Deep Freezing of Concentrated Boar Semen for Intra-Uterine Insemination

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

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To the memory of my Grandmother Rosa

ABSTRACT

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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 suboptimal, which results in low farrowing rates and small litter sizes when conventional cervical AI is performed, are among the reasons why FT boar semen is rarely used in AI. Compensatory attempts include AI with large sperm numbers, which, however, reduces the number of doses produced per ejaculate. New procedures for intra-uterine AI are now available, opening up possibilities for the deposition of smaller volumes (thus diminishing semen reflux) and therefore lower total sperm numbers per dose, provided these spermatozoa (spz) can be packed in a higher density and sustain cryopreservation (i.e. provided they have an acceptable survival rate post-thaw). This thesis includes a summarised review of available literature on the history, as well as constraints on and future prospects of the use of frozen boar semen in AI. Also, a trial is reported whose aim was to concentrate (2×10^9 spz/mL) and freeze boar spermatozoa packed in either 0.5 ml volume medium straws (MS) or a multiple FlatPack (MFP) (four 0.7 ml volume segments of a single FlatPack [SFP]) intended as AI doses for intra-uterine AI. A single, well-proved freezing protocol was followed using a SFP (5×10^9 spz/5 ml) as control package and sperm concentration. Sperm motility was measured by computer-assisted sperm analysis (CASA) while plasma membrane integrity was assessed with the SYBR-14/PI assay combined with flow cytometry, and with a rapid hypo-osmotic swelling test (sHOST), in order to establish sperm viability. Sperm motility did not differ statistically (NS) between the test-packages and the control, neither in terms of overall sperm motility (range of means: 37-46%) nor sperm velocity. Percentages of linear motile spermatozoa (LIN), however, were significantly higher in controls (SFP) than in the test packages. Spermatozoa frozen in the SFP (control) and MFP depicted the highest percentages of PMI (54 and 49% respectively) compared to straws (38 %, $p<0.05$) when measured with flow cytometry. In absolute numbers, more viable spermatozoa were present in the MFP dose post-thaw than in the MS ($p<0.05$). Inter-boar variation was present, albeit only significant for MS (sperm motility) and SFP (PMI). In conclusion, the results indicate that boar spermatozoa can be successfully frozen if concentrated in a small volume.

Key words: spermatozoa; high sperm count artificial insemination (AI) doses; computer-assisted sperm analysis (CASA); flow cytometry; sHOST; boars

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Contents

Background

Artificial insemination

Artificial insemination (AI) is referred as firstly performed at least 600 years ago. Undocumented Arabic writings, thought to date from 1322, describe (in an epic manner) procedures for collecting stallion semen from a mated mare in order to breed other mares. More than 450 years later, in 1784, Lazzaro Spallanzani scientifically documented a successful AI in a female dog, which whelped three pups 62 days later, making AI the first reproductive biotechnology to have ever been applied to domestic animals (Flowers, 1999).

Artificial insemination is a process of collecting spermatozoa, extending them with appropriate fluids for either short- or long-term preservation, and then manually placing them into the reproductive tract of sexually receptive females. The procedure was primarily designed to avoid spreading of venereal diseases by natural mating, a goal reached beyond expectations. Furthermore, AI is applied to increase the dissemination of genetic material to large numbers of farm animals. Also, AI as the most successful reproductive biotechnology thus far has provided impetus for developing other technologies such as sperm cryopreservation, sperm sorting for chromosomal sex, procedures for semen deposition, control of the oestrous cycle, embryo transfer, embryo production *in vitro* and associated technologies, such as cloning (Foote, 2002). The exponential use of AI has resulted in an accelerated rate of genetic selection and production improvement, most notably in dairy cattle but also, in pigs (Medeiros *et al.*, 2002).

Last but not least, sperm preservation in association with AI is one of the cornerstones of procedures for the preservation and dissemination of genetic resources. It has contributed to the international exchange of genetic lines as well as the conservation of superior genetic individuals, helping to establish genetic resource banks, reserve supplies in response to disease-related disasters and contribute to the conservation of threatened or endangered species (Yoshida, 2000).

Artificial insemination in swine was initiated by Ivanow in Russia in the early 1900s. Russian state farms started to use AI by 1931 (Rodin and Lipatov, 1936). Sows were inseminated with 100–150 mL of fresh, extended semen on the second day of oestrus. Average conception rates of 70% were reported. Despite the importance of these achievements, it was not until after World War II that fresh boar semen was used successfully (Ito *et al.*, 1948; Polge, 1956), extensively from 1975 onwards (Reed, 1985). Use of liquid boar semen for AI, preserved for up to 3–5 days at 16–20ºC, constitutes the core of the AI routinely performed by the pig breeding industry, covering more than 99% of AIs in pigs done around the world (Wagner and Thibier, 2000). The worldwide tendency indicates an increase in the number of AIs in pigs over the next few years (Weitze, 1999) and it is predicted that by 2005, 80% of all pig breeding will be by AI (Burke, 1999).

Using conventional AI, pigs are often inseminated several times (i.e. two or three times) during oestrus. A large volume of semen (80–100 mL) containing high numbers of spermatozoa (usually 2–3 x 10^9 total spz) is deposited into the posterior region of the cervix by a disposable catheter, emulating the situation that occurs in this species during natural mating. In general, the insemination starts 12 hours after oestrus detection and is repeated every 12–18 hours until the standing oestrous reflex is not longer shown. The standing oestrus can vary widely among individual females (20–64 hours or longer). When proper detection of oestrus is performed, the farrowing rate and litter sizes are comparable with rates/sizes achieved with AI with liquid semen and natural mating, reaching over 90% of farrowing and mean litter sizes of ten to twelve piglets (Johnson *et al.,* 2000).

Use of frozen semen in porcine artificial insemination

In the early trials of freezing boar semen, procedures (Polge, 1956) and extenders (Roy, 1955; King and Macpherson, 1967) as for cattle AI and bull semen were used. Most of the time boar spermatozoa did not reach the rates of post-thaw survival achieved in bovines; furthermore, the surviving spermatozoa seemed unable to keep their fertilising ability intact. In 1970, using surgical insemination in the oviduct, the first successful fertilisation using frozen-thawed (FT) boar semen was reported (Polge, Salamon and Wilmut, 1970), and one year later the first successes, of live offspring from cervical AIs using FT boar semen, were reported by three different groups (Crabo and Einarsson, 1971; Graham *et al.*, 1971; Pursel and Johnson, 1971).

At present, while use of FT semen for AI is considered a basic component for cattle breeding worldwide (Curry, 2000) the situation in the pig industry is diametrically different; use of FT boar semen is limited to no more than 1% of the AIs performed around the world (Wagner and Thibier, 2000). Among the reasons behind this restricted use of FT boar semen are the low survivability of spermatozoa in the face of the freezing-thawing process and the shorter lifespan of the surviving spermatozoa, both resulting in lower farrowing rates and smaller litter sizes compared with AI using semen preserved in liquid form.

To compensate for the effects of a suboptimal freezing methodology an AI dose usually contains excessive spermatozoa, in the order of 5 x 10^9 total spermatozoa (Johnson, 1985; Johnson *et al*., 2000). The procedure for AI follows the above described pattern, a large re-extension of the FT semen dose (to 80–100 ml) with two or more AIs per oestrus. The aim is to deposit a maximal number of short-lived spermatozoa as close as possible to the moment of ovulation (Reed, 1985). Obviously, few (six to eight) AI doses are usually obtained from a single ejaculate, thus contributing to the low efficacy of the methodology.

