are considered to be required (particularly in sows) to elicit sperm transport after cervical AI (Baker *et al.*, 1968). Although thawing and re-extension can inflict damages to boar spermatozoa (Bwanga, 1991; Bwanga *et al.*, 1991a; Bwanga *et al.*, 1991b) few attempts have been made to establish whether low semen volumes could be conventionally inseminated and yet transported to the sperm reservoir (Hancock, 1959; Stratman & Self, 1960; Hancock & Hovel, 1961).

Many domestic animals have been successfully artificially inseminated with intra-uterine body/horn sperm deposition such as cattle (Seidel *et al.*, 1997; Hunter, 2003; Verberckmoes *et al.*, 2004), horse (Petersen *et al.*, 2002; Squires *et al.*, 2002), sheep (Gillan *et al.*, 1999; Wulster-Radcliffe *et al.*, 2004), dog (Linde-Forsberg *et al.*, 1999), cat (Chatdarong *et al.*, 2001), and pig (Krueger & Rath, 2000). Moreover use of intra-uterine AI is of interest for conservation of exotic species such as the ferret (Kidder *et al.*, 1998), the killer whale (Robeck *et al.*, 2004)**,** or the leopard (Howard *et al.*, 1996).

There are basically two procedures for depositing spermatozoa into the pig uterus; surgically (Krueger *et al.*, 1999; Krueger & Rath, 2000; Rath, 2002) or non-surgically. The latter includes semen deposition either in the uterine body (Levis *et al.*, 2002; Watson & Behan, 2002) or deep into the uterine horn (Martinez *et al.*, 2001a, 2002).

An intra-uterine AI with low numbers of spermatozoa is surgically accomplished by placing a small semen volume (0.5 mL) close to the UTJ of each uterine horn (Krueger *et al.*, 1999). Surgical AI in synchronized female pigs with a 150 fold reduction of sperm numbers ($1 \times 10^7$ sperm) appeared sufficient to ensure fertility in gilts (farrowing rate 88.9%, litter size 7.8$\pm$3.6, Krueger *et al.*, 1999) and sows (farrowing rate 85.7%, litter size 10$\pm$4.2, Krueger & Rath, 2000). Since the surgical intra-uterine AI has no room in practice for animal welfare reasons, only detailed descriptions of the non-surgical procedures follow.

*Non-surgical intra-uterine body AI (IUB-AI)*

Non-traumatic transcervical catheters that allow an easy penetration of the cervix and deposition of semen in the uterine body of the sow have been designed. Briefly, a conventional catheter (outer catheter) is placed toward/locked in the cervix (See Figure A, 1). An inner tube (around 4 mm outer Ø) is passed through the outer catheter, along the cervical lumen, to reach the uterine body or the posterior part of one of the uterine horns (about 200 mm beyond the tip of the outer catheter, See Figure A, 2). Under commercial conditions, use of an IUB-AI catheter can reduce sperm numbers to 0.5 (Mezalira *et al.*, 2004) or 1 billion spermatozoa (Watson & Behan, 2002; Rozeboom *et al.*, 2004) per AI-dose without detrimental effects on fertility. The IUB-AI catheter can be used with minimal training and it does not seriously delay the process of insemination. Although there does not seem to be any animal welfare implication of this type of AI, it can only be safely used in sows (Levis *et al.*, 2002; Watson & Behan, 2002). The use of IUB-AI does, however, not overcome significant losses during the transport of spermatozoa towards the oviduct (Levis, 2002; Dallanora *et al.*, 2004). Furthermore, the price of a IUB-AI catheter is still higher than of a conventional AI-catheter (Levis, 2002).
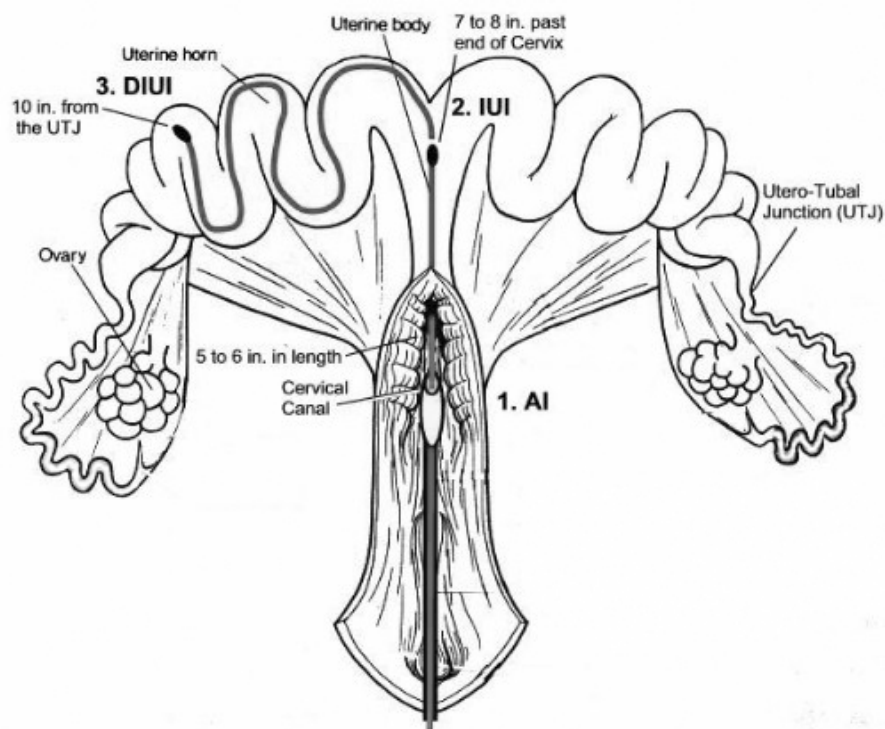


**Figure A**: Intra uterine insemination in pigs:
(1) AI. Conventional insemination in the cervix
(2) IUB-AI. Intrauterine Insemination in the uterine body
(3) DIU-AI. Deep intra-cornual insemination, 2/3 of the way up on a uterine horn
(modified from Belstra, 2002).

*Non-surgical deep intra-uterine AI (DIU-AI)*

Non-surgical deep intra-uterine artificial insemination (DIU-AI) has been performed in non-sedated pigs using a flexible fibreoptic endoscope (length: 1.35 m, outer Ø: 3.3 mm) (Martinez *et al.*, 2001a) inserted via the vagina and cervix to reach the upper segment of one uterine horn. With this technique, the semen dose could be deposited close to an UTJ in 96.7% of sows and 88.5% of gilts. The procedure required 3 to 5 min in 90% of the females. After this DIU-AI, only 1 % of sows showed signs of uterine infection.

However, as the endoscope is a highly expensive instrument and therefore unpractical for routine use, a flexible catheter was developed on the basis of the propulsion force and flexibility of the fibroendoscope (Martinez *et al.*, 2002). This catheter is now commercially available (Firflex®, Magapor, Spain, see Figure A, 3) and can be used for DIU-AI, requiring a similar time as for traditional AI (Martinez *et al.*, 2001b). The method allows deposition of low sperm doses of either fresh (Martinez *et al.*, 2002) or frozen-thawed (Roca *et al.*, 2003) spermatozoa. Moreover, the technology can be successfully used to produce piglets with sex-sorted spermatozoa (Vazquez *et al.*, 2003), or for embryo transfer (Martinez *et al.*, 2004). When FT-semen, frozen in medium straws (0.5 mL) at concentrations of $1 \times 10^9$ spermatozoa per mL and re-extended to a 7 mL dose, was deeply inseminated with these catheters, promising results were obtained. With hormonally-induced ovulation and a single DIU-AI, the farrowing rate was 77.5%, with $9.31 \pm 0.41$ live piglets born, while with spontaneous ovulation and two DIU-AIs, the farrowing rate was 70%, with $9.25 \pm 0.23$ piglets born (Roca *et al.*, 2003). Since the use of hormones is refrained from commercial practice and the re-extension of the FT-semen appears deleterious for sperm survival, there is a need for the intra-uterine deposition of un-extended, frozen-thawed semen, cryopreserved in concentrated form in small volumes (0.5 mL).

## Optimal time of AI: a pre-requisite for fertility?

The lifespan of FT-boar semen in the female reproductive tract is shorter (2 to 8 h) than that of its liquid-preserved counterpart (Einarsson & Viring, 1973; Pursel *et al.*, 1978). In general, pre-ovulatory AI is recommended to establish a sperm reservoir in the oviduct and thus ensure that sufficient numbers of spermatozoa are available to fertilize the ovulated oocytes. The critical interval for FT-spermatozoa to achieve acceptable fertility is also narrow, and the AI must be done within 4 to 6 h before ovulation, whereas AI 4 h after ovulation conducts to a significant decrease in fertilization and pregnancy rate (Einarsson & Viring, 1973; Waberski *et al.*, 1994). Taking into consideration that the fertilising ability of the ovulated oocytes is also short, not more than 6 to 8 hours, proper timing of sperm deposition in relation to the moment of ovulation when using FT-boar semen, is critical (Holt, 2000).

Ovulation time can be precisely monitored by non-invasive methods such as the determination of luteinizing hormone, oestrogen or progesterone (Soede *et al.*, 1994). However, such methods are of no practical value for AI application. Non-invasive transcutaneous ultrasonography (Weitze *et al.*, 1989; Waberski *et al.*, 1994) and transrectal ultrasonography (Soede *et al.*, 1992; Mburu *et al.*, 1995) of

the ovaries have been used to monitor the occurrence of ovulation. On average, ovulation occurs when 2/3 of the standing oestrus has passed, which means for most sows 35 to 45 h after onset of oestrus (OO). However; the variability between sows is large, and occurrence of ovulation has been reported to occur between 10 to 85 h (Waberski *et al.*, 1994), 10 to 58 h (Soede *et al.*, 1995) or 35 to 43 h (Mburu *et al.*, 1995) after OO. The average timing of ovulation varies thus between 64 and to 72% of the duration of oestrus (Soede & Kemp, 1997).

