Strategies to improve the fertility of frozen-thawed boar semen for artificial insemination

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Although cryopreservation of boar semen for artificial insemination (AI) was developed 35 years ago, cryopreservation conditions and AI strategies are still considered sub-optimal. AI with excessive numbers of frozen-thawed sperm (5-6 $x10^9$ cells), still does not achieve fertility levels similar to AI using liquid semen because of reduced sperm survival. Frozen-thawed (FT) spermatozoa have therefore not been the preferred option for commercial breeding programmes. However, substantial progress has been made regarding boar sperm cryopreservation. Adjustment of cooling and re-warming rates to biophysical properties of boar spermatozoa, new sperm package systems and the achievement of accurately consistent freezing of large numbers of samples using programmable freezers have contributed to post-thaw survival rates above 50%, a threshold similar to that used for bull AI-semen. Moreover, these post-thaw sperm survival rates are consistent within a large population of boars selected for sperm freezability potential, as occurs with AI-bull sires. When such post-thaw boar semen is deposited intra-utero, acceptable fertility (in terms of farrowing rates and litter size) is obtained. Currently, the most effective application of FT-semen for AI is achieved using deep uterine-AI (DUI) which allows placement of a minimal semen dose (in volume 0.5 to 10 mL and sperm number 0.5 to 1 $x10^9$ total spermatozoa) into the anterior 1/3 of one uterine horn, with levels of fertility close to AI with liquid semen. However, owing to their shorter life span, FT-boar spermatozoa require an AI-to-ovulation interval not longer than 4-6 h, making peri-ovulatory AI a pre-requisite to obtain the highest possible fertility. Spontaneous ovulation most often occurs when two-thirds of oestrus has passed. Estimation of the duration of oestrus, taking into account the weaning-to-oestrus interval, is helpful when establishing appropriate AI-schedules. However, as the length of oestrus varies within and between farms, different AI strategies should be established *a priori*. The

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development of bio-sensors for spontaneous ovulation will widen the use of AI with frozen-thawed frozen semen.

Introduction

Artificial insemination (AI) is the most world-widely applied breeding tool in pig farms (Weitze, 2000). It is well established that AI with fresh or cooled boar semen is a simple, successful and economical procedure, able to achieve high farrowing rates (> 80%) and litter sizes (> 10 piglets born per litter) comparable to those obtained after natural mating. However, to maximise the advantages of AI, it is important to include cryopreserved spermatozoa, as this is the only practical alternative for global transport of sperm and long-lasting preservation of genetic material. Such strategies could restore male populations lost by removal of infected sires or the creation, or improvement, of nucleus breeding herds. Freezing-thawing (FT) of boar semen for cervical AI, a technique initiated 35 years ago, can be conducted to cover these expectations, but it is not practical and inexpensive enough for full application in commercial breeding.

Following the birth of piglets conceived by cervical AI using FT-spermatozoa at the beginning of the 1970's (Crabo and Einarsson, 1971; Graham *et al.*, 1971; Pursel and Johnson, 1971), several trials were conducted during the same decade, some of them achieving high fertility levels (reviewed by Johnson, 1985). In spite of these promising results accomplished under controlled experimental conditions, there was a little use of FT-semen in commercial farms. The variability in freezing success between boars, the high sperm numbers required per dose and the low fertility levels achieved compared to those obtained with liquid semen, were among the major limiting factors (Johnson, 1985; Reed, 1985). From a statistical viewpoint, less than 1% of total AIs were, at that time, performed worldwide with FT-semen (Reed, 1985). During the following years, a wealth of new information about different aspects of frozen storage of boar semen has accumulated, and many attempts have been made to enhance fertility with AI using FT-semen (for more details, see reviews of Bwanga, 1991; Woelders, 1997; Johnson *et al.*, 2000). Despite these efforts, at the beginning of the new millennium the percentage of sows inseminated with FT-semen was, surprisingly, still less than 1% (Wagner and Thibier, 2000) and the limiting factors remained those listed above (Johnson et al., 2000). This apparent lack of progress could relate to the continuous use of empirical approaches when cryopreserving boar spermatozoa (see review of Watson, 1995) as well as the absence of research-oriented technological development aiming to improve the transport of FT-spermatozoa through the genital tract of sows (see review of Martínez *et al.*, 2001).