The lifespan of FT boar semen, then, is shorter than that of its liquid-preserved counterpart, often considered about half that of fresh semen. Properly controlled studies have indicated that FT boar spermatozoa do not usually survive for more than 4 hours after deposition in the genital tract of sows (Waberski *et al.,* 1994)

this is in accord with earlier studies that determined that FT boar spermatozoa survive between 2–6 hours in the genital tract (Larsson, 1976). Taking into consideration that the fertilising ability of oocytes after ovulation is also short, no more than 6–8 hours, proper timing of sperm deposition in relation to the moment of ovulation is critical when using FT semen. The success of AI programmes is therefore highly dependent upon the availability of good techniques for predicting, controlling and detecting the ovulation (Holt, 2000b). In general, pre-ovulatory insemination is recommended to establish a sperm reservoir in the oviduct and thus ensure that sufficient numbers of spermatozoa are available to fertilise the ovulated oocytes. As mentioned before, the duration of oestrus in sows varies, influenced by different factors, between individuals. Moreover, spontaneous ovulation could occur anywhere between 10 and 85 hours after onset of oestrus (Soede and Kemp, 1997). Obviously, onset of oestrus is not a good predictor for the timing of AI with FT semen. Although it is still practised in some places of the world induction of ovulation through exogenous hormones is not an option, for the sake of safe production of food of animal origin and for animal welfare reasons. Monitoring occurrence of ovulation, for instance by use of transrectal ultrasonography, provides information on the interval between onset of standing oestrus and spontaneous ovulation, a period that is usually maintained between consecutive oestruses within animals; generally, ovulation occurs when two-thirds of the standing oestrus has passed (Soede *et al.*, 1992; Dalin *et al.*, 1995; Mburu *et al.*, 1995).

Cryopreservation of boar semen

Freezing and thawing procedures

Following serendipitous discoveries, spermatozoa were the first mammalian cells to be successfully cryopreserved (Polge, Smith and Parkes, 1949). Following the discovery that glycerol can be used as a cryoprotectant, empirically devised technical adjustments were introduced in sperm cryopreservation protocols with the objective of extending the methodology and improve the efficiency of the process in different species (Graham, 1996; Leboeuf, Restall and Salamon, 2000; Salamon and Maxwell, 2000) including the pig (for a review, see Johnson *et al.*, 2000).

Cryopreservation of boar semen involves subjecting the spermatozoa to a series of closely related steps, mainly extension and reconcentration, temperature reduction, cellular dehydration, freezing and thawing.

Interestingly, the most accepted and successful procedures currently in use for freezing boar semen have common features (Bwanga, 1991; Johnson *et al.*, 2000). Briefly, the steps include: (i) a reduced (1:1) extension with buffer and a period of equilibration in the presence of homologous seminal plasma (SP) to promote the capacity of spermatozoa to withstand thermal shock events (Pursel, Johnson and Schulman, 1973; Watson and Plummer, 1985), usually done while cooling the semen to 15ºC; (ii) a separation of the bulk of SP by centrifugation, thus reconcentrating the spermatozoa; (iii) inclusion of chilling protectants (lactose and

egg yolk [LEY]) during cooling to 5ºC; followed by (iv) inclusion of penetrating cryoprotectants (the most commonly used being glycerol at a 3% final concentration) immediately prior to freezing to enable rapid decreases of temperature, which are usually effected by programmable freezers. Boar spermatozoa are frozen at concentrations considered optimal, of about 1 x 10^9 spz/mL.

The proportion of glycerol is kept fairly low compared with other domestic species, in order to preserve both sperm motility and acrosome integrity (Almlid and Johnson, 1988). Also, these concentrations allow for certain rates of cooling which are closely dependent on the cryogenic quality of the container used to pack the semen. For instance, for 0.5 mL plastic straws, the optimum cooling rate appears to be 30ºC/min (Fiser and Fairfull, 1990) while for the 5 mL FlatPack, it seems to be 50ºC/min (Eriksson and Rodriguez-Martinez, 2000b). A relatively quick thawing rate, 200ºC/min, seems to be required for boar spermatozoa independently of the container used (0.5 mL straws [Fiser *et al.*, 1993]; 5 mL Maxi-straws [Westendorf, Richter and Treu, 1975]). In the case of the 5 mL FlatPack the best thawing regime in terms of post-thaw motility has been reported to be around 900ºC/min (Eriksson and Rodriguez-Martinez, 2000b).

Cryo-injuries

Boar spermatozoa seem not well adapted to endure cooling to low temperatures. There is a reduction of their post-thaw viability and consequently their fertility, as a consequence of capacitation-like changes in their plasma membrane (Green and Watson, 2000; 2001; Watson, 2000), as well as accumulated cellular injuries that arise throughout the cryopreservation process (Watson, 2000).

At the beginning of the freezing procedure, the first cellular injury results from rapid cooling from the temperature at which the semen is ejaculated, to temperatures near the freezing point of water. Freshly ejaculated boar spermatozoa in the whole ejaculate will not survive even slow cooling below 15ºC, with an increase in the number of cells affected and the magnitude of the damage as the temperature approaches 0ºC. Such damage can often be significantly reduced (but not entirely prevented) by either adding protective compounds to the semen extenders (Parks, 1997) or cooling the spermatozoa very slowly (by 10ºC/h to around 4ºC; Watson, 1981). Spermatozoa from ungulates appear to be particularly sensitive to cold shock (Wales and White, 1959), with those from boar being most sensitive (Watson, 1981; Parks, 1997). Cold shock aetiology involves damage to the cellular membranes and alterations in the metabolism of the cells, primarily caused by changes in the arrangement of plasma membrane constituents, the phospholipids in particular (Parks, 1997). The most obvious sign of cold shock injury is an irreversible loss of motility upon rewarming (Lasley and Bogart, 1944; Parks, 1997). Other effects are the loss of selectivity in sperm permeability and, ultrastructurally, the disruption of the acrosomal membrane, indicating that although the plasma membrane is the primary site of cooling damage, the phenomenon also affects other sperm organelles, including the mitochondria. Boar spermatozoa seem to acquire a certain resistance to cooling stress upon incubation in their own SP at low extension rates for 1–5 hours at ambient temperature (Pursel, Johnson and Schulman, 1972; 1973; Butler and Roberts, 1975). Holding

periods of over 3 hours have been reported to not show detrimental effects on FT spermatozoa in terms of fertilising ability (Eriksson *et al.*, 2001).

Spermatozoa have very limited biosynthetic activity and are dependent on catabolic processes in order to function (Hammerstedt and Andrews, 1997). Therefore, a decrease in temperature reduces their metabolic activity, allowing some extension of their lifespan. However, the remaining metabolic activity of the spermatozoon eventually leads to death because thermally driven chemical reactions are not stopped unless cells are deeply cooled. To halt metabolism, the cells need to be cooled to below –130ºC.

The biggest challenge to spermatozoa during cryopreservation is the lethality of an intermediate temperature zone of between –15ºC and –60ºC, which the spermatozoon must traverse during both cooling and thawing (Mazur, 1963). As extended semen is cooled to about –5ºC the spermatozoa and the surrounding medium remain unfrozen and super-cooled. Between –5ºC and –10ºC ice crystals form in the extracellular medium, either spontaneously or as a result of seeding the solution. The cytoplasm of the spermatozoon remains unfrozen or super-cooled, presumably because most of the water is bound to other molecules and not free as in the extracellular medium. The super-cooled water inside the spermatozoon has, by definition, a higher chemical potential than does the water in the partially frozen, extracellular medium, and thus water flows osmotically out of the spermatozoon and freezes externally, increasing the size of the extracellular ice.

The subsequent physical events depend on the cooling rate. If spermatozoa are cooled too rapidly, water is not lost fast enough to maintain equilibrium and the spermatozoa become increasingly super-cooled, eventually attaining equilibrium by freezing inside the cell (Mazur, 1963; 1990). In most cases spermatozoa that do not dehydrate eventually form intracellular ice crystals, being lethally damaged. If cooling is sufficiently slow, the spermatozoa will lose water rapidly enough avoiding formation of intracellular ice. However, if the spermatozoa are cooled too slowly, they will experience severe volume shrinkage and long-time exposure to high-solute concentrations before eutectic equilibrium is achieved, both of which may cause irreversible membrane collapse (Gao, Mazur and Crister, 1997). The changes causing cell injury as a result of deleterious concentrations of solutes have been collectively named “solution effects” (Mazur, Leibo and Chu, 1972).

The mechanical stress and ice formation around the spermatozoa can also convey a certain degree of damage since spermatozoa are pressed, together with highly concentrated unfrozen solutes, in narrow veins of very limited space in between the lakes of frozen ice (Gao, Mazur and Crister, 1997). Sperm viability, as a function of rate of freezing, corresponds graphically to an inverted U-shaped curve, where the most appropriate freezing rate is the fastest rate that allows extracellular water freezing without intracellular ice formation.

Those spermatozoa that survive cooling to low subzero temperatures still face challenges during warming and thawing, which can exert effects on survival comparable to those of cooling (Mazur, 1984). These effects depend on whether the prior rate of cooling has induced intracellular freezing or dehydration. In the

former case, rapid thawing can rescue many cells, possibly because it can prevent the harmful formation of small intracellular ice crystals by recrystallisation.