Estimation of ovulation can be performed by determining the interval between OO and ovulation time of the previous cycle (Mburu *et al.*, 1995). Most of the studies of optimal timing for AI have been done with cervical catheters and liquid semen, and few studies have considered use of FT-semen. Taken together, there is a need to determine the optimal schedule for AI using DIU-AI with small doses of highly concentrated FT-spermatozoa by estimating the interval between onset of standing oestrus and spontaneous ovulation.

14

# Introduction to the research report

Frozen-thawed (FT) boar semen for artificial insemination (AI) in commercial pig production is still only used in exceptional cases, mostly for incorporation of top genetics. Low sperm survival after a freezing procedure that is still considered sub-optimal leads to the use of excessive sperm numbers per AI-dose. In any case, AI with FT-boar semen still results in low farrowing rates and small litter sizes when conventional cervical AI is performed. Boar spermatozoa can now be successfully frozen concentrated in a small volume ($1x \, 10^9$ spz in 0.5 mL) and new procedures for deep intra-uterine AI are now available. These technological improvements open up possibilities for testing the fertility after the deposition of fewer FT-boar spermatozoa per dose in small volumes. With insemination time being among the most relevant factors constraining fertility when using FT-boar semen, the determination of the most suitable AI-schedule becomes imperative.

# Aim of the study

The overall aim of the study was to increase our knowledge about the use of deep intra-uterine artificial insemination (DIU-AI) with frozen-thawed boar semen. Particularly, it aimed to determine the fertility of sows following DIU-AI of small volumes of highly concentrated FT-boar semen. The semen was packed either in medium-straws (MS) or multiple FlatPacks (MFP), and deposited once or twice during oestrus in relation to spontaneous ovulation. Ovulation was estimated by transrectal ultrasonography (TUS), to determine an optimal scheme for AI with frozen-thawed semen in weaned sows.

# Research report

## Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses

Tuempong Wongtawan[1], Fernando Saravia[1], Margareta Wallgren[1,2], Ignacio Caballero[1,3], Heriberto Rodríguez-Martínez[1]

[1]*Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Ullsvägen 14 C, Clinical Centre, PO Box 7039 Ultuna, SE-750 07 Uppsala, Sweden*

[2]*Quality Genetics, Kävlinge, Sweden*

[3]*Department of Medicine and Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain*

## ABSTRACT

Boar semen can be successfully frozen- highly packed- in small containers (medium-straw, MS or multiple FlatPack, MFP). The use of deep intra-uterine artificial insemination (DIU-AI) can make possible the deposition of small volumes of this thawed, non re-extended semen deeply intra-uterine, close to the sperm reservoir. The present experiments studied the fertility achieved after single or double DIU-AI per oestrus, with special attention to the interval between AI and spontaneous ovulation. Semen from two boars of proven fertility was frozen in MS or MFP holding $1 \times 10^9$ total spermatozoa. Multiparous (2-5 parity, n= 42) crossbred sows were checked for oestrous behaviour after weaning and the occurrence of spontaneous ovulation was checked with transrectal ultrasonography (TUS) to establish the interval between onset of oestrus (OO) and ovulation. The sows were, in the following standing oestrus, subjected to DIU-AI using thawed semen from either MS (n= 20) or MFP (n= 22), inseminated without further re-extension. The sows were randomly allotted to one of 3 groups: (1) Single DIU-AI 8 h before expected ovulation (Control group, n= 19), (2) Single DIU-AI 4 h before expected ovulation (Treatment group S, n= 15) and (3) Double DIU-AI 12 and 4 h before expected ovulation (Treatment group D, n= 8). Pregnancy was confirmed by TUS 28 days after OO in those sows not returning to oestrus. These sows were slaughtered (30 to 45 days of pregnancy), and the appearance of the reproductive tract and ovaries, the number of live and dead foetuses, of implantation sites and of corpora lutea (CL) were recorded. The sows (n= 9) returning to oestrus ("open") were re-inseminated (either once [n=4] or twice [n=5]) the following oestrus with either MFP (n=5) or MS (n=4) and slaughtered 12 to 14 h post-ovulation for recovery of spermatozoa from the uterotubal junctions (UTJ, sperm reservoir) and of tubal oocytes, to disclose the effectiveness of sperm transport. Post-thaw sperm motility was 44.3 ± 3.21% in MFP and 42.8 ± 0.72 % for MS (LSMean±SEM, n.s.), and did not significantly change from thawing to AI. The DIU-AI could be performed in all sows, but insertion was

difficult (slow >5 mins) in 5/42 sows. Four of these sows returned to oestrus. Pregnancy rate averaged 35% (Group D: 25%, Group S: 40%, Control: 36%, n.s.). The interval between DIU-AIs and ovulation varied largely, ranging from -13 to -3 h for group C, for group S from -11 to + 3h and for group D from -17 to -4 h. Pregnancy rates were clearly related to the interval DIU-AI and ovulation, being highest (60%, 12/20) when AI occurred between 8 and 4 h before ovulation. The number of implantation sites ranged 6 to 22 (n.s. among groups), and the number of alive foetuses 2 to 11 (n.s. among groups). Fertilization rate (total number of implantations/CL) ranged 48.0 to 69.7% being highest in the D-group (P<0.05). The examination of the "open" sows slaughtered 12 to 14h post ovulation showed low sperm numbers (approx 4,000) in the sperm reservoirs (UTJs). Few recovered oocytes were fertilized (approx. 10%) and only 40% of oocytes had spermatozoa bound to the zona pellucida, not more than 2 spermatozoa per oocyte, irrespective of using single or double DIU-AI (n.s.). The highest values (P<0.05) for these variables were recorded when DIU-AI (either single or double [second AI]) was done 4 to 8 h before ovulation, especially when MFP-semen was used (P<0.05). In conclusion; (1) DIU-AI can be easily performed in most sows, (2) pregnancies can be obtained by the DIU-AI of low volumes of highly concentrated frozen-thawed boar semen, once or twice during oestrus, but fertility is still low, probably owing to an insufficient replenishment of the sperm reservoirs, and (3) fertility is related to the interval DIU-AI and ovulation which should be -8 to -4 h of spontaneous ovulation and to the package, MFP having shown better results *in vivo*.

**Key words:** frozen semen, low volume AI, deep intra-uterine AI, boar, pig.


# Introduction

Frozen-thawed (FT) boar semen is not regularly used for artificial insemination (AI) in the swine industry; apart from the gene banking of valuable pig genetics, for export or for research purposes. The sub-optimality of the freezing procedure (high cell mortality) and the short-life of the surviving spermatozoa post-thaw accounts, despite the use of large sperm numbers per AI-dose, for the well-documented lower fertility achieved after conventional AI (Curry, 2000; Johnson *et al.*, 2000).

Deposition of reduced sperm numbers through deep intra-uterine AI (DIU-AI) is a relatively new reproductive practice that has drawn the attention of the swine industry. With this technique, the number of frozen-thawed spermatozoa has been substantially reduced, still achieving acceptable fertility by single AI in sows during hormonally-induced oestrus or by two AIs during spontaneous oestrus (Roca *et al.,* 2003). The DIU-AI method, despite being more invasive than the conventional cervical AI, does not seem to affect animal welfare, with a low risk of inducing genital damage or infections (Martinez *et al.,* 2001). Moreover, the use of this method for AI implies that fewer boars are needed to produce the same number of doses of semen compared with cervical insemination. The DIU-AI might, therefore, help to widespread those genetically most valuable boars by the global distribution of frozen packages.

18

The deposition of a low volume of highly concentrated spermatozoa during a deep intra-uterine insemination close to the utero-tubal junction (UTJ), seems more convenient than using larger volumes. Primarily, because it diminishes the backflow of the inseminate and the significant sperm losses it represents. Secondly, because it also diminishes the sperm damage seen with the customary re-extension post-thaw. Freezing of highly concentrated boar semen in small packages has proven possible, considering *in vitro* results of sperm viability and functionality (Saravia *et al*., 2004). However, although some indications of in *vivo* fertility have been reported, there are no conclusive studies yet available.

The fertile life span of oocytes and spermatozoa in the female genital tract is limited. To achieve high fertility, the timing of insemination relative to ovulation is crucial. Sub-optimal time for insemination before and after ovulation leads to low rates of fertilization and pregnancy, and to small litter sizes (Waberski *et al.*, 1994; Soede *et al.*, 1995a; Nissen *et al.*, 1997). Frozen-thawed boar spermatozoa have a shorter life span (2-8 h) than freshly maintained spermatozoa (Einarsson & Viring, 1973; Pursel *et al.*, 1978), thus requiring sperm deposition as close as possible to the moment of ovulation. Achievement of acceptable fertility post-AI is reported as best obtained when frozen-thawed boar spermatozoa is deposited 4-6 h before ovulation (Waberski *et al.*, 1994). However, the optimal time for sperm deposition using DIU-AI in relation to spontaneous ovulation in sows has not yet been reported.

The aim of the present study was to determine the fertility of sows following deep intra-uterine artificial insemination (DIU-AI) of high numbers of frozen-thawed boar spermatozoa in small volumes. The semen was packed in medium-straws (MS) or in multiple FlatPacks (MFP), and deposited once or twice during oestrus in relation to spontaneous ovulation, estimated by transrectal ultrasonography.