At present, there is an extensive requirement for establishing sperm cryobanks for both export and conservation of superior genetic lines, and for reserving supplies in response to disease-related disasters. In response, our laboratory has created four cryobanks, with more that 20,000 AI doses, during the past 3 years. In addition, FT boar semen is a pre-requisite for the best use of some of emergent reproductive biotechnologies (for details see Sommer *et al.*, 2002; Gerrits *et al.*, 2005).

The present review centres on the improvement of sperm cryosurvival using standardised methods for freezing and the development of efficient AI strategies; these are important pre-requisites to achieve fertility levels that would increase the use of FT-boar spermatozoa in commercial pig breeding.

Improvement of sperm cryosurvival

Increasing the survival of spermatozoa subjected to freezing and thawing will require optimisation of methods for cryopreservation as well as minimising existing intra- and inter-animal variation

in sperm freezability present in a boar population unselected for this purpose. The obvious objective is to retrieve maximal numbers of spermatozoa with preserved potential fertilising capacity post-thaw after using a single freezing method.

Optimisation of sperm cryopreservation methodology

Poor sperm survival after thawing has traditionally represented the major limitation to the successful application of cryopreserved boar semen in commercial AI programmes, since it largely explained the low fertility results achieved. Thus, the primary objective of any strategy to enhance the fertility after AI with FT-spermatozoa must be to improve cryopreservation and maximise sperm cryosurvival. During the past few years, specific cryobiological studies focussing on the adaptation of cooling and rewarming rates to biophysical properties of boar spermatozoa, changes of sperm package systems as well as the accurate and consistent freezing of large numbers of samples led to the optimisation of cryopreservation protocols (see review of Holt, 2000a).

Cooling and re-warming rates are the most critical variables influencing sperm cryosurvival and thus a pre-requisite for an optimal sperm cryopreservation protocol. Furthermore, spermatozoa from different species respond differently to cooling and re-warming, requiring specific knowledge. Determination of the rates of water permeability using differential scanning calorimetry has confirmed, for instance, that the optimal cooling rate for freezing boar spermatozoa is 30 °C/min when a standard concentration of glycerol is used (Devireddy *et al.*, 2004). Unfortunately, similar non-empirical studies are yet lacking considering re-warming and its effects on sperm cryosurvival. The general consideration is that the response of spermatozoa to rewarming depends on the cooling rate used. With a cooling rate of 30 °C/min, a rapid rewarming of 1,200 °C/min- seems most suitable (Fiser *et al.*, 1993).

New packaging systems have also improved sperm survival post-thaw. Historically, boar spermatozoa were routinely packaged in maxi (5-6 mL) plastic tubes or straws containing the most appropriate number of sperm as one AI-dose (5-6 $x10^9$ cells). However, the use of macrotubes or maxi-straws involves certain cryobiological problems as the cooling and thawing rates may differ across the straw. Weitze *et al.* (1987) found that extended boar spermatozoa packaged in maxi-straws froze 3.75 times faster at the periphery than in the centre, resulting in a significant lower post-thaw sperm survival compared to sperm packaged in medium-straws (0.5 mL). Later, optimal freezing and thawing were obtained by packing boar spermatozoa into French medium-straws (Fiser and Fairfull, 1990) and now most boar semen is packed for freezing into 0.5 mL straws. These are not only better from a cryobiological viewpoint, they are also more practical, given the relative high number of spermatozoa (2 $x10^9$ cells/mL) that can be packaged per straw (Saravia *et al.*, 2005), the good post-thaw sperm survival (Carvajal *et al.*, 2004) and the acceptable *in vivo* fertility levels achieved (Bussiere *et al.*, 2000). An alternative package system containing either 5 $x10^9$ cells in 5 mL (FlatPack®) has also been developed. The promising results of post-thaw sperm survival (Eriksson and Rodriguez-Martínez, 2000), *in vitro* penetrability (Eriksson *et al.*, 2001) and *in vivo* fertility (Eriksson *et al.*, 2002), together with their possibilities to package a large number of spermatozoa, indicate, from a practical and commercial viewpoint, that FlatPack® is a very suitable method for packaging commercial single doses of frozen boar spermatozoa for cervical AI. Recently, a modification of the procedure (holding 1.4 $x10^9$ cells in 0.7 mL, MiniFlatPack) has been devised and tested for deep uterine-AI (DUI; Saravia *et al.*, 2005; Wongtawan *et al.*, 2005).