Extenders

The survival of ejaculated spermatozoa in SP alone is limited to a few hours. In order to freeze semen, extending the sperm suspension with a protective solution is compulsory. In general, semen extenders are composed of sugars, proteins and lipoproteins, buffers, additives and cryoprotective agents. Based on their constituents they may be divided into extenders without buffer, such as egg yolk-glucose (Polge, Salamon and Wilmut, 1970), egg yolk-lactose (Richter *et al.*, 1975; Westendorf, Richter and Treu, 1975) and egg yolk-saccharose-ethylenediamine tetra-acetic acid (EDTA), Mg and Ca salts (Milovanov *et al.*, 1974), and extenders with buffering capacity, such as glycine-phosphate and glucose-phosphate (Iida and Adachi, 1966), egg yolk-glucose-citrate (Serdiuk, 1970), egg yolk-glucose-citrate-EDTA-potassium-unitol-urea (Shapiev, Moroz and Korban, 1976), Beltsville F3 (Pursel and Johnson, 1971), Beltsville F5 (Pursel and Johnson, 1975), Tes-tris-fructose-citrate-egg yolk, TEST (Graham *et al.*, 1971), Tes-NaK-glucose-egg yolk (Crabo and Einarsson, 1971; Larsson, Einarsson and Swensson, 1977), Tris-fructose-EDTA-egg yolk (Salamon and Visser, 1973) and Tris-glucose-EDTA-egg yolk (Park *et al.*, 1977).

Many cryoprotective agents other than glycerol have been tested but none has proved better at preserving boar spermatozoa (Watson, 1995). The portions of the extender that contain glycerol are usually characterised by a low concentration of this cryoprotective, quickly penetrating agent. As stated previously, concentrations around 3% are commonly used since they have demonstrated the best results in terms of post-thaw viability (Pursel and Johnson, 1975; Westendorf, Richter and Treu, 1975; Wilmut and Polge, 1977; Scheid, Westendorf and Treu, 1980) when added after the semen has been chilled to 5ºC (Almlid and Johnson, 1988).

Among the most commonly used extender additives are sugars, proteins and lipoproteins, detergents, anti-oxidants and SP. The osmotic properties of the sugars provide extracellular protection and their combination with glycerol has been found indispensable in providing protection during freezing (Salamon, Wilmut and Polge, 1973). Sugars do not penetrate plasma membranes and probably act by either raising the percentage of unfrozen water at a given temperature or reducing the concentration of salts in the unfrozen extracellular water (Mazur, 1984). Glucose and lactose are the most commonly used sugars (Pursel and Johnson, 1975; Westendorf, Richter and Treu, 1975; Paquignon and Courot, 1976). Phospholipids and the low-density lipoprotein fraction from egg yolk are considered to be responsible for the cryoprotection of the plasma membrane of bull spermatozoa (Gebauer *et al.*, 1970; Foulkes, 1977) and though it has still not been proved these components could have the same effect on boar spermatozoa. The mechanism of action is unclear (Paquignon, 1985) but the process could be mediated by either a less intense cellular dehydration (Courtens and Paquignon, 1985) or stabilisation of the spermatozoa plasma membrane (Watson, 1975).

Another additive that is included in most egg yolk-containing extenders (Pursel and Johnson, 1975; Westendorf, Richter and Treu, 1975) is a synthetic detergent

and wetting agent based on sodium and triethanolamine lauryl sulphate. Its commercial name is "Orvus Es Paste" (OEP), and it is also known as "Equex STM" (Nova Chemical, Scituate, MA, USA). It was introduced by Graham and co-workers (1971) who found that this detergent decreases post-thaw damage to boar spermatozoa. Although its action is still not fully known, it appears to act by altering some components of the egg yolk rather than by a direct effect on the sperm plasma membrane (Pursel, Schulman and Johnson, 1978; Strzezek *et al.*, 1984).

Inter-boar variation

Another problem constraining the design of a successful freezing protocol is the variability of response of different boars to the process of cryopreservation. Why this difference is present is not yet fully known but a recent report provided evidence that the existence of good and bad freezers has a genetic basis and that freezing quality could be identified prospectively (Thurston *et al.*, 2002). To maximise the use of FT semen, a selection of the males both by genetic merit and according to freezability should be considered.

Evaluation of the freezing and thawing process: sperm viability

The fertilising potential of a sperm population subjected to freezing and thawing depends upon the preservation of a series of sperm attributes, all of which are sooner or later related to fertilisation of the oocyte(s). The intactness of these attributes is dramatically affected after cryopreservation, leading to the death of a large number of spermatozoa, clearly evidenced by the loss of motility shown in the sample, a reflection of the loss of sperm viability. Assessment of sperm motility is therefore still used as the method of choice to quantify sperm survival. Such assessment is most often done subjectively, which is a very quick and inexpensive method. Since the subjectivity of the method has always been regarded as a minus, computer-assisted instruments (the so-called "computer-assisted sperm analysis [CASA]" instruments) have been designed. Such instruments can not only examine larger sperm numbers for their motility but also, discriminate among a series of motility patterns and velocities, all of which provide a series of valuable details for analysing the post-thaw *in vitro* sperm viability (Holt *et al.*, 1997; Eriksson and Rodriguez-Martinez, 2000b).

Evidence gathered over the past 10 years clearly indicates that, in general, spermatozoa surviving a freezing-thawing process have an altered membrane structure or altered membrane properties, which may render them functionally similar to capacitated and/or acrosome-reacted spermatozoa (Maxwell and Johnson, 1997; Thundathil *et al.*, 1999; Green and Watson, 2000; Holt, 2000a; Schembri *et al.*, 2000; 2002; Watson, 2000). Apart from its key structural function, the status of the sperm plasma membrane is of utmost importance due to its role during the interactions between spermatozoa, the epithelium of the female tract and the oocyte and its vestments (Rodriguez-Martinez, 2001). Our capability to assess the status of the plasma membrane of spermatozoa subjected to cryopreservation has increased dramatically over the past decade with the

development of fluorophore markers for cytoplasmic enzymes, deoxyribonucleic acid (DNA) and/or membrane potential (Rodriguez-Martinez, Larsson and Pertoft, 1997; Peña, Johannisson and Linde-Forsberg, 1999; Johnson *et al.*, 2000). A drawback of the use of these methods is the cost of the reagents and, when flow cytometry rather than fluorescence microscopy is the instrument used to analyse sperm numbers, the cost and availability of the equipment.

An alternative to the above-mentioned assays is the use of a hypo-osmotic swelling test (HOST). Since only spermatozoa with a biochemically active membrane will swell when hypo-osmotically stressed, following influx of water through an intact membrane the HOST can be used to evaluate functional integrity of the sperm membrane (Schrader *et al.*, 1986). Recently a short exposure test (the so-called "sHOST") was used to assess boar spermatozoa and was presented as cheap, sensitive and sufficiently reproducible (Perez-Llano *et al.*, 2001). Obviously the value of the sHOST has to be validated against other types of membrane integrity tests (e.g. fluorophores; see Vazquez *et al.*, 1997) to determine whether the measurements are reliable.

Increasing the use of frozen boar semen: not an easy task

Since boar semen has to be preserved in large volumes, when performing AI researchers have tried, for the sake of practicality, different cooling and thawing regimes, none substantially superior and many dependent upon the type of container used (Pursel and Johnson, 1975; Bamba and Cran, 1985; 1988; Weitze, Rath and Baron, 1987; Almlid and Johnson, 1988; Fiser and Fairfull, 1990; Almlid and Hofmo, 1996; Eriksson and Rodriguez-Martinez, 2000b). Because of the dependency on the container type, attempts have been made to adapt various packaging systems other than the conventional medium (0.5 mL) or large plastic straw (5–6 mL), aiming to find one that is cryogenically best suited as well as being practical at the moment when the semen is to be thawed and inseminated. Cryogenically, the smaller the straw the better so that for instance mini-straws are suitable; however, there is the drawback that several mini- or medium-straws (MS) are needed to make up a conventional AI dose. One of the more recently developed packages has been the FlatPack (Eriksson and Rodriguez-Martinez, 2000b) whose use as single AI dose has yielded acceptable fertility rates in the field (Eriksson, Petersson and Rodriguez-Martinez, 2002).