# Materials and Methods

### Animals

Two mature boars (one Swedish Yorkshire and one Swedish Landrace) 2-5 years old and selected for normal semen quality and proven fertility were used as semen donors. The boars were kept in individual pens with females in the close neighbourhood. Multiparous crossbred sows (Swedish Landrace x Swedish Yorkshire, (n = 42) with a lactation of five weeks and a mean parity of 3.5 (range of 2-5 farrowings) were purchased from a commercial herd and brought directly after weaning to the Division of Comparative Reproduction, Obstetrics and Udder Health, Swedish University of Agriculture Science (SLU), Uppsala, Sweden. Before the study, the sows showed normal reproductive performance. All sows were clinically examined at arrival, and individually housed in straw-bedded pens with optimal ventilation and fed according to Swedish standard (Simonsson, 1994) twice daily with water provided *ad libitum*. The experimental protocol had

previously been reviewed and approved by the Local Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

## Semen Processing

Semen collected from the two boars was processed and frozen according to Eriksson & Rodriguez-Martinez (2000). Only ejaculates with at least 70% motile spermatozoa were used. Semen was extended (1:1, v/v) in Beltsville thawing solution (BTS, IMV, L' Aigle, France; here named Extender I). The BTS-diluted semen was refrigerated to +16ºC for 3 h. Thereafter, the semen was centrifuged twice at 800 g for 10 min (Centra MP4R, IEC, MN, USA), and the supernatant was discarded. Sperm concentration was determined in a haemocytometer (Bürker) and the spermatozoa were re-extended at a ratio 2:1 with a second extender (Extender II; 80 mL [80% v/v 310 mM] of ß-lactose and 20 mL Egg yolk). The semen was further cooled to +5ºC for 2 h, at which temperature the semen was slowly mixed at a ratio 2:1 with a third extender (Extender III; 89.5 mL Extender II, 9 mL glycerol and 1.5 mL of Equex STM, Nova Chemical Sales Inc., Scituate, MA, USA), yielding a final concentration of 3% glycerol and $2x10^9$ spermatozoa/mL. The spermatozoa were packed at +5 ºC in a cold cabinet (IMV, L' Aigle, France) in either one of two packages; 0.5 mL polyvinyl chloride medium-straw (MS) (Minitube, Germany) or multiple FlatPack (MFP) (four 0.7 mL volume of Single Flat Pack (SFP) (Saravia *et al.,* 2004). The MSs were sealed with PVC powder while the MFPs were heat-sealed. After sealing, the packages were transferred to a programmable freezer (Mini Digitcool 1400; IMV, L' Aigle, France) set to +5ºC. The cooling/ freezing rate was as follow: 3ºC/min from +5 ºC to -5ºC, 1 minute of holding time for crystallization, and thereafter 50ºC/min from -5ºC to -140ºC. The samples were then immediately plunged into liquid nitrogen (-196ºC) for storage.

## Detection of oestrus

Oestrous detection started from day 2 after weaning. The sows were observed for signs of proestrus every 12 h primarily by inspection of the vulva for eventual reddening and swelling. When the sows revealed signs of proestrus, oestrous detection was performed every 4 h until the end of oestrus. Oestrous detection was performed by allowing snout-to-snout contact of the sow with a mature boar; and the appearance of standing reflex after applying manual backpressure by an operator. Sows that revealed standing reflex were considered to be in oestrus. The time of onset of oestrus was defined as the first time a sow revealed a standing response minus 2 h. The time of end of oestrus was defined as the last time a sow revealed a standing response plus 2 h.

## Detection of ovulation

Transrectal ultrasonography (TUS) of the ovaries was performed 18 h after onset of the first oestrus post-weaning and the ovaries were scanned every 4 h until completion of ovulation. An annular array sector scanner (Scanner 250, Pie Medical b.v. Maastricht, Netherlands) with a 5-MHz multiple angle transducer was used. The sows were immobilized in a specially-constructed wagon during the

TUS-scanning (Dalin et al., 1995). At each TUS-scanning, the estimates of the number of follicles and the average diameter of presumptive ovulatory follicles (eg > 4 mm) were recorded. Ovulation time was defined as the first scanning when no presumptive ovulatory follicles were seen minus 2 h. If the follicular count was not zero, but lower than previously, the ovulation was assumed to have just started (t=0) (Soede *et al.,* 1992).

## Time of insemination

Inseminations were performed in the following oestrus in relation to the expected spontaneous ovulation, which was estimated from the recorded ovulation time on the previous oestrous cycle (Mburu *et al.,* 1995). Sows were randomly allotted for DIU-AI in one of three groups: (1) Single DIU-AI 8 h before expected ovulation (Control group, n= 19), (2) Single DIU-AI 4 h before expected ovulation (Treatment group S, n= 15) and (3) Double DIU-AI 12 and 4 h before expected ovulation (Treatment group D, n= 8).

## Semen thawing and assessment of sperm motility

The frozen semen was thawed in warm water at 35° C for 20 seconds. Post-thaw sperm motility was assessed subjectively by using a phase contrast microscope as well as with a computer-assisted sperm analysis (CASA, SM-CMA, MTM Medical Technologies, Montreaux, Switzerland), as described by Saravia *et al* (2004). Before examination, a drop of semen from the thawed package was re-extended (1:20) in BTS to give as concentration $50\text{-}60 \times 10^6$ spermatozoa/mL. The re-extended-FT semen was incubated (38° C) for 30 min before 5 μL of semen were placed in a Makler counting chamber (Sefi Medical Instrument, Haifa, Israel) on a heated stage (38° C). For each sample, optical fields around the centre of the Makler chamber were used to count a minimum number of 200 spermatozoa per sample, recording percentages of motility, motility patterns and velocities of displacement for linearly motile spermatozoa.

## Deep intra-uterine insemination technique

Deep intra-uterine inseminations (DIU-AI) were performed as described by Martinez *et. al.* (2002). Sows were inseminated without sedation or immobilization (during spontaneous standing reflex in front of a boar). The flexible intra-uterine catheter (Firflex®, Magapor, Spain) was removed from its package, and soaked externally and internally with BTS at 35° C before insertion. A conventional cervical catheter (outer catheter, IMV, L' Aigle, France) was inserted through the vagina and locked into the cervical canal to serve as a guide for the flexible intra-uterine catheter. The flexible catheter was then manually inserted through the outer catheter, passed through the cervical canal and moved forward along one uterine horn until its total length was inserted. Frozen-thawed semen from MFP (n= 22) or MS (n= 20) at 0.5 mL volume ($1\times10^9$ spermatozoa/dose) was then injected into the flexible catheter by use of a temperated syringe. Since the volume of the intra-uterine AI-catheter inner lumen was previously measured to approximately 2 mL, the same volume of BTS extender was used to flush the catheter clean of semen. The degree of easiness of the DIU-AI was recorded for each sow.

## Pregnancy detection

Confirmation of pregnancy was performed by TUS at day 28 from onset of oestrus (first day of oestrus: day 1) in those sows not returning to oestrus. The pregnant sows were then slaughtered between days 30 and 45 of pregnancy, and the appearance of the reproductive tract and ovaries were recorded. The number of implantation sites, of live and dead foetuses, and of corpora lutea (CL) were also counted.

## Fertilization and sperm reservoir

A series of sows (n= 9) that had returned to oestrus ("open sows") were re-inseminated once (n= 4) or twice (n= 5), using MFP (n= 5) or MS (n= 4) semen, in the following oestrus and slaughtered 12 to 14 h after spontaneous ovulation. The aspect of the genital tracts and the number of visible ovulations were recorded. One of the sows, inseminated twice with semen frozen in MS, presented an aplasia of one of the uterine horns (*uterus unicornis*) and was deleted from the population. The UTJs were removed and flushed twice with 5 mL of warm BTS into a 10 mL tube. Each flushing was centrifuged to concentrate the spermatozoa and the sperm number counted using a Bürker chamber. Each oviduct was flushed from the *isthmus* towards the *infundibulum* with 20 mL of Dulbecco phosphate-buffered saline (DPBS, Sigma, Germany). The flushing was placed in a petri dish and searched for presence of oocytes/presumptive zygotes under a stereomicroscope. The oocytes/presumptive zygotes were searched for presence of spermatozoa attached to the zona pellucida (ZP) using phase contrast microscopy. To evaluate occurrence of fertilization, the oocytes were fixed in 25% (v/v) acetic acid in ethanol at room temperature for 48-72 h, stained with 1% lacmoid (Sigma, USA) in 45% (v/v) acetic acid, and examined under a light microscope (400x).

## Statistics

This experiment used a 3x2 factorial design. Data were analyzed using the Statistical Analysis System package (SAS Institute Inc., Cary, NC, USA, 1994). Data of oestrus and ovulation time are shown as mean ± SD, and/or ranges. Data of semen analysis, number of foetuses, number of spermatozoa in the oviductal sperm reservoir and of accessory spermatozoa per oocyte are presented as least square means and were analyzed using the GLM model, while comparison between treatments was done using the Student's t-test. Pregnancy rate, fertilization rate and percentage of oocytes with spermatozoa bound to their ZP are presented as percentages and were analyzed using the Chi-square test. $P<0.05$ was set as level of significance.

# Results

## Oestrus and ovulation

All sows in this experiment showed normal signs of oestrus including occurrence of spontaneous ovulation. The average weaning-oestrus interval (WOI) was $4.8 \pm 1.01$ days (Mean $\pm$ SD), with a range of 3 to 6 days. The average duration of oestrus was $62.5 \pm 13.16$ h, ranging from 38 to 92 h. The average interval onset of oestrus (OO) to ovulation was $41.4 \pm 13.10$ h, with a range of 22 to 72 h. The average length of the oestrous cycle was $22.2 \pm 2.03$ days, with a range of 20 to 28 days. Ovulation occurred as when $66 \pm 8\%$ of the oestrous period had passed. The mean diameter of the pre-ovulatory follicle was $0.8 \pm 0.24$ cm with a range 0.6 to 1.2 cm. The mean number of CL per sow (both ovaries) was $21.5 \pm 3.94$, ranging from 17 to 29 CL.