From a practical viewpoint, the replacement of the traditional freezing in a static gradient of nitrogen-cooled air (LN_2 vapours) by controlled-rate freezers (also using N_2-cooled air but whose rate of cooling is controlled by a computer) has been an important advance for commercial

production of frozen AI-doses. Although the overall effectiveness of some programmable freezing devices can be questionable (for more details see Holt, 2000b; Thurston *et al.*, 2003), those with a precise temperature control system capable of measuring both internal and external temperatures of straws can most effectively control the rate of cooling (Medrano *et al.*, 2002; Kumar *et al.*, 2003). Thus, effective controlled-rate freezers optimise freezing, providing accurate cooling rates for a large batch of samples and minimising sample-to-sample variability. Together, these developments allow production of frozen doses with an improved and consistent post-thaw sperm survival.

Selection of AI boars for good freezability

Optimal sperm cryosurvival depends not only on cryopreservation methodologies, but also on the individual variation each boar shows regarding susceptibility of the ejaculated spermatozoa to sustain cryoinjury. An essential difference between liquid and FT-semen is the different response of the ejaculated spermatozoa when cooled. While spermatozoa of practically all boars respond similarly well to liquid storage, where the cooling is marginal, the response varies greatly when more intense cooling is used. So, irrespective of breed or genetic line, important boar differences on sperm freezability have been well established (Thurston *et al.*, 2001). When these researchers objectively classified boars as "good", "moderate" or "bad" freezers according to their post-thaw sperm quality, a large percentage (32.6%, 42 boars from of 129 tested) were identified as bad freezers.

In a recent experience in our laboratory, during the creation of a sperm cryobank for commercial purposes, a total of 255 ejaculates from 85 boars (3 ejaculates per boar) were frozen. To establish their post-thaw sperm quality, 2 straws of each ejaculate were thawed to undergo computer-assisted assessment of motility (CASA) and microscopic evaluation of plasma membrane and acrosome integrity after staining with a triple fluorescent staining (R123 + PI + FITC-PNA). Following the same criteria as Thurston *et al.* (2002), the ejaculates and boars were classified as "good", "moderate" or "bad", with 44.3% being found as "good" and 27.8% as "bad" ejaculates, respectively (for details see Fig. 1). These data clearly demonstrated important differences in sperm freezability among ejaculates, corroborating earlier results (Thurston *et al.*, 2002). However, the most relevant finding of our study was that whereas all ejaculates from 29 boars were classified as "good", all ejaculates from 18 boars were classified as "bad" (Fig. 1a), showing that the spermatozoa from some boars had consistent difficulties to overcome the freezing and thawing cycle.

From a commercial viewpoint, eliminating these "bad" freezers as providers of ejaculates for cryopreservation and AI is the most practical way to improve the fertility levels of FT-spermatozoa. Any boar selected as a sire in an AI programme using FT-semen should, in addition to his genetic merits, also be selected according to the freezability potential of his semen. However, there is currently no single predictive test to determine the freezability potential of an ejaculate, because post-thaw sperm survival has been traditionally based on unpredictable pre-freeze values (Roca *et al.*, 2000). The identification of different molecular markers linked to genes controlling sperm freezability in a genomic analysis of 22 Large White boars, indicated the individual variability in freezability among boars has a genetic basis (Thurston *et al.*, 2002). This important finding is the first step to develop a simple and feasible molecular or genetic test capable of marker-identification of "good" or "bad" freezer boars from a blood sample. Unfortunately, such a test is not yet commercially available.