The thawed semen is re-extended to volumes of 80–100 mL, since such volumes are considered to be required to elicit sperm transport after cervical AI (Baker, Dziuk and Norton, 1968). Although thawing and re-extension can inflict damages to boar spermatozoa (for a review, see Bwanga, 1991) few attempts have been made to establish whether low semen volumes can be conventionally inseminated and transported to the sperm reservoir despite the low volume (Hancock, 1959; Hancock and Hovell, 1961).

Owing to the large sperm losses present post-thaw, boar semen is frozen at concentrations of 1 x 10^9 spz/mL in 5–6 mL maxi-straws (single AI dose) (Westendorf, Richter and Treu, 1975), 0.5 mL MS (multiple AI doses) (Mileham

et al., 1997) or 5 mL FlatPacks (single AI dose) (Eriksson and Rodriguez-Martinez, 2000b; Eriksson, Petersson and Rodriguez-Martinez, 2002). After re-extension to 80–100 mL the FT semen (with a surplus of spermatozoa; 5–6 x 10^9) is deposited in the posterior cervical opening using disposable catheters. To achieve acceptable fertility under commercial conditions at least two AIs are performed per oestrus. Unfortunately cervical insemination in pigs with FT spermatozoa frozen in straws has been hampered by low pregnancy rates or low litter size. Inseminating FT semen packaged in FlatPacks (5 x 10^9 spz/dose), twice during spontaneous oestrus, resulted in a farrowing rate above 70% and an average of 10.7 live born piglets (Eriksson, Petersson and Rodriguez-Martinez, 2002). Despite these good results such numbers of spermatozoa per AI dose allow no more than ten doses per ejaculate (Almlid, Stave and Johnson, 1987; Almlid and Hofmo, 1996; Eriksson, Petersson and Rodriguez-Martinez, 2002). Provided that a 7-day period of sexual rest between collections is allowed, the number of doses could be increased for some boars, up to 15–20 doses per ejaculate. Use of smaller doses may obviously be the solution, provided they are inseminated in a location that ensures effective sperm transport to the fertilisation site, a condition that was not met previously, which may explain the low results obtained thus far with FT semen (Kemp and Soede, 1997).

Procedures for intra-uterine semen deposition have been recently presented. One is a transcervical procedure using a novel inseminating device (Watson and Behan, 2002). The transcervical device allows the cervix to be penetrated in multiparous sows and the insemination dose to be deposited in the uterine body. Using this catheter, an AI dose of liquid semen can be reduced to 1 x 10^9 spermatozoa (but still in 80 mL of extender) without significant reduction of fertility (Watson and Behan, 2002). Another recently described procedure for transcervical AI in sows is non-surgical, deep intra-uterine catheterisation (Martinez *et al.*, 2001a; 2002). Using a flexible, long catheter passed through a conventional AI catheter locked in the cervix, semen can in a majority of sows be deposited deep into one of the uterine horns, often close to the utero-tubal junction (UTJ), requiring a similar time as used for traditional AI (Martinez *et al.*, 2001b). Using fresh liquid semen this method would allow a 20 to 60-fold reduction in the number of spermatozoa inseminated, with an eight- to tenfold reduction in dose volume, without jeopardising fertility (Martinez *et al.*, 2001b). When FT semen, frozen in MS (0.5 mL) at concentrations of 1 x 10^9 spermatozoa per mL and re-extended to a 7 mL dose, was deeply inseminated with these catheters promising results were obtained. With induced ovulation and a single insemination (with 2 straws per dose) the farrowing rate was 77.5%, with 9.31 ± 0.41 live piglets born; with spontaneous ovulation and two inseminations, the farrowing rate was 70%, with 9.25 ± 0.23 piglets born (Roca *et al.*, 2003).

Sperm transport seems to be effective in either case of intra-uterine insemination, thus calling for the use of smaller AI doses. With conventional AI a distension of the uterine lumen is caused by the infusion of 80–100 mL of fluid (either neat semen or semen with extenders), producing a stimulatory effect on myometrium contractility (Viring and Einarsson, 1980a). Such contractility enables distribution of spermatozoa into both uterine horns and a colonisation of both reservoirs (Viring and Einarsson, 1980b). With intra-uterine catheters it is probable that the deep insertion of an AI catheter could trigger a similar stimulatory effect on

uterine musculature and thus cause the redistribution of the smaller volumes of spermatozoa and the colonisation of the sperm reservoirs. A possible concomitant advantage using such small volumes of undiluted semen is the avoidance of any harmful re-extension of the semen dose post-thaw. In addition, use of smaller doses would lead to a higher number of AI doses produced per ejaculate, which would mean cheaper production and a more effective use of genetically superior sires. A cryopreservation method for freezing highly concentrated spermatozoa with acceptable sperm survival rates post-thaw needs to be developed before such strategy can be satisfactorily applied.

Introduction to the research report

Cryopreservation of boar semen is still considered to be a suboptimal methodology, since it kills a substantial part of the processed spermatozoa. As a result there is the need to inseminate compensatory high numbers of spermatozoa per AI dose, which in turn yields few doses produced per ejaculate. Now that new insemination strategies have been devised to deposit semen into the uterus, there is an opportunity for freezing small volumes containing high sperm numbers, which could result in similar or perhaps better fertility output. A basic requirement is, however, that the spermatozoa packed and processed in this manner properly sustain cryopreservation, with an acceptable survival rate post-thaw.

Aim of the study

The aim of the present study was to determine the feasibility of freezing high numbers of spermatozoa in small volumes (2 x 10^9 spz/mL, packaged in 0.5–0.7 mL containers) as potentially usable in intra-uterine AI in pigs.

Research report

Deep Freezing of Concentrated Boar Semen for Intra-Uterine Insemination: Effects on Sperm Viability

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ABSTRACT

The use of deep-frozen boar semen for artificial insemination (AI) is constrained by the need for high sperm numbers per dose, yielding few doses per ejaculate. With the advancement of new, intra-uterine insemination strategies, there is an opportunity for freezing small volumes containing high sperm numbers, provided the spermatozoa properly sustain cryopreservation. The present study aimed to concentrate (2 x 10^9 spz/mL) and freeze boar spermatozoa packed in a 0.5 ml volume plastic straw (MS) or a multiple FlatPack (MFP) (four 0.7 ml volume segments of a single FlatPack [SFP]) intended as AI doses for intra-uterine AI. A single freezing protocol was employed using a FlatPack (SFP) (5 x 10^9 spz/5 mL volume) as control package and concentration. Sperm viability post-thaw was monitored as sperm motility measured by computer-assisted sperm analysis (CASA) and also, plasma membrane integrity (PMI) either assessed by SYBR-14/PI, combined with flow cytometry, or a rapid hypo-osmotic swelling test (sHOST). Sperm motility did not differ statistically (NS) between the test-packages and the control, neither in terms of overall sperm motility (range of means: 37-46%) nor sperm velocity. The percentages of linearly motile spermatozoa were, however, significantly higher in controls (SFP) than in the test packages. Spermatozoa frozen in the SFP (control) and MFP depicted the highest percentages of PMI (54 and 49%, respectively) compared to MS (38 %, $p<0.05$) when assessed with flow cytometry. In absolute numbers, more viable spermatozoa were present in the MFP dose post-thaw than in the MS ($p<0.05$). Inter-boar variation was present, albeit only significant for MS (sperm motility) and SFP (PMI). In conclusion, the results indicate that boar spermatozoa can be successfully frozen when concentrated in a small volume.

Key words: spermatozoa; high sperm count artificial insemination (AI) doses; computer-assisted sperm analysis (CASA); flow cytometry; sHOST; boars.

INTRODUCTION

Artificial insemination (AI) with frozen-thawed (FT) bull semen is considered to be a core biotechnology of animal breeding and an integral component of the cattle industry worldwide (Curry, 2000). In species such as the pig the use of frozen semen for AI is limited to no more than 1% of the AIs in pigs performed around the world. The other 99% are performed with semen preserved in liquid form at 16–20ºC (Wagner and Thibier, 2000). Among the reasons behind this restricted use of frozen boar semen are the low survivability of spermatozoa in the face of freezing-thawing and the shorter lifespan of the surviving spermatozoa, both resulting in lower farrowing rates and smaller litter sizes than achieved with AI using semen preserved in liquid form. To compensate for the effects of a suboptimal freezing methodology, insemination doses usually contain excessive spermatozoa in the order of 5 billion total spermatozoa which, following re-extension with 80–100 mL extender, are deposited in the cervix of the female (Johnson, 1985). It is also routine to perform two or more AIs per oestrus to deposit a maximal number of short-lived spermatozoa as close as possible to the moment of ovulation (Reed, 1985). As a consequence of both these practices, few (six to eight) AI doses can be obtained from a single ejaculate, thus contributing to the low efficacy of the methodology.