## Post-thaw sperm motility

Post-thaw sperm motility is depicted in **Table 1**. Sperm motility did not statistically ($P>0.05$) differ between MFP and MS, neither in terms of overall sperm motility nor sperm velocities. Sperm motility did not vary significantly (n.s.) when measured in semen from the package, the syringe or the catheter used for DIU-AI (data not shown). Semen used to inseminate sows that rendered pregnant or not-pregnant did not differ in sperm quality post-thaw ($P>0.05$).

**Table 1**: Post-thaw sperm motility patterns obtained by CASA analysis of the semen doses used (MS: 0.5 ml Medium Straw; MFP: Multiple Flatpack; LSMeans ± SEM)

| Package | n | Motility % | LIN % | VSL (µm/s) | VAP (µm/s) | VCL (µm/s) |
|---|---|---|---|---|---|---|
| **MFP** | 22 | 44.3±3.21 | 29.7±3.12 | 54.3±1.66 | 70.4±2.74 | 129.4±6.80 |
| **MS** | 20 | 42.8±0.72 | 30.7±3.43 | 58.9±2.43 | 76.8±3.71 | 134.4±8.14 |

LIN: linearly motile spermatozoa, VSL: straight linear velocity, VAP: average path velocity, VCL: curvilinear velocity. No significance differences between packages ($P>0.05$)

## Easiness of the DIU-AI

The insemination procedure implied that the intrauterine catheter would have been completely inserted (e.g. the back tip was to reach the posterior opening of the outer cervical catheter). The intrauterine catheter could in all sows be inserted to an average length of $172.5 \pm 15$ cm (mean $\pm$ SD), thus implying different degrees of insertion per sow. The DIU-AI could then be performed in all sows, but insertion was slow (>5 mins) in 5/42 sows, indicating difficulties for the procedure. Four of these sows returned to oestrus. Neither vaginal/cervical bleedings nor abnormal vaginal discharges were noticed after the DIU-AI.

## Fertility post-DIU-AI

The fertility of the sows after single or double DIU-AI is depicted in **Figure 1**. Pregnancy rate averaged 35% (Group D: 25%, Group S: 40%, Control: 36%, n.s.). As the ovulations were spontaneous, the interval between the DIU-AIs to ovulation varied largely, ranging for group C: -13 to -3 h, for group S: -11 to + 3h and for group D: -17 to -4 h. Pregnancy rates clearly related to the interval DIU-AI and ovulation, being highest (60%, 12/20) when ovulation occurred between -8 and -4 h later (Figure 1).

**Figure 1**: Fertility of the sows after single or double DIU-AI (grey arrows marked the intended moment of DIU-AI in each C-, S- or D-group) in relation to when they spontaneously ovulated (white horizontal bars) and their status (white circle: empty sow, black circle: pregnant sow). The scale shows hours before (-) or after (+) ovulation (black arrow). Numbers of pregnancies/number of AI-animals are presented for each group (n).
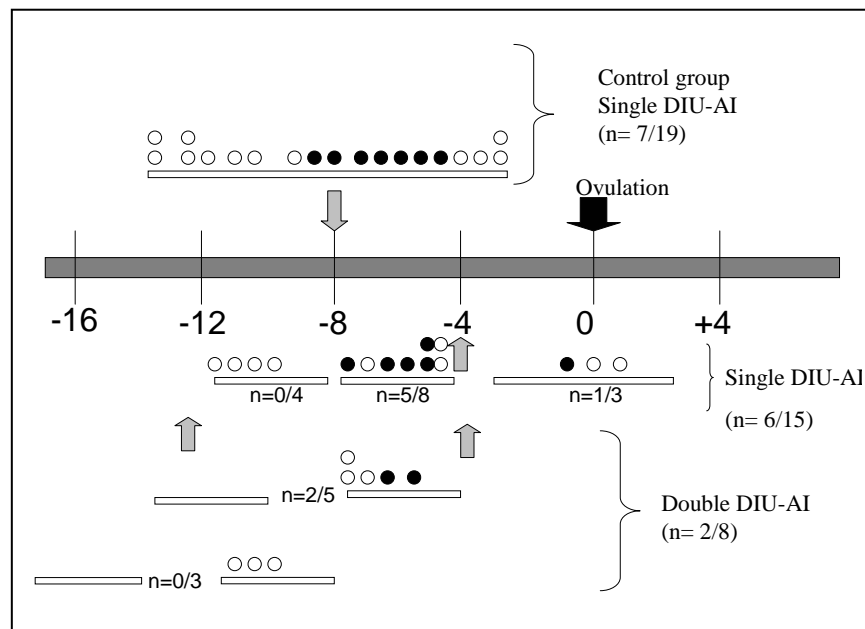


The overall pregnancy rate, number of apparent implantation sites, of live foetuses and a calculated fertilization rate for the different groups (Control, S and D) of DIU-AI are presented in **Table 2**. There were no significant differences between Control or treatment groups (S-D) for pregnancy rate (P>0.05), ranging 25 to 40% (Table 2). The number of apparent implantation sites ranged 6-22 (n.s. among groups), and of live foetuses ranged 2-11 (n.s. among groups), while fertilization rate (total number of implantations/CL) ranged 48.0-69.7% being highest (P<0.05) in the D-group, compared with Control group (P<0.05).

**Table 2**: Pregnancy rate, number of live foetuses, implantation sites and fertilization rate (Total implantation sites/ number of CL) obtained after DIU-AI in the control, single and double DIU-AI groups.

| Group of DIU-AI | n | Pregnancy rate % | Number of live foetuses LSM (range) | Total implantation sites LSM (range) | Fertilization rate (%) |
|---|---|---|---|---|---|
| C* | 19 | 36.8 (7/19)[a] | 5 (2-11)[a] | 9.5 (6-16)[a] | 48.0[a] |
| S ** | 15 | 40.0 (6/15)[a] | 5.5 (5-6)[a] | 12 (8-16)[a] | 58.2[ab] |
| D *** | 8 | 25.0 (2/8)[a] | 7 (4-10)[a] | 16.5 (11-22)[a] | 69.7[b] |

* one DIU-AI 8 h before expected ovulation, ** single DIU-AI 4 h before expected ovulation, *** double DIU-AI 12 and 4 h before expected ovulation
[a-b] Different superscripts indicate statistically significant differences between groups (P<0.05)

**Table 3** describes the same variables but discriminated for the type of package used (MS or MFP), indicating that there was no statistical difference between package type (MFP or MS) for any of the variables (n.s.). Neither were there any statistical interactions between time of insemination and semen packages (P>0.05).

**Table 3**: Pregnancy rate, number of live foetuses, implantation sites and fertilization rate (Total implantation sites/ number of CL) obtained after DIU-AI with frozen-thawed boar semen either packed in MFP or MS (MS: 0.5 ml medium straw; MFP: Multiple Flatpack; LSMeans).

| Package | n | Pregnancy rate (%) | Live foetuses LSM (range) | Total implantation sites LSM (range) | Fertilization rate (%) |
|---|---|---|---|---|---|
| MFP | 22 | 31.8 (7/22) | 6.2 (2-11) | 12.6 (6-16) | 62.6 |
| MS | 20 | 40.0 (8/20) | 4.8 (2-10) | 10.4 (8-22) | 46.2 |

No significant differences (P>0.05) were present between rows (packages).

## Sperm numbers in tubal reservoir and fertilization rate in sows that had returned to oestrus after the DIU-AI

Those sows re-inseminated after returning to oestrus ("open") were searched for spermatozoa in the oviductal sperm reservoir and the presence of fertilized oocytes in the oviduct. These data are presented in **Tables 4** to **6**. Spermatozoa were recovered from only one sperm reservoir (one of the UTJs) in 62.5% of the sows while in 12% of sows there were no spermatozoa in the UTJs, irrespective of having being inseminated once or twice. The total number of spermatozoa recovered 12 to 14 h post ovulation from the UTJs was low, around 4,000. Fertilization of the recovered oocytes was also low (approx. 10%) with only 40% of oocytes having spermatozoa bound to the ZP. This number was restricted to 1-2 spermatozoa per oocyte. No differences were seen for these variables between single or double DIU-AI (Table 4).

**Table 4**: Sperm and oocyte recovery from the oviducts of sows that had returned to oestrus after a primary session of DIU-AI and thereafter re-inseminated either by single or double DIU-AI and slaughtered 12-14h after spontaneous ovulation (UTJs: sperm reservoirs, ZP: zona pellucida).

| DIU-AI | n | Sperm in UTJs | Oocytes | Fertilization rate (%) | Oocytes with spermatozoa in ZP (%) | Accessory sperm/ oocyte |
|---|---|---|---|---|---|---|
| Single | 4 | 4,025[a] | 61 | 12.5[a] | 43.3[a] | 1.5[a] |
| Double | 4 | 3,988[a] | 68 | 11.8[a] | 33.5[a] | 1.7[a] |

No significant differences were recorded between single or double DIU-AI (P>0.05)

Differences were noticed between MS and MFP, the latter showing significantly higher values (Table 5). The number of spermatozoa in the UTJs (P<0.05), fertilization rates and percentages of oocytes holding spermatozoa in their ZP as well as presence of accessory spermatozoa appeared higher when the DIU-AI (either single or double [second AI]) was done 4-8 h before ovulation (Table 6).