An alternative strategy, effective to identify "bad" freezers, could be to implement a suitable freezability test. Based on our experience, cryopreserving 5-10 mL of the sperm-rich fraction of an ejaculate and assessing the post-thaw sperm quality (evaluation of objective

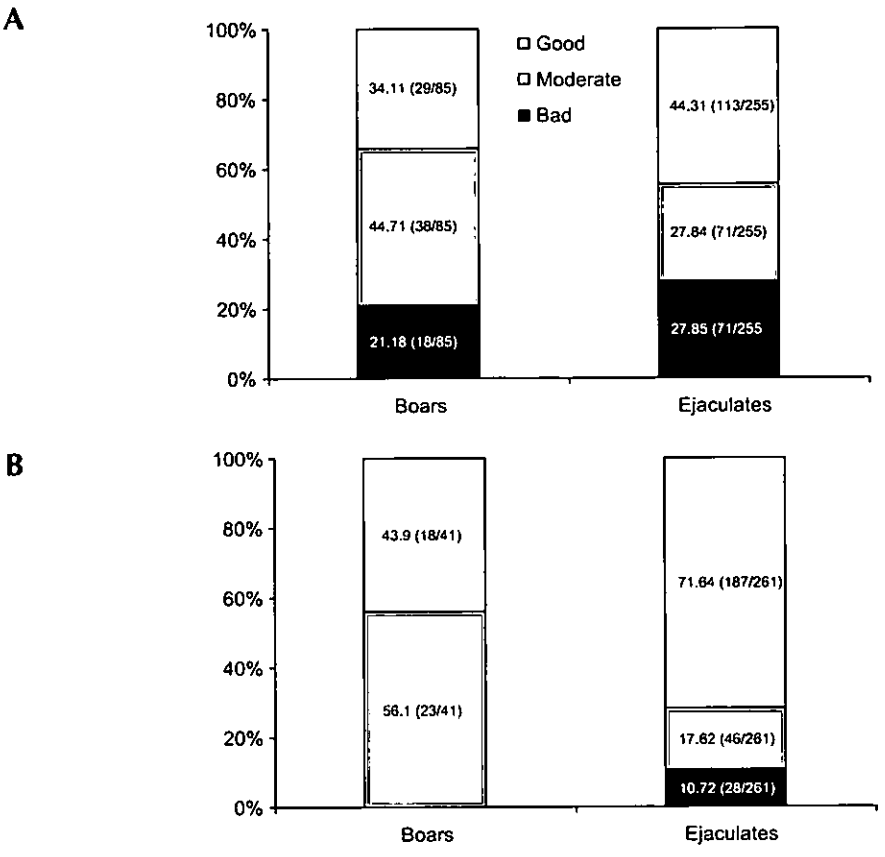

Fig. 1 Distribution of boar and ejaculates of two different sperm cryobanks founded with frozen ejaculates collected from unselected (a) or selected (b) boars according to sperm freezability. Boar and ejaculates were classified according their post-thaw sperm quality (motility and viability) assessed from two thawed Beltsville Thawing Solution (BTS)-resuspended straws per ejaculate held 30 min in a water bath at 37°C. The ejaculate classification criteria were: "good" (≥50 % of spermatozoa are motile and viable after thawing), moderate (36-49%) or "bad" (≥35%). Boar classification criteria were: "good" (all ejaculates had good post-thaw sperm quality), "moderate" (ejaculates with different post-thaw sperm quality) or "bad" (all ejaculates had bad post-thaw sperm quality). Viable sperm were those that after incubation with three fluorescent probes (deoxyribonucleic acid specific fluorochrome propidium iodide, the mitochondria specific fluorochrome rhodamine-123 and the acrosome specific fluorochrome fluorescein isothiocyanate-labeled peanut agglutinin), showed green stain only on the midpiece under fluorescent microscopy (Roca et al., unpublished).