Because of the need for preserving large volumes of semen, researchers have tried different cooling and thawing protocols, often empirically (Pursel and Johnson, 1975; Bamba and Cran, 1985; 1988; Weitze, Rath and Baron, 1987; Almlid and Johnson, 1988; Fiser and Fairfull, 1990; Almlid and Hofmo, 1996; Eriksson and Rodriguez-Martinez, 2000b), as well as various packaging systems other than the conventional medium (0.5 mL) or large (5–6 mL) plastic straw. The aim was to find the system that is cryogenically most suitable. One of these recently developed packages has been the FlatPack (Eriksson and Rodriguez-Martinez, 2000b) whose use as single AI dose has provided acceptable fertility rates in the field (Eriksson, Petersson and Rodriguez-Martinez, 2002). In either case the thawed semen, either in multiple FlatPacks (MFPs) or as single-straw FlatPacks (SFPs), is customarily re-extended to a volume considered necessary for eliciting sperm transport through the uterine horns (Baker, Dziuk and Norton, 1968). Thawing and re-extension can inflict damages on boar spermatozoa (for a review, see Bwanga, Ekwall and Rodriguez-Martinez, 1991).

Early protocols for cryopreservation, most still in use, did not consider freezing high sperm concentrations, since they were considered to be of poor performance after thawing (Bwanga, 1991). Spermatozoa are stored in the terminal segment of the ductus epididymis at high concentrations. A physical component accompanies the mechanisms for sperm quiescence at this segment of the male genital tract (for a review, see Rodriguez-Martinez, 1991). To the best of our knowledge, there have been no studies aiming to explore the possibility of freezing concentrations of boar spermatozoa at ranges similar to those present in the epididymal reserves (in the order of 3×10^9 spz/mL) (Einarsson, 1971). Because of the above constraints on freezing large semen volumes and also, with a potential use of sex-sorted spermatozoa in mind, procedures for intra-uterine semen deposition have recently been presented, with semen being placed either transcervically using a novel

inseminating device (Watson and Behan, 2002) or deep into one uterine horn, often close to the tip, using a long, flexible catheter (Martinez *et al.*, 2002). Using semen frozen in MS (0.5 mL) at concentrations of 1 x 10^9 spz/mL and a final volume of 7 mL, the latter method yielded promising fertility results (Roca *et al.*, 2003). Although they are still re-extended, these low volumes of semen seem able to provide enough numbers of fertilising spermatozoa during AI. Although it has not yet been proved, the deep insertion of the AI catheter may convey a similar stimulatory effect on myometrium contractility as does distension with 80–100 mL of fluid (Viring and Einarsson, 1980a), allowing for a redistribution of spermatozoa onto the contralateral horn and a colonisation of both reservoirs (Viring and Einarsson, 1980b). If such an effect is present, using a very small dose of highly concentrated spermatozoa may be enough to supersede the deleterious effects of re-extension and permit the colonisation of the tubal sperm reservoirs. Furthermore, more AI doses per ejaculate than customary can be produced, which means a saving in time and hence, in cost, as well as better use of certain sires. However, before such a strategy can be employed, a cryopreservation method of highly concentrated spermatozoa must be developed, with acceptable sperm survival rates post-thaw.

The present study aimed to freeze high numbers (1 x 10^9 total), nearly two-thirds of the concentration present in the cauda epididymis (Einarsson, 1971), of spermatozoa per AI dose and establish their survival post-thaw, as potentially usable for intra-uterine AI. The semen was frozen using a single freezing protocol in two different test packages, a 0.5 ml volume plastic straw (MS) and a multiple FlatPack (MFP) (four 0.7 ml volume segments of a single FlatPack (SFP), 1 x 10^9 total spz/segment) against a control package (SFP, 5 ml volume, 1 x 10^9 spz/mL, total 5 x 10^9 total spz). Sperm viability post-thaw was monitored as sperm motility and also using computer-assisted sperm analysis (CASA, Eriksson and Rodriguez-Martinez, 2000b); as plasma membrane integrity (PMI), assessed by the SYBR-14/PI assay using flow cytometry (Johnson *et al.*, 2000) and a rapid hypo-osmotic swelling test (sHOST) (Perez-Llano *et al.*, 2001), respectively.

MATERIALS AND METHODS

Animals

Five mature boars (three Swedish Yorkshire and two Swedish Landrace) 2–5 years old and selected according to normal semen quality and proved fertility were used. No preselection for semen freezability was performed. All boars were kept on straw beds in individual pens at the Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, with females in the close neighbourhood and were fed according to Swedish standards (Simonsson, 1994) and provided with water *ad libitum*. The experimental protocol had previously been reviewed and approved by the Local Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Semen processing

On a weekly basis, each boar was allowed to mount a dummy and the sperm-rich fraction of the ejaculate was collected with the gloved-hand technique in a plastic

bag inside an insulated thermos flask. Only ejaculates with at least 70% motile spermatozoa and 75% morphologically normal spermatozoa were used. From a total of 57 ejaculates collected, only two were discarded, both from the same boar, owing to an occasional low concentration in those particular ejaculates. Semen was processed primarily according to Eriksson and Rodriguez-Martinez (2000a; 2000b). Approximately 60 minutes post-collection, holding time at room temperature (r.t.), 20–22ºC, the semen was extended (1:1, v/v) in Beltsville thawing solution (BTS(+)®, IMV, L'Aigle, France). The BTS-extended semen was allowed to stand in a programmable refrigerated centrifuge (Centra MP4R, IEC, MN, USA) set at +16ºC for 3 hours, after which it was centrifuged at 800 x g for 10 minutes. Sperm concentration and motility were determined in aliquots. After double centrifugation at 800 x g for 10 minutes, the supernatant was discarded and the volume (graduated vial) and concentration were measured in a Bürker haemocytometer. The remaining pellets were re-extended with a lactose-egg yolk (LEY) extender (80 mL (80% v/v 310 mM) of β-lactose + 20 mL egg yolk, at a ratio of one to two parts semen to one part extender). After thorough mixing the semen was further cooled to +5ºC for 2 hours in the centrifuge. At this temperature the semen was slowly mixed with a third extender consisting of 89.5 mL LEY extender, 9 mL glycerol and 1.5 mL of Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA), which is equivalent to Orvus Es Paste (Graham *et al.*, 1971), at a ratio of two parts of semen to one part of extender, yielding a final concentration of glycerol of 3%.

Spermatozoa were packaged at 5ºC in a cool cabinet (IMV, L'Aigle, France) in either of two test packages, 0.5 mL polyvinyl chloride (PVC) straws (MS) (Minitüb, Germany) or MFP (four 0.7 mL volume segments of a SFP), at concentrations of 2 x 10^9 spz/mL. As a control, semen was packed in SFP at a concentration of 1 x 10^9 spz/mL. The MS were sealed with PVC powder while the FlatPacks were heat-sealed (either end-sealed as SPFs or sealed as segments for the MFP) and placed either in conventional racks (straws) or standing vertically along their long axis on specially made freezing racks (Eriksson and Rodriguez-Martinez, 2000b).

After sealing, all racks were transferred to the chamber of a programmable freezer (Mini Digitcool 1400; IMV, L'Aigle, France) set at 5°C. The cooling/freezing rate was as follows: 3ºC/min from 5ºC to –5ºC, 1 minute for crystallisation, and thereafter 50ºC/min from –5ºC to –140ºC. The samples were then plunged into liquid N_2 (–196ºC) for storage.