**Table 5**: Sperm and oocyte recovery from the oviducts of sows that had returned to oestrus after a primary session of DIU-AI, and thereafter re-inseminated either with frozen boar semen packed in MFP (Multiple Flatpack) or MS (medium plastic straw) and slaughtered 12-14h after spontaneous ovulation (UTJs: sperm reservoirs, ZP: zona pellucida)(LSMeans).

| Package | n | Sperm in UTJs LSM | Oocytes | Fertilization rate (%) | Oocytes with sperm in ZP (%) | Accessory sperm/ oocyte |
|---|---|---|---|---|---|---|
| **MFP** | 5 | 2,567[a] | 76 | 19.4[c] | 48.4[a] | 2.0[a] |
| **MS** | 3 | 217[b] | 53 | 6.0[d] | 21.8[b] | 1.8[a] |

Different superscripts indicate statistically significant differences between rows ([a-b] P<0.05, [c-d] P<0.01)

**Table 6**: Sperm and oocyte recovery from the oviducts of sows that had returned to oestrus after a primary session of DIU-AI, and thereafter re-inseminated at different intervals in relation to spontaneous ovulation and slaughtered 12-14h thereafter (UTJs: sperm reservoirs, ZP: zona pellucida)(LSMeans).

| Interval last DIU-AI to ovulation (h) | n | Sperm in UTJs (LSM) | Oocytes | Fertilization rate (%) | Oocytes with sperm in ZP (%) | Accessory sperm/ oocyte |
|---|---|---|---|---|---|---|
| <-4 | 3 | 166[a] | 48 | 0[c] | 41.1[a] | 1.3[a] |
| -4 to -8 | 3 | 4,000[b] | 47 | 8.2[d] | 52.6[a] | 1.7[a] |
| >-8 | 2 | 624[a] | 34 | 0[c] | 13.1[b] | 0.6[a] |

Different superscripts indicate statistically significant differences between groups
([a-b] P<0.05, [c-d] P<0.001)

# Discussion

In the present study, deep intrauterine insemination of small volumes of frozen-thawed, non re-extended boar semen either once or twice per oestrus yielded pregnancy in a certain proportion of sows. Fertility was low, and depended on the interval between sperm deposition and ovulation, indicating either that sperm transport was inefficient, sperm numbers too low or the life-span of the spermatozoa in the dose too short to ensure that a sufficient number of spermatozoa colonized the sperm reservoirs in the oviducts. A concerted effect of these variables is most likely.

Post-thaw sperm motility averaged 40% for either packaging system, and did not significantly change from thawing to AI. Such sperm viability has been considered above the threshold for AI (Eriksson *et al*., 2000). The fact that sperm motility (as an indirect evidence of viability) did not decrease from thawing to AI suggests an average number of $400 \times 10^6$ live spermatozoa were deposited once or twice. A too high number of spermatozoa deposited close to one UTJ might increase the incidence of polyspermic fertilization whereas a too low sperm number would also affect fertility, by not providing with enough numbers of spermatozoa to ensure maximal fertilization. Which dose is optimal for FT-boar semen has not yet been determined, since fertility depends of many different -albeit concerted- factors. Using a dose as low as $250 \times 10^6$ frozen-thawed boar spermatozoa for DIU-AI, has led to acceptable fertility (50% pregnancy rate) in terms of farrowing rate (43%) and litter size (7.2) (Bathgate *et al.,* 2003). Use of $1,000 \times 10^6$ live spermatozoa yielded better results (70% of farrowing rate and $9.25 \pm 0.23$ piglets born, Roca *et al.,* 2003). These data and our present results indicate sperm numbers are of utmost importance, but sperm numbers per insemination dose do not necessarily need to be dramatically increased.

Not only sperm number, but also its relationship to the volume of the insemination dose seems to be important for successful inseminations. Conventional AI with different volume affects fertility, sperm transport (Baker *et al.,* 1968) and embryo survival (Stratman & Self, 1960). Inseminations of 10 or 20 mL volume into the distal uterine horn yielded similar pregnancy rates (Wolken *et al.,* 2002). The insemination of a larger volume might increase the passive flow of the sperm suspension along the uterine horns by the concerted myometrial contractions that a distension of the lumen would trigger. Myometrial contractility is an autonomous activity of this smooth muscle and is the major importance to effectuate the first phase of sperm transport in the female pig (Rodriguez-Martinez *et al.*, 2001). Distension by an optimum large volume may stretch the myometrial receptors and initiate a local contractile response or a neurological signal to stimulate the neurohypophysis to stimulate uterine contractions by oxytocin release as it occurs in the mare (Arthur, 1970; Ginther & Meckley, 1972; Nikolakopoulos *et al*., 2000). Intrauterine infusion of 80 mL of saline solution increased the number, amplitude and duration of uterine contractions in the mare (Campbell & England, 2004), but discordant results have been described for the sow (Langendijk *et al.*, 2002) perhaps owing to the lack of effect of intra-uterine infusion of saline solution on blood oxytocin levels as earlier reported by Claus (1990). Myometrial contractions in pigs can also be influenced by other factors (boar stimuli, seminal

plasma) (Langendijk, 2001; Langendijk *et al.*, 2004). For instance, cervical stimulation during intra-cervical insemination seem to stimulate uterine activity through an adrenergic or cholinergic pathway, as oxytocin was not always released (Pitjkanen & Prokofjev, 1964; Claus *et al.*, 1989; Claus & Schams, 1990; Langendijk *et al.*, 2003). In the light of the present study, the insertion of the catheters (the cervical guide and the internal flexible long DIU-AI-catheter) can mechanically stimulate the cervix and myometrium, respectively (Martinez *et al.*, 2002). Such signalling could elicit muscle contractions by direct stimuli or trigger the release of other hormones than oxytocin, such as prostaglandins.

The DIU-AI could be performed in all sows, but insertion was difficult in about 10% of the animals taking longer time than expected. The fact that 4/5 of these sows returned to oestrus suggests that the easiness of passing the catheter along the uterine cavity is of utmost importance. Perhaps the excessive manipulation of the catheter could overstimulate the myometrium and cause excessive contractions or disarray the normal contractility of the uterus thus impairing proper sperm transport to the contralateral uterus and the oviducts. Detailed studies of the effect of intrauterine insemination procedures on uterine motility are needed, since extrapolation from other species would not be accurate.

Pregnancy rate averaged 35%, without significant differences between the different insemination moments devised. The hereby named control group was defined for practical reasons as a single insemination to be done about 8h before ovulation, based on the concept that spontaneous ovulation would occur when 70% of the oestrous period had passed (Soede *et al.,* 1992; Dalin *et al.,* 1995; Mburu *et al.,* 1995). Group S (single AI) was designed to inseminate 4 h before expected ovulation and group D (Double AI) to deposit the semen 12 and 4 h before expected ovulation. Such treatment AI-schedules were chosen owing to the current knowledge that best fertility post-AI has been obtained when frozen-thawed boar spermatozoa was inseminated 4-6 h before ovulation (Waberski *et al.*, 1994).

However, since the sows ovulated spontaneously, the interval between the DIU-AIs and ovulation varied largely, ranging for group C: from -13 to -3 h, for group S: from -11 to + 3h and for group D: from -17 to -4 h, and thus yielding low pregnancy rates (Group D: 25%, Group S: 40%, Control: 36%). There were no significant differences between groups, but when explored for the distribution of pregnancy and non-pregnancy along the periods, it appeared evident that pregnancy rates clearly related to the interval DIU-AI and ovulation, being highest (60%, 12/20) when ovulation occurred between 8 and 4 h later. Interesting enough, there were no differences between single and double DIU-AI. Neither were there such differences when accounting for fertilization rate (total number of implantations/CL) a variable that appeared highest in the D-group ($p<0.05$), in relation to the short interval between AI and ovulation. Our results confirm, therefore, that there is a minimum AI and ovulation interval to be respected so that maximal fertilization (Soede *et al.*, 1995a; Soede *et al.*, 1995b), farrowing rates and litter size (Terqui *et al.,* 2000) can be obtained. It could be concluded that the interval between last AI and ovulation time is the most important single factor for both conventional and deep intra-uterine insemination.

28

The present study reports for the first time the fertility after single DIU-AI with concentrated low-volume frozen-thawed boar semen in spontaneous ovulated sows. However, the pregnancy rate and number of foetuses were lower than the results obtained by Roca *et al* (2003), with 80% of pregnancy rate and 9.25 piglets per litter. These differences might result from differences in sperm numbers inseminated, as well as the differences in volume. Moreover, they had used hormonal induction of ovulation, which might impose differences in uterine physiology and thus differences in sperm transport.

In the present study, a volume of 0.5 mL for the inseminate was considered more convenient than using large volumes. This was done in order to diminish sperm losses due to back flow as well as reducing risk of sperm damage due to the re-extension to a larger dose implies (Maxwell & Johnson, 1999). However, the fertility of the hereby tested DIU-AI was lower than expected. In "open" sows (e.g. sows that had returned to oestrus post AI and were re-inseminated), spermatozoa were recovered, 12-14h post ovulation, from only one sperm reservoir (UTJ) in 62.5% of the sows. In 12% of sows there were no spermatozoa in the UTJs, irrespectively of single or double insemination. The total number of spermatozoa recovered from the UTJs was low, around 4,000. Such scenario indicates there was (at least in these sows) an ineffective sperm transport that constrained fertilization of the recovered oocytes. Only a 10% of these recovered oocytes were fertilized and only 40% of all oocytes had some accessory spermatozoa bound to the ZP. It is most likely that the low sperm concentration, the small volume and the site of deposition have all contributed to an insufficient migration of spermatozoa to both the ipsi-lateral sperm reservoir as well as to the contra-lateral horn (Fantinati *et al.,* 2004).