Computer-assisted sperm analysis

Sperm doses were thawed in a circulating water-bath at 50°C for 13 seconds (SFP) or 35ºC for 20 seconds (MS and MFP). After thawing, the semen suspension was extended at room temperature (20–25°C) with a 1:20 ratio in prewarmed BTS(+)® to give a sperm concentration of about 50–60 x 10^6 spz/mL, which is considered optimal for the automated CASA equipment. The inclusion of the LEY extender prevented the spermatozoa from sticking to the chamber glass during motility evaluation. The re-extended, thawed semen was placed into a 38°C incubator for 30–55 minutes before being examined for motility patterns with CASA (SM-CMA, MTM Medical Technologies, Montreaux, Switzerland). Five µL of semen

were placed in a prewarmed 10 µm deep Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and warmed to 38ºC. Sperm motility was assessed in a microscope equipped with 38°C microscope stage and phase contrast optics (x 200) (Optiphot-2, Nikon, Japan), both subjectively and using the CASA instrument. For each sample, eight predetermined optical fields around the central reticulum of the chamber were used to count a minimum number of 200 spermatozoa per sample. In conjunction with the percentage of motile spermatozoa, the following motility patterns were recorded: linearly motile spermatozoa (LIN) (%); straight linear velocity (VSL) (µm/s); average path velocity (VAP) (µm/s); and curvilinear velocity (VCL) (µm/s). The parameter settings for the SM-CMA software were: 32 frames with a spermatozoon present in at least 16 in order to be counted; time resolution 20 msec (50 Hz). An object with VAP <10 µm/s was considered immotile and objects with a velocity >25 µm/s were deemed as motile. Spermatozoa deviating <10% from a straight line were designated as linearly motile, and those spermatozoa with a radius <25 µm were classified as circularly motile.

Assessment of sperm plasma membrane integrity

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit L-7011 (Molecular Probes Inc., Eugene, OR, USA). Frozen semen samples were thawed and suspended in prewarmed BTS+® to approximately 5 x 10^6 spz/ml. An amount of 1 000 µl of the sperm suspension was supplemented with 1 µL SYBR-14 and 5 µL PI from a working solution containing 100 nM of SYBR-14 and 12 µM PI, components A and B, respectively, of the sperm viability kit in Falcon tubes (Becton Dickinson, San José, CA, USA) and incubated at 38°C for at least 10 minutes before cytometric analysis.

Flow cytometric analysis was conducted using a LSR flow cytometer (Becton Dickinson, San José, CA, USA). The SYBR-14 and PI dyes were excited by a 20 mW Argon ion 488 nm laser. Whereas SYBR-14 fluorescence was detected on detector FL 1 (530/28 nm BP), PI was detected on detector FL 3 (670 nm LP). Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale. Acquisitions were done using the CellQuest 3.3 software (Becton Dickinson, San Jose, CA, USA). The flow cytometer was used at low flow rate (6–24 µL/min). Acquisition data were stored for further analyses in list mode. Gates were set according to forward and side scatters to eliminate particles smaller than spermatozoa or cell aggregates. Using SYBR-14 and PI a further gating was used, according to the green and red fluorescent intensities, to eliminate debris particles the size of spermatozoa. Fluorescent data from 10 000 gated events per sample were collected in list mode. Dot plots for offline analyses were drawn by WinMDI 2.8 (free software by J. Trotter, available for downloading at http://facs.scripps.edu/software.html).

Assessment of sperm plasma membrane functionality

The *in vitro* membrane functionality was evaluated using the rapid hypo-osmotic swelling test (sHOST) as previously described for use in boar spermatozoa (Perez-Llano *et al.*, 2001). The evaluation was performed using the same semen dose as utilised for flow cytometric analysis. After thawing, 100 µL of semen was

immediately added to 1 000 µL of BTS(+)®, adjusted with distilled water to obtain a hypo-osmotic test solution (75 mOsm) and incubated at 38ºC for 5 minutes. Following this incubation time, 200–300 µL of the semen-hypo-osmotic solution was fixed in 1 000 µL of a solution of hypo-osmotic BTS(+)® plus 5% formaldehyde, for later evaluation. For the sHOST evaluation, two trained operators counted 100 spermatozoa each, using guidelines as described by Jeyendran and co-workers (1984) at 40 x magnification in a microscope equipped with phase-contrast optics (Laborlux 12, Leitz, Jena, Germany). The outcome was accepted only if a <10% difference was encountered between operators. An average obtained from both counts was used for the analyses.

Statistical analysis

Data, as mean values (using angular transformation where necessary) for post-thaw CASA parameters, PMI and sHOST, were examined by analysis of variance (ANOVA) using the general linear model (GLM) procedure of the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC, USA, 1994). The statistical model used included the effects of boar, the packaging system and the interaction between boar and package. A Student's *t*-test was used to determine differences between PMI tests. Differences were considered significant at $p<0.05$.

RESULTS

A mean of 61.7 ± 7.3 x 10^9 spermatozoa were collected per ejaculate, with significant variation among boars ($p<0.05$). An ejaculate could therefore provide for approximately 50 sperm doses (as MS or MFP) or 10 sperm doses (as SFP), considering that the sperm losses during the processing could approximately reach 20%.

The results post-thaw for the sperm parameters evaluated in the three packages used in the study are summarised in Table 1. The statistical model showed that there was no interaction between males and packaging system ($p>0.05$). Consequently, the variables male and package were analysed separately. The results are summarised in Tables 2–5.

Post-thaw sperm motility measurements

Sperm motility post-thaw did not differ statistically (NS) between the test-packages and the control, neither in terms of overall sperm motility nor sperm velocity (either VSL, VAP or VCL). The percentages of linearly motile spermatozoa (LIN) differed significantly ($p<0.05$), however, among packages; the SFP (control group) displaying the highest percentage of spermatozoa with linear motility, and spermatozoa frozen in MS depicting the lowest mean values (Table 1).

The results for the different packages, discriminated per boar, are summarised in Tables 2 (0.5 mL MS), 3 (MFP) and 4 (SFP) regarding CASA variables while PMI values are presented in Table 5.

Overall sperm motility post-thaw varied significantly ($p<0.05$) among boars when semen was frozen in MS (Table 2). Such variation was not detected when using MFP or SFP for freezing (Tables 3 and 4, respectively). The frequency of linearly motile spermatozoa also varied ($p<0.05$) among boars, but solely for MS and MFP (Tables 2 and 3).

Post-thaw sperm plasma membrane intactness

Sperm Viability Test (SYBR-14/PI)

Spermatozoa frozen in the SFP (control) and MFP depicted the highest percentages of plasma membrane intactness (PMI) compared to straws ($p<0.05$) when assessed with flow cytometry and SYBR-14/PI (Table 1). Consequently, more viable spermatozoa e.g. spermatozoa that maintained an intact plasma membrane after thawing, were (in absolute terms) present in the MFP ($494.5 \pm 13.5 \times 10^6$/spz, mean ± SEM) than in the MS ($381.6 \pm 22.1 \times 10^6$/spz, mean ± SEM, $p<0.05$).

Short Hypo-Osmotic Swelling Test (sHOST)

The sHOST did not shed significant differences in terms of membrane functionality between either package (Table 1) or boar (Table 5).

Comparing the two *in vitro* tests used (SYBR/PI and sHOST), the outcome was similar between MS and MFP, but differed in the SFP for some boars (control, Tables 1 and 5, $p<0.05$), probably owing to the lower number of spermatozoa assayed per sample with sHOST than with flow cytometry.

DISCUSSION

The results of the present study show that it is possible to successfully freeze high numbers of boar spermatozoa in small volumes (0.5–0.7 mL). Post-thaw, these spermatozoa showed comparable viability, in terms of sperm membrane integrity and functionality, to semen frozen at lower concentrations in a higher volume of recognised cryogenic suitability. Between the test-packages, the MFP, of which about 50 doses could be produced per ejaculate, gave the highest total number of post-thaw intact spermatozoa per dose.

One of the major inconveniences of the extensive use of FT boar semen is the large volume and high total number of spermatozoa of a single insemination dose; currently about 5×10^9 total spermatozoa are required in a final volume of 80–100 mL. This implies freezing the semen at a concentration rate of 1×10^9 spz/mL and using several MSs (0.5 mL) or a SFP (5 mL) to obtain a single AI dose. This procedure is time-consuming and implies a redilution of the spermatozoa after thawing, which causes a decrease in their viability (Larsson and Einarsson, 1976). A major goal of a more extensive use of FT semen by the porcine industry is to design a freezing method that permits the use of a unique AI dose with a high sperm concentration and, hopefully, a low volume that would allow easy storage and handling under current conditions.

During natural mating in pigs the volume of the ejaculate is between 150 mL and 200 mL, with a mean concentration of 100–200 x 10^6 spz/mL. The semen is deposited in the cervix and immediately reaches the inside of the sow/gilt's uterus. The deposited volume increases the uterine lumen and triggers a series of contractions that help a minor percentage of the spermatozoa to reach the utero-tubal junction (UTJ), the sperm reservoir site where spermatozoa await ovulation, but also, to reflux through the vagina (see below). Recent evidence has indicated that spermatozoa present in the initial portion of the sperm-rich fraction of the ejaculate (i.e. the first 10 mL) largely contribute to building the sperm reservoir (Rodriguez Martinez *et al.*, 2003). This physiological condition may be important when we consider the design of protocols for porcine AI with FT semen. Based on the physiological regulation of sperm transport to the oviduct, it may be advantageous to reduce sperm losses and obtain more AI doses per ejaculate and a high fertilisation rate by depositing the spermatozoa closer to the UTJ and depositing them at a high concentration and in a small volume. Using highly concentrated/low-volume sperm doses, the number of doses produced per ejaculate could be increased 5-6 fold, compared to the $5x10^9$ spermatozoa-containing doses, used at present.

The basic essentials about a high volume and sperm concentration of the AI doses have been maintained for more than 30 years, without important modifications, mainly because the pig semen industry is not focused on frozen semen. At present, with the development of deep-insemination methods and the advent of sexed semen, it is imperative to find new freezing protocols that will achieve a higher sperm survival post-thaw and thus improve the fertility results when using FT semen.

The question is which of the packages used is the most suited for storing the spermatozoa. The results on sperm post-thaw PMI obtained from the present flow cytometric analysis showed clear statistical differences ($p<0.05$) between semen frozen in MS and semen frozen in either of the two FlatPack packages, favouring the FlatPacks. If we take into account only the MFP the sperm PMI was similar to the results obtained in previous studies (Eriksson *et al.*, 2001; Eriksson, Petersson and Rodriguez-Martinez, 2002) where semen was frozen in SFP but at a lower concentration (1 x 10^9 spz/mL) and where PMI was established by optic microscopy, a less objective method than flow cytometry. The difference seen between MS and MFP may not be attributable to the number of spermatozoa frozen, since MS and MFP had the same sperm concentration and quite the same volume. The results confirm that the FlatPack concept provides the best cryogenics for freezing and thawing. The average post-thaw sperm motility was about 36% for MS, and between 42 to 46% for either FlatPack used. Using this variable as a measure of sperm viability, the MFP appears superior to the MS in terms of cryogenic ability. Cryopreservation dramatically affects sperm motility, impairing sperm function and destroying a series of sperm components as a result of cooling and rewarming (Watson, 2000). Therefore, although thawed boar spermatozoa often show low motility when examined in ordinary diluents (Larsson, 1985), the motility achieved using the MFP is higher than what is generally considered acceptable for FT semen (Johnson *et al.*, 1981). There are several explanations for the low sperm motility recorded, including sperm damage due to a lower amount of water to reflow into the spermatozoa post-thaw, leading to solute-effect damage.

Also, a high sperm concentration could cause a major agglutination between spermatozoa and probably require a longer incubation time to separate from each other after thawing. The latter speculation could explain that the few spermatozoa frozen in the SFP had a better linearity ($p<0.05$) than the spermatozoa frozen at a high concentration. The linearity found in the SFP was similar to that reported in previous studies (Eriksson *et al.*, 2001; Eriksson, Petersson and Rodriguez-Martinez, 2002). Moreover, linearity was the variable that presented the highest variation among boars when the semen was frozen in MS or MFP, but not when it was frozen in the control package (i.e. the SFP).

A relevant boar effect on post-thaw sperm viability variables, such as *in vitro* fertilising capacity, embryo survival and fertility *in vivo*, has been repeatedly reported (Larsson and Einarsson, 1976; Larsson and Ersmar, 1980; Johnson *et al.*, 1981; Almlid, Stave and Johnson, 1987; Almlid and Johnson, 1988; Berger and Parker, 1989; Martinez *et al.*, 1993; Eriksson and Rodriguez-Martinez, 2000a; 2000b; Eriksson, Petersson and Rodriguez-Martinez, 2002). This variation in post-thaw semen quality between males can be related to different semen handling procedures and methods of freezing and thawing but may also have a genetic background (Thurston *et al.*, 2002). Therefore, the existence of good and bad freezers has to be always considered until a selection procedure for semen freezability is carried out, as has been the case with AI bull sires. The boars used for these experiments were not preselected for freezing capability and clearly responded differently to the freezing method used, as determined *in vitro*. These differences were particularly significant in the high concentration protocol. Since sperm membranes are very susceptible to cryo-injury (Watson, 2000) their evaluation is an appropriate indicator of the success of freezing-thawing. Membrane integrity can be assessed using supravital fluorescent stains such as the combination of deoxyribonucleic acid (DNA)-binding fluorophores and SYBR-14/PI. Used in combination with flow cytometry, these allow the assessment of thousands of cells within a short interval and the methodology appears invaluable. The major drawback, however, is the high cost of the flow cytometer. Other techniques such as hypo-osmotic tests, which analyse the ability of the plasma membrane to respond to media with other osmolarity than the spermatozoa, discriminate between dead and living cells (for a review, see Rodriguez Martinez and Larsson, 1998). The sHOST used in the present study often showed statistically comparable results to the SYBR/PI assay for the PMI of the semen frozen in MS or MFP, indicating that either method can be used for this purpose, although the sHOST is subjective and only hundred/s of spermatozoa are counted, in contrast to the 10,000 accounted for in the flow cytometer. The advantage of the sHOST is that the procedure is cheaper and could be easily performed at any laboratory equipped for basic sperm analysis.

In general these *in vitro* results are comparable to those obtained with semen frozen in the SFP and tested for fertility either *in vitro* (Eriksson *et al.*, 2001) or *in vivo* (Eriksson, Petersson and Rodriguez-Martinez, 2002). A major point to emphasise is that this method of freezing highly concentrated boar spermatozoa, particularly in MFP, provides a suitable sperm number of viable spermatozoa that can be deposited in the female at a single time, in a low volume, close to the oviduct, if intra-uterine deep insemination is practiced. Obviously, there is a need to check the fertility of these small volumes of highly concentrated sperm doses.

We recently used deep intra-uterine insemination catheters (Martinez *et al.*, 2002) to test the fertility of boar semen frozen by this method. Inseminations were performed in weaned sows, once during a spontaneous oestrus, to deposit the semen close to the expected spontaneous ovulation (monitored by transrectal ultrasonography). Semen doses (MS or MFP) were thawed and inseminated without any re-extension but since the volume of the dose did not fill the channel of the catheter, 2 mL of BTS extender were used to flush the catheter clean and ensure that the entire dose was deposited deep inside the uterus. Preliminary results collected thus far show that normal pregnancies could be obtained in this way (seven pregnancies, four with MFP and three with MS, resulted; with an average of 9.5 and three foetuses present, respectively, when sows were slaughtered at 35–55 days of gestation) (Saravia *et al.*, unpublished data). Although these are only preliminary data, they demonstrate that the FT spermatozoa reached the sperm reservoir at the UTJ despite the low volume of unextended FT semen.

These preliminary results are comparable to results of another study using FT semen and deep intra-uterine insemination with small (5 mL)-volume doses (Roca *et al.*, 2003). This group achieved a 72.5% pregnancy rate and 9.25 piglets per litter born on average. However, they used two MSs, and rediluted a volume of 5 mL of extender by an additional 2 mL of extender to flush the catheter, plus two AIs per oestrus (Roca *et al.*, 2003). Boar spermatozoa traverse the uterus and reach the sperm reservoirs aided by myometrial contractions, mainly elicited by the distension of the uterine lumen. This is a rational explanation for the requirement of 80–100 mL volume of current cervical AI doses (Baker, Dziuk and Norton, 1968). How the present low volumes can be transported is as yet unknown, but the stimulation of the myometrium could simply be elicited by the insertion of the long, deep AI catheter. Furthermore, the frequently seen reflux of semen inseminated when cervical AI of large volumes is performed (attaining up to 35% of the volume, with the subsequent sperm losses it implies (Viring and Einarsson, 1981) is not present with these intra-uterine AIs, an advantage that should not be disregarded. Further research is needed to explain the degree of intra-uterine sperm transport using unilateral deep inseminations.