When pig spermatozoa were deposited -using DIU-AI- close to one UTJ, spermatozoa were able to reach the contra-lateral oviduct and fertilize the oocyte (Martinez *et al.*, 2002). Such findings have been done in other species such as the cat (Tsutsui *et al.*, 2000b), the rabbit (Kanayama *et al.*, 1992), the dog (Tsutsui *et al.*, 2000a), and the cow (Lopez-Gatius, 1997). In the present study, highly concentrated FT-spermatozoa could migrate contra-laterally in some sows. The pathway of sperm contra-lateral transport can either be via trans-uterine (Viring & Einarsson, 1980; Viring *et al.*, 1980) or trans-peritoneal (Hunter, 1978; Martinez *et al.*, 2002; Yaniz *et al.*, 2002). Since sperm numbers are related to fertilization rates, insufficient sperm numbers might account for the lower fertilization rates encountered in the present study. However, other factors such as the quality of the deposited spermatozoa have to be considered. The fact that no differences were seen for these variables between single or double DIU-AI suggests that the time interval between deposition and ovulation is still the most crucial factor.

The present study revealed that the fertility after deep intra-uterine artificial insemination did not differ between packages (MS or MFP). However, more spermatozoa were flushed from the sperm reservoirs, and higher fertilization rates were found following AI with MFP-packed spermatozoa. As well, there was a tendency for more foetuses after DUI-AI with MFP compared to MS. These *in vivo* results of sperm quality are comparable to previous *in vitro* sperm tests (Saravia *et al.*, 2004). Unfortunately, the number of specimens pregnant in the present study was low and we must, therefore, refrain from further analyses.

# Acknowledgements

# References

Arthur, G.H. 1970. The induction of oestrous in mares by intrauterine infusion of saline. *Vet Rec* 86, 584-586.

Baker, R.D., Dziuk, P.J. & Norton, H.W. 1968. Effect of volume of semen, number of sperm and drugs on transport of sperm in artificially inseminated gilts. *J Anim* 27, 88-93.

Bathgate, R., Eriksson, B., Maxwell, W.M. & Evans, G. 2003. Low dose deep intrauterine insemination of sows with fresh and frozen-thawed spermatozoa. In: 5th International Conference on Boar semen PreservationDoorwerth, Netherland

Campbell, M.L. & England, G.C. 2004. Effect of teasing, mechanical stimulation and the intrauterine infusion of saline on uterine contractions in mares. *Vet Rec* 155, 103-110.

Claus, R., Ellendorff, F. & Hoang-Vu, C. 1989. Spontaneous electromyographic activity throughout the cycle in the sow and its change by intrauterine oestrogen infusion during oestrus. *J Reprod Fertil* 87, 543-551.

Claus, R. & Schams, D. 1990. Influence of mating and intra-uterine estradiol infusion on peripheral oxytocin concentrations in the sow. *J Endocrinol* 126, 361-365.

Curry, M.R. 2000. Cryopreservation of semen from domestic livestock. *Rev Reprod* 5, 46-52.

Dalin, A.M., Nanda, T., Hulten, F. & Einarsson, S. 1995. Ovarian activity at naturally attained oestrus in the sow. An ultrasonographic and LH study. *Acta Vet Scand* 36, 377-382.

Einarsson, S. & Viring, S. 1973. Distribution of frozen-thawed spermatozoa in the reproductive tract of gilts at different time intervals after insemination. *J Reprod Fertil* 32, 117-120.

Eriksson, B.M. & H. Rodriguez-Martinez. 2000. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in large 5 ml packages (FlatPack). *Anim. Reprod. Sci* 63, 205–220.

Fantinati, P., Zannoni, A., Bernardini, C., Webster, N., Lavitrano, M., Forni, M., Seren, E. & Bacci, M.L. 2004. Laparoscopic insemination technique with low numbers of spermatozoa in superovalated prepubertal gilts for biotechnological application. *Theriogenology* in press.

Hunter, R.H. 1978. Intraperitoneal insemination, sperm transport and capacitaion in the pig. *Anim Reprod Sci* 1, 167-179.

Ginther, O.J. & Meckley, P.E. 1972. Effect of intrauterine infusion on length of diestrous in cows and mares. Vet Med Small Anim Clin 67, 751-754.

Johnson, L.A., Weitze, K.F., Fiser, P. & Maxwell, W.M. 2000. Storage of boar semen. *Anim Reprod Sci* 62, 143-172.

Kanayama, K., Sankai, T., Nariai, K., Endo, T. & Sakuma, Y. 1992. Implantation in both of the uterine horns after the unilateral intrauterine insemination in rabbits. *J Vet Med Sci* 54, 1199-1200.

Langendijk, P. 2001. Is there a future for the boar?: The role of boar stimuli in reproductive processes around estrus in the pig. Ph.D Thesis thesis, Wageningen University.

Langendijk, P., Bouwman, E.G., Schams, D., Soede, N.M. & Kemp, B. 2003. Effects of different sexual stimuli on oxytocin release, uterine activity and receptive behavior in estrous sows. *Theriogenology* 59, 849-861.

Langendijk, P., Bouwman, E.G., Soede, N.M., Taverne, M.A. & Kemp, B. 2002. Myometrial activity around estrus in sows: spontaneous activity and effects of estrogens, cloprostenol, seminal plasma and clenbuterol. *Theriogenology* 57, 1563-1577.

Langendijk, P., Soede, N.M. & Kemp, B. 2004. Uterine activity, sperm transport, and the role of boar stimuli around insemination in sows. *Theriogenology* in press.

Lopez-Gatius, F. 1997. Transuterine sperm transport is not affected by bilateral asymmetry of the reproductive system in dairy cows. *Theriogenology* 47, 1319-1325.

Martinez, E.A., Vazquez, J.M., Roca, J., Lucas, X., Gil, M.A., Parrilla, I., Vazquez, J.L. & Day, B.N. 2002. Minimum number of spermatozoa required for normal fertility after deep intrauterine insemination in non-sedated sows. *Reproduction* 123, 163-170.

Martinez, E.A., Vazquez, J.M., Roca, J., Lucas, X., Gil, M.A. & Vazquez, J.L. 2001. Deep intrauterine insemination and embryo transfer in pigs. *Reprod Suppl* 58, 301-311.

Maxwell, W.M. & Johnson, L.A. 1999. Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. *Theriogenology* 52, 1353-1362.

Mburu, J.N., Einarsson, S., Dalin, A.M. & Rodriguez-Martinez, H. 1995. Ovulation as determined by transrectal ultrasonography in multiparous sows: relationships with oestrous symptoms and hormonal profiles. *Zentralbl Veterinarmed A* 42, 285-292.

Nikolakopoulos, E., Kindahl, H., Gilbert, C.L., Goode, J. & Watson, E.D. 2000. Release of oxytocin and prostaglandin F2 alpha around teasing, natural service and associated events in the mare. *Anim Reprod Sci* 63, 89-99.

Nissen, A.K., Soede, N.M., Hyttel, P., Schmidt, M. & D'Hoore, L. 1997. The influence of time of insemination relative to time of ovulation and farrowing frequency and litter size in sows, as investigated by ultrasonography. *Theriogenology* 47, 1571-1582.

Pitjkanen, I.G. & Prokofjev, M.I. 1964. Modification of the sexual processes of sows by neurotropic substances and stimulation of the receptors of the uterine cervix. *Anim Breed* 35, 124.

Pursel, V.G., Schulman, L.L. & Johnson, L.A. 1978. Distribution and morphology of fresh and frozen-thawed sperm in the reproductive tract of gilts after artificial insemination. *Biol Reprod* 19, 69-76.

Roca, J., Carvajal, G., Lucas, X., Vazquez, J.M. & Martinez, E.A. 2003. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. *Theriogenology* 60, 77-87.

Rodriguez-Martinez, H., P. Tienthai, K. Suzuki, H. Funahashi, H. Ekwall & A. Johannisson. 2001. Oviduct involvement in sperm capacitation and oocyte development. Reproduction, Suppl 58, Control of Pig Reproduction VI, 129-145.

Saravia, F., Wallgren, M., Nagy, S., Johannisson, A. & Rodriguez-Martinez, H. 2004. Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. *Theriogenology* in press.

Simonsson, A. 1994. 'Näringsrekommendationer och fodermedelstabeller till svin (Nutritional recomendations and feedstuff for swine), vol 75.' Swedish University of Agriculture Sciences, Research Information Centre, Uppsala, Sweden.

Soede, N.M., Noordhuizen, J.P. & Kemp, B. 1992. The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. *Theriogenology* 38, 653-666.

Soede, N.M., Wetzels, C.C., Zondag, W., de Koning, M.A. & Kemp, B. 1995a. Effects of time of insemination relative to ovulation, as determined by ultrasonography, on fertilization rate and accessory sperm count in sows. *J Reprod Fetil* 104, 99-106.

Soede, N.M., Wetzels, C.C., Zondag, W., Hazeleger, W. & Kemp, B. 1995b. Effects of a second insemination after ovulation on fertilization rate and accessory sperm count in sows. *J Reprod Fer*til 105, 135-140.

Stratman, F.W. & Self, H.L. 1960. Effect of semen volume and number of sperm on fertility and embryo survival in artificially inseminated gilts. *J Anim Sci* 19, 1081-1089.

Terqui, M., Guillouet, P., Maurel, M.C. & Martinat-Botte, F. 2000. Relationship between peri-oestrus progesterone levels and time of ovulation by echography in pigs and influence of the interval between ovulation and artificial insemination (AI) on litter size. *Reprod Nutri Dev* 40, 393-404.

Tsutsui, T., Hase, M., Tanaka, A., Fujimura, N., Hori, T. & Kawakami, E. 2000a. Intrauterine and intravaginal insemination with frozen canine semen using an extender consisting of orvus ES paste-supplemented egg yolk tris-fructose citrate. *J Vet Med Sci* 62, 603-606.

Tsutsui, T., Tanaka, A., Takagi, Y., Nakagawa, K., Fujimoto, Y., Murai, M., Anzai, M. & Hori, T. 2000b. Unilateral intrauterine horn insemination of frozen semen in cats. *J Vet Med Sci* 62, 1247-1251.

Viring, S. & Einarsson, S. 1980. Influence of boar seminal plasma on the distribution of spermatozoa in the genital tract of gilts. *Acta Veterinaria Scandinavica* 21, 598-606.

Viring, S., Einarsson, S., Jones, B. & Larsson, K. 1980. Transuterine transport of small- and medium-sized molecules deposited in the uterus gilts. *J Reprod Fertil* 59, 459-462.

32

Wolken, A., Rath, D., Bortolozzo, I. & Marquetti, A. 2002. Sows can successfully be inseminated non-surgically into the distal uterine horn with a highly reduced number of sperm cells. *Theriogenology* 57, 392.

Waberski, D., Weitze, K., Gleumes, T., Schwarz, M., Willmen, T. & Petzoldt, R. 1994. Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology* 42, 831-840.

Yaniz, J.L., Lopez-Bejar, M., Santolaria, P., Rutllant, J. & Lopez-Gatius, F. 2002. Intraperitoneal insemination in mammals: a review. *Reprod Domes Anim* 37, 75-80.

# Future prospects

An optimal prospect for using frozen-thawed boar semen in the commercial swine industry would be the use of a single deep intra-uterine insemination, with a minimum of spermatozoa per dose on an optimal insemination time, to yield acceptable fertility and litter size. Such ideal prospect is still far out of reach and requires further research to determine:

1. A method to determine the imminence (within few hours) of spontaneous ovulation.

2. The minimum number of spermatozoa per insemination required for acceptable fertility.

2. The optimal volume of insemination that supports sperm transport without negative effect on sperm viability.

4. The best location for a deep DIU-AI so that a sufficient number of spermatozoa reaches both uterine horns.

# General references

Baker, R.D. & Degen, A.A. 1972. Transport of live and dead boar spermatozoa within the reproductive tract of gilts. *Journal of Reproduction & Fertility* 28, 369-377.

Baker, R.D., Dziuk, P.J. & Norton, H.W. 1968. Effect of volume of semen, number of sperm and drugs on transport of sperm in artificially inseminated gilts. *Journal of Animal Science* 27, 88-93.

Belstra, B.A. 2002. 'Review: Intrauterine (transcervical) and fixed-time artificial insemination in swine.' Department of Animal Sciences, College of Agriculture & Life Science, North Carorina state University, North Carorina.

Burke, P. 1999 Productivity assessment of liquid boar semen usage. In: IVth International conference on boar semen preservation (Eds L.A. Johnson&H.D. Guthrie). Allen Press, Inc., Beltsville, Maryland, USA, pp. 149-152

Bwanga, C.O. 1991. Cryopreservation of boar semen. I: A literature review. *Acta Veterinaria Scandinavica* 32, 431-453.

Bwanga, C.O., Einarsson, S. & Rodriguez-Martinez, H. 1991a. Cryopreservation of boar semen. II: Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini- and maxi-straws and plastic bags. *Acta Veterinaria Scandinavica* 32, 455-461.

Bwanga, C.O., Ekwall, H. & Rodriguez-Martinez, H. 1991b. Cryopreservation of boar semen. III: Ultrastructure of boar spermatozoa frozen ultra-rapidly at various stages of conventional freezing and thawing. *Acta Veterinaria Scandinavica* 32, 463-471.

Chatdarong, K., Lohachit, C., Ponglowhapan, S. & Linde-Forsberg, C. 2001. Transcervical catheterization and cervical patency during the oestrous cycle in domestic cats. *Journal of Reproduction & Fertility Supplement* 57, 353-356.

Curry, M.R. 2000. Cryopreservation of semen from domestic livestock. *Reviews of Reproduction* 5, 46-52.

Dallanora, D., Mezalira, A., Katzer, L.H., Bernadi, M.L., Bortolozzo, F.P. & Wentz, I. 2004. Volume and sperm number in the semen backflow after intrauterine or cervical insemination in sows. In: 15th International Congress on Reproduction (Eds L.R. Franca, H.P. Godinho, M. Henry&M.I.V. Melo). Brazilian college of animal reproduction, Porto Seguro, BA- Brazil, p. 387

Dube, C., Beaulieu, M., Reyes-Moreno, C., Guillemette, C. & Bailey, J.L. 2004. Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. *Theriogenology* 62, 874-886.

Einarsson, S. & Viring, S. 1973. Distribution of frozen-thawed spermatozoa in the reproductive tract of gilts at different time intervals after insemination. *Journal of Reproduction & Fertility* 32, 117-120.

Eriksson, B.M., Petersson, H. & Rodriguez-Martinez, H. 2002. Field fertility with exported boar semen frozen in the new flatpack container. *Theriogenology* 58, 1065-1079.

Eriksson, B.M. & Rodriguez-Martinez, H. 2000. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. *Animal Reproduction Science* 63, 205-220.

First, N.L., Short, R.E., Peters, J.B. & Stratman, F.W. 1968. Transport and loss of boar spermatozoa in the reproductive tract of the sow. *Journal of Animal Science* 27, 1037-1040.

Gillan, L., Skovgold, K., Watson, P.F., Evans, G. & Maxwell, W.M. 1999. Fate and functional integrity of fresh and frozen-thawed ram spermatozoa following intrauterine insemination. *Reproduction, Fertility and Development* 11, 309-315.

Hancock, J.L. 1959. Pig insemination technique. *The Veterinary Record* 71, 527.

Hancock, J.L. & Hovel, C.J. 1961. The effect of semen volume and number of spermatozoa on the fertility of intrauterine insemination of pigs. *Animal Production* 3, 153-161.

Holt, W.V. 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* 53, 47-58.

Howard, J., Byers, A.P., Brown, J.L., Barrett, S.J., Evans, M.Z., Schwartz, R.J. & Wildt, D.E. 1996. Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (Neofelis nebulosa). *Zoo Biology* 15, 55-69.

Hunter, R.H. 1990. Fertilization of pig eggs in vivo and in vitro. *Journal of Reproduction & Fertility Supplement* 40, 211-226.

Hunter, R.H. 2003. Advances in deep uterine insemination: a fruitful way forward to exploit new sperm technologies in cattle. *Animal Reproduction Science* 79, 157-170.

Johnson, L.A. 1985 Fertility results using frozen boar spermatozoa: 1970 to 1985. In: Deep Freezing of Boar Semen (Ed. K. Larsson). Uppsala, Sweden, pp. 199-222

Johnson, L.A., Weitze, K.F., Fiser, P. & Maxwell, W.M. 2000. Storage of boar semen. *Animal Reproduction Science* 62, 143-172.

Kidder, J.D., Foote, R.H. & Richmond, M.E. 1998. Transcervical artificial insemination in the domestic ferret (Mustela putorius furo). *Zoo Biology* 17, 393-404.

Krueger, C. & Rath, D. 2000. Intrauterine insemination in sows with reduced sperm number. *Reproduction, Fertility and Development* 12, 113-117.

Krueger, C., Rath, D. & Johnson, L.A. 1999. Low dose insemination in synchronized gilts. *Theriogenology* 52, 1363-1373.

Levis, D.G. 1999. Liquid boar semen production: current extender technology and where do we go from here. In: Boar semen preservation IV (Eds L.A. Johnson&H.D. Guthrie). Allen Pres, Inc., Beltsville, Maryland, USA., pp. 121-128

Levis, D.G. 2002. New reproductive technologies for the AI industry. In: Proceedings National Swine Improvement Federation (NSIF) Conference and Annual MeetingNational Swine Improvement Federation (NSIF), Nashville, TN, U.S.A., pp. 1-25

Levis, D.G., Burroughs, S. & Willium, S. 2002. Use of intra-uterine insemination of pigs: Pros, Cons and Economics. In: Proceedings Reproductive Pharmacology and Technology, Annual Meeting American Association of Swine Veterinarians American Association of Swine Veterinarians, Kansas, MO, U.S.A., pp. 39-62

Linde-Forsberg, C., Strom Holst, B. & Govette, G. 1999. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. *Theriogenology* 52, 11-23.

Martinez, E.A., Caamano, J.N.*, et al.* 2004. Successful nonsurgical deep uterine embryo transfer in pigs. *Theriogenology* 61, 137-146.

Martinez, E.A., Vazquez, J.M., Roca, J., Lucas, X., Gil, M.A., Parrilla, I., Vazquez, J.L. & Day, B.N. 2001a. Successful non-surgical deep intrauterine insemination with small numbers of spermatozoa in sows. *Reproduction* 122, 289-296.

Martinez, E.A., Vazquez, J.M., Roca, J., Lucas, X., Gil, M.A. & Vazquez, J.L. 2001b. Deep intrauterine insemination and embryo transfer in pigs. *Reprod Suppl* 58, 301-311.

Martinez, E.A., Vazquez, J.M., Roca, J., Lucas, X., Gil, M.A., Parrilla, I., Vazquez, J.L. & Day, B.N. 2002. Minimum number of spermatozoa required for normal fertility after deep intrauterine insemination in non-sedated sows. *Reproduction* 123, 163-170.

Matthijs, A., Engel, B. & Woelders, H. 2003. Neutrophil recruitment and phagocytosis of boar spermatozoa after artificial insemination of sows, and the effects of inseminate volume, sperm dose and specific additives in the extender. *Reproduction* 125, 357-367.

Mburu, J.N., Einarsson, S., Dalin, A.M. & Rodriguez-Martinez, H. 1995. Ovulation as determined by transrectal ultrasonography in multiparous sows: relationships with oestrous symptoms and hormonal profiles. *Zentralblatt fur Veterinarmedizin. Reihe A* 42, 285-292.