Semen frozen in FlatPacks was used in a field fertility study (Eriksson, Petersson and Rodriguez-Martinez, 2002), yielding satisfactory results with two inseminations per oestrus. In another field fertility study (Roca *et al.*, 2003), using deep intra-uterine insemination with FT semen, results similar to results achieved with the controls (fresh semen) were only achieved when ovulation was hormonally induced in the sows. Further research is, therefore, required to determine the most appropriate moment of insemination in relation to spontaneous ovulation, as well as whether more than one AI is required to reach optimal fertilisation. The ultimate goal is to use the present (or a better) freezing method and inseminate a single, undiluted AI dose into the uterus once per spontaneous oestrus. Such practice requires the use of frequent transrectal ultrasonography to establish the interval from the onset of oestrus to ovulation and confirm the occurrence of the ovulation. Such strategy is still far from being put into commercial practice.

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Table 1: Post-thaw sperm motility patterns obtained by CASA analysis and plasma membrane intactness (PMI) monitored by SYBR-14/PI and sHOST, of boar semen frozen in three different packages (MS: 0.5 mL plastic straw; MFP: Multiple FlatPack; SFP: Single Flat-Pack, control; means ± SEM).

[a-c]: Values with different superscripts in the same column differ significantly.
[A-B]: Values for PMI with different subscripts in the same row differ significantly.
CASA: Computer-Assisted Sperm Analysis, sHOST: short Hypo Osmotic Swelling Test.
LIN: linearly motile spermatozoa, VSL: straight linear velocity, VAP: average path velocity, VCL: curvilinear velocity.

Table 2: Post-thaw sperm motility parameters (CASA) of the semen frozen from individual boars in medium straws (MS, means ± SEM).

Boar	Replicate (n)	Motility (%)	LIN (%)	VSL (µm/s)	VAP (µm/s)	VCL (µm/s)
343	6	51.1±4.0^{a}	10.5±1.3^{ab}	75.9±1.4^{a}	80.2±1.4^{a}	118.8±1.4^{a}
407	7	39.9±4.7^{ab}	7.0±1.0^{a}	71.1±4.4^{a}	75.6±4.5^{a}	122.7±5.6^{a}
415	7	29.8±5.0^{b}	14.1±0.8^{b}	71.0±2.2^{a}	74.7±2.4^{a}	119.3±5.9^{a}
1303	7	29.0±5.1^{b}	10.0±1.2^{ab}	69.2±3.3^{a}	72.8±3.6^{a}	114.4±6.5^{a}
1352	6	34.1±5.8^{ab}	28.7±3.0^{c}	77.5±2.5^{a}	81.0±2.5^{a}	116.7±2.2^{a}

$^{a-c}$: Values with different superscripts in the same column differ significantly.
CASA: Computer-Assisted Sperm Analysis
LIN: linearly motile spermatozoa, VSL: straight linear velocity, VAP: average path velocity, VCL: curvilinear velocity.

Table 3: Post-thaw sperm motility parameters (CASA) of the semen frozen from individual boars in a Multiple Dose Flat-Pack (MFP, means ± SEM).

Boar	Replicate (n)	Motility (%)	LIN (%)	VSL (µm/s)	VAP (µm/s)	VCL (µm/s)
343	5	41.6±6.0^{a}	17.1±2.4^{a}	69.3±5.0^{a}	73.4±5.3^{a}	114.8±6.9^{a}
407	5	49.3±3.5^{a}	13.5±5.6^{a}	76.2±4.6^{a}	80.2±4.9^{a}	128.5±5.7^{a}
415	5	45.5±7.1^{a}	14.0±1.4^{a}	70.4±3.4^{a}	74.2±3.6^{a}	117.0±4.8^{a}
1303	5	31.1±7.5^{a}	14.5±1.3^{a}	68.5±6.2^{a}	72.3±6.7^{a}	114.3±8.6^{a}
1352	3	41.7±4.1^{a}	36.5±6.3^{b}	73.7±5.5^{a}	76.9±5.5^{a}	114.5±5.6^{a}

$^{a-b}$: Values with different superscripts in the same column differ significantly.
CASA: Computer-Assisted Sperm Analysis
LIN: linearly motile spermatozoa, VSL: straight linear velocity, VAP: average path velocity, VCL: curvilinear velocity.

Table 4: Post-thaw sperm motility parameters (CASA) of the semen frozen from individual boars in a Single Dose Flat-Pack (SFP, means ± SEM).

Boar	Replicate (n)	Motility (%)	LIN (%)	VSL (µm/s)	VAP (µm/s)	VCL (µm/s)
343	2	33.7±29.5[a]	29.8±8.3[a]	67.3±8.2[a]	70.9±8.7[a]	103.0±13.1[a]
407	2	54.6±7.7[a]	27.8±5.4[a]	78.3±0.2[a]	82.4±0.1[a]	123.3±7.6[a]
415	2	46.4±14.2[a]	32.1±14.9[a]	68.8±3.6[a]	72.2±4.0[a]	112.0±12.7[a]
1303	3	41.3±6.7[a]	22.9±1.3[a]	64.5±0.1[a]	68.0±0.3[a]	104.3±1.9[a]
1352	2	54.9±19.4[a]	49.6±8.1[a]	76.0±2.9[a]	78.9±3.0[a]	104.7±0.0[a]

[a]: Values within column did not differ significantly.
CASA: Computer-Assisted Sperm Analysis.
LIN: linearly motile spermatozoa, VSL: straight linear velocity, VAP: average path velocity, VCL: curvilinear velocity.

Table 5: Post-thaw sperm plasma membrane intactness (PMI) assessed with SYBR-14/PI and sHOST, respectively, for the different boars and package systems used (means ± SEM).

Boar	Medium-straws (MS)		Multiple FlatPack (MFP)		Single FlatPack (SFP)	
	SYBR-14/PI (%)	sHOST (%)	SYBR-14/PI (%)	sHOST (%)	SYBR-14/PI (%)	sHOST (%)
343	49.6±4.7[a]$_A$	48.7±3.7[a]$_A$	48.8±3.5[a]$_A$	44.1±2.5[a]$_A$	44.9±9.0[a]$_A$	45.8±6.3[a]$_A$
407	41.5±6.0[a]$_A$	47.8±2.2[a]$_A$	51.1±2.4[a]$_A$	50.0±3.7[a]$_A$	59.2±6.0[a]$_A$	39.8±2.3[a]$_A$
415	30.8±4.6[a]$_A$	37.9±4.7[a]$_A$	42.7±5.6[a]$_A$	44.1±4.2[a]$_A$	42.9±10.1[a]$_A$	46.8±9.3[a]$_A$
1303	32.5±5.2[a]$_A$	45.4±5.6[a]$_A$	46.8±3.6[a]$_A$	37.1±4.9[a]$_A$	56.5±1.5[a]$_A$	42.7±2.4[a]$_B$
1352	38.2±7.4[a]$_A$	39.6±4.8[a]$_A$	52.4±4.9[a]$_A$	49.8±4.3[a]$_A$	63.6±2.4[a]$_A$	42.8±1.3[a]$_B$

[a]: Values within column did not differ significantly.
$_{A\text{-}B}$: Values for PMI between methods within packages. Values with different subscripts in the same row differ significantly.
sHOST: short Hypo-Osmotic Swelling Test.

Future prospects

A major goal for widespread application of frozen boar semen in commercial pig AI would be to use a single, small AI dose, inseminated once per oestrus, close to spontaneous ovulation. Obviously several points need to be further researched and developed before this goal can be reached. Some of these points are listed below:

1. The survivability of the spermatozoa after freezing should be increased to yield better results than obtained in this study.

2. The minimal numbers of spermatozoa needed for maximal fertility should be determined, taking into consideration the need to decrease the numbers of spermatozoa in the AI doses.

3. The best location for deep AI needs to be determined, in terms of delivering a minimal amount of spermatozoa able to colonise both sperm reservoirs.

4. To increase sperm viability and other parameters, and augment the viscosity of the inseminate in order to promote factors such as the sperm transport through the uterine horns, the placing of additives before freezing should be considered.

5. There should be more specific selection of boars for semen freezability.

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