Mburu, J.N., Einarsson, S., Lundeheim, N. & Rodriguez-Martinez, H. 1996. Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. *Animal Reproduction Science* 45, 109-121.

Mburu, J.N., Rodriguez-Martinez, H. & Einarsson, S. 1997. Changes in sperm ultrastructure and localisation in the porcine oviduct around ovulation. *Animal Reproduction Science* 47, 137-148.

Melrose, D.R. & O'Hagen, C. 1961. Investigations into the technique of artificial insemination in the pig. In: Proceedings of the fouth international congress on the animal reproductionThe Hague, pp. 855-859

Mezalira, A., Dallanora, D., Bernadi, M., Wentz, I. & Bortolozzo, F.P. 2004. Reproductive performance of sows submitted to intrauterine insemination with different sperm cell dose. In: Proceeding of the 18th International Pig Veterinary Society Congress (IPVS) (Eds T.G. Blaha&C. Pahlitzsch). Hambrug, Germany, p. 477

Nissen, A.K., Soede, N.M., Hyttel, P., Schmidt, M. & D'Hoore, L. 1997. The influence of time of insemination relative to time of ovulation and farrowing frequency and litter size in sows, as investigated by ultrasonography. *Theriogenology* 47, 1571-1582.

Petersen, M.M., Wessel, M.T., A., S.M., Liu, I.K.M. & Ball, B.A. 2002. Embryo recovery rates in mares after deep intrauterine insemination with low number of cryopreserved equine spermatozoa. *Theriogenology* 58, 663-665.

Pursel, V.G. 1982. Effect of uterine ligation and cervical plugs on retention of frozen-thawed boar sperm. *Journal of Animal Science* 54, 137-141.

Pursel, V.G., Schulman, L.L. & Johnson, L.A. 1978. Distribution and morphology of fresh and frozen-thawed sperm in the reproductive tract of gilts after artificial insemination. *Biology of Reproduction* 19, 69-76.

Rath, D. 2002. Low dose insemination in the sow--a review. *Reproduction in Domestic Animals* 37, 201-205.

Rigby, J.P. 1966. The persistance of spermatozoa at the uterotubal junction of the sow. *Journal of Reproduction & Fertility* 11, 153-155.

Robeck, T.R., Steinman, K.J., Gearhart, S., Reidarson, T.R., McBain, J.F. & Monfort, S.L. 2004. Reproductive physiology and development of artificial insemination technology in killer whales (Orcinus orca). *Biology of Reproduction* 71, 650-660.

Roca, J., Carvajal, G., Lucas, X., Vazquez, J.M. & Martinez, E.A. 2003. Fertility of weaned sows after deep intrauterine insemination with a reduced number of frozen-thawed spermatozoa. *Theriogenology* 60, 77-87.

Rodin, I.M. & Lipatov, V.I. 1936. Artificial insemination of pigs. *Animal Breeding* 4, 205.

Rodriguez-Martinez, H., Tienthai, P., Suzuki, K., Funahashi, H., Ekwall, H. & Johannisson, A. 2001. Involvement of oviduct in sperm capacitation and oocyte development in pigs. *Reproduction Supplement* 58, 129-145.

Rozeboom, K.J., Reicks, D.L. & Wilson, M.E. 2004. The reproductive performance and factors affecting on-farm application of low-dose intrauterine deposit of semen in sows. *Journal of Animal Science* 82, 2164-2168.

Saravia, F., Wallgren, M., Nagy, S., Johannisson, A. & Rodriguez-Martinez, H. 2004. Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. *Theriogenology* in press.

Seidel, G.E., Jr., Allen, C.H., Johnson, L.A., Holland, M.D., Brink, Z., Welch, G.R., Graham, J.K. & Cattell, M.B. 1997. Uterine horn insemination of heifers with very low number of nonfrozen and sexed spermatozoa. *Theriogenology* 48, 1255-1264.

Soede, N.M., Helmond, F.A. & 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. *Journal of Reproduction & Fertility* 101, 633-641.

Soede, N.M. & Kemp, B. 1997. Expression of oestrus and timing of ovulation in pigs. *Journal of Reproduction & Fertility Supplement* 52, 91-103.

Soede, N.M., Noordhuizen, J.P. & Kemp, B. 1992. The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. *Theriogenology* 38, 653-666.

Soede, N.M., Wetzels, C.C., Zondag, W., de Koning, M.A. & Kemp, B. 1995. Effects of time of insemination relative to ovulation, as determined by ultrasonography, on fertilization rate and accessory sperm count in sows. *Journal of Reproduction & Fertility* 104, 99-106.

Squires, E.L., Reger, H.P., Maclellan, L.J. & Bruemmer, J.E. 2002. Effect of time of insemination and site of insemination on pregnancy rates with frozen semen. *Theriogenology* 58, 655-658.

Steverink, D.W., Soede, N.M., Bouwman, E.G. & Kemp, B. 1998. Semen backflow after insemination and its effect on fertilisation results in sows. *Animal Reproduction Science* 54, 109-119.

Steverink, D.W., Soede, N.M., Groenland, G.J., van Schie, F.W., Noordhuizen, J.P. & Kemp, B. 1999. Duration of estrus in relation to reproduction results in pigs on commercial farms. *Journal of Animal Science* 77, 801-809.

Stratman, F.W. & Self, H.L. 1960. Effect of semen volume and number of sperm on fertility and embryo survival in artificially inseminated gilts. *Journal of Animal Science* 19, 1081-1089.

Vazquez, J.M., Martinez, E.A., Parrilla, I., Roca, J., Gil, M.A. & Vazquez, J.L. 2003. Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa. *Theriogenology* 59, 1605-1614.

Verberckmoes, S., Van Soom, A., De Pauw, I., Dewulf, J., Vervaet, C. & de Kruif, A. 2004. Assessment of a new utero-tubal junction insemination device in dairy cattle. *Theriogenology* 61, 103-115.

Viring, S. & Einarsson, S. 1980. Influence of boar seminal plasma on the distribution of spermatozoa in the genital tract of gilts. *Acta Veterinaria Scandinavica* 21, 598-606.

Viring, S. & Einarsson, S. 1981. Sperm distribution within the genital tract of naturally inseminated gilts. *Nordisk Veterinarmedicin* 33.

Vyt, P., Maes, D., Dejonckheere, E., Castryck, F. & Van Soom, A. 2004. Comparative study on five different commercial extenders for boar semen. *Reproduction in Domestic Animals* 39, 8-12.

Waberski, D., Weitze, K., Gleumes, T., Schwarz, M., Willmen, T. & Petzoldt, R. 1994. Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology* 42, 831-840.

Wagner, H.G. & Thibier, M. 2000. World statistics for artificial insemination in small ruminants and swine. In: Fourteenth International Congress on Animal Reproduction Stockholm, Sweden

Watson, P.F. & Behan, J.R. 2002. Intrauterine insemination of sows with reduced sperm numbers: results of a commercially based field trial. *Theriogenology* 57, 1683-1693.

Weitze, K.F. 1999 Update on the worldwide application of swine AI. In: IVth International conference on boar semen preservation (Eds L.A. Johnson&H.D. Guthrie). Allen Press, Inc., Beltsville, Maryland, USA, pp. 141-146

Weitze, K.F., Habeck, O., Willmen, T. & Rath, D. 1989. Detection of ovulation in the sow using transcutaneous sonography. *Zuchthygiene* 24, 40-42.

Woelders, H. & Matthijs, A. 2001. Phagocytosis of boar spermatozoa in vitro and in vivo. *Reproduction Supplement* 58, 113-127.

Wulster-Radcliffe, M.C., Wang, S. & Lewis, G.S. 2004. Transcervical artificial insemination in sheep: effects of a new transcervical artificial insemination instrument and traversing the cervix on pregnancy and lambing rates. *Theriogenology* 62, 990-1002.

# Acknowledgements

This study was carried at the Division of Comparative Reproduction, Obstetrics and udder health (ex. Dept of Obstetrics and Gynaecology), Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden.

I would like to express my deeply sincere gratitude to all those direct or indirect support, guidance, and wishes made it possible for me to complete my studies.

Professor Hans Kindahl, former head of the Department of Obstetrics and Gynaecology, for giving me an opportunity to work in the Department.

Professor Heriberto Rodriguez Martinez, former head of the Department of Obstetrics and Gynaecology and present head of the Division of Reproduction, my scientific supervisor. I am very grateful that you accepted me as your student, which has given me an invaluable experience. Thanks for your encouragement and strong guidance.

Dr.Margareta Wallgren, my scientific co-supervisor, for your guidance and support. I appreciate when you helped me during my night and holiday duties. I have felt you are my Swedish sister, thank you for taking care of me not only in matters of research but also in other personal matters.

Fernando Saravia and Ignacio Caballero, my scientific colleagues, I am very thankful for your research assistance, especially in the final spurt of my studies.

Professor Stig Einarsson and Associate professor Anne-Marie Dalin for your valuable discussions about sperm transport, ultrasonography and reproductive immunity.

My thai friends, especially the Suriyasomboon and Kampa families, I'm very grateful for you taking care and supporting me in Sweden.

The MSc student, Suresh, Visah, Amitha and Carlos for nice friendship

Ylva Brandt my unofficially beautiful teacher in ultrasonography

Annika Rikberg, Karin Selin-Wretling and Åsa Jansson for many laboratory technical assistances and for teaching me Swedish.

Kjell-Ove Eklund, for technical assistance with my computer and network nightmares. Bo fred, for technical help and the provision of my orange bicycle.

Carola Jansson and Mari Wallbring for taking do good care of my sows and for teaching me so many nice Swedish expressions.

40