motility and viability of two thawed straws using the above procedures) could be enough to identify "bad" freezers. So, before the creation of a second sperm cryobank, we checked the freezability potential of all boar candidates using such a test. Out of 51 boar candidates, 10 (19.6%) were classified as "bad" freezers and therefore eliminated as ejaculate providers. Finally, the cryobank was founded with 261 ejaculates from 41 selected boars (5 ejaculates minimum per boar). According to post-thaw sperm quality, 28 ejaculates (10.7%) were classified as "bad". Regarding boars, whereas in 18 boars (43.9%) all their ejaculates were classified as "good", in the remaining 23 boars only isolated ejaculates were classified as "bad" (Fig. 1b).

These data showed clearly that a simple freezability test may be suitable for both minimising ejaculate variability among boars and enhancing the overall post-thaw sperm quality of frozen ejaculates.

Differences in sperm cryosurvival rates can also be found within the same boar (i.e. among ejaculates). This intra-boar variability could be attributed to boar health status, reproductive management, frequency of ejaculation or differences in ejaculate processing before or during the cryopreservation process. Thus good boar management and careful processing of the ejaculate would help to minimise intra-boar ejaculate variability on post-thaw sperm survival.

More efficient insemination strategies

The lower fertility of FT-semen compared to liquid semen is traditionally related to the low proportion of functional spermatozoa that survive cryopreservation. However, with the recent improvements mentioned above, survival levels of thawed, cryopreserved boar spermatozoa are now quite satisfactory. Together with the possibility to select "good" freezers as semen providers, it is now possible to achieve reasonably high and consistent post-thaw sperm survival rates; over 50% of viable and motile spermatozoa in a large population of boar sires. It is noteworthy that such sperm survival rates are similar to those obtained in AI-bull sires, which have undergone selection pressure for freezability over decades in commercial dairy cattle breeding programmes (Vishwanath and Shannon, 2000). Therefore, the answer to the lower fertility might be more in relation to the site of sperm deposition in the female, as was the case in the bovine, where intrauterine AI contributed dramatically to the fertility rates achieved with FT-semen.

The profitable application of FT-semen on commercial pig AI programmes requires more efficient insemination strategies than at present, the final objective being to obtain high fertility levels using the lowest possible number of FT-spermatozoa. To maximise the efficiency of FT-semen, in terms of maximal fertility and prolificacy, critical factors such as proper placement of the semen in the sow´s genital tract and a proper timing of insemination ought to be considered.

Insemination procedure: proper semen deposition

The wealth of research performed with the aim of improving boar sperm cryopreservation protocols has, unfortunately, not been matched by the development of more efficient insemination techniques for FT-semen. The current insemination procedure includes the deposition of a large number of spermatozoa (5-6 $x10^9$ cells) into the cervical canal, mimicking natural mating and using the same equipment as for liquid semen. Overall, the resulting fertility levels are usually low, indicating the poor efficiency of this insemination procedure, rather than the presumable lower quality of the semen. Therefore, from a practical viewpoint, other AI-procedures are to be tested to overcome the two major hurdles to the routine application of FT-semen in commercial farms, namely the high numbers of spermatozoa required per dose and the low fertility levels achieved.

The low efficiency of FT-semen after cervical deposition is not only a matter of concern for the pig; it has also been observed in other species where alternative insemination procedures are available, such as ovine. Direct intrauterine sperm deposition by laparoscopy is usual as an AI procedure in ewes, and fertility of FT-semen deposited in this way is higher than that obtained after cervical deposition (King et al., 2004). Moreover, fertility differences cannot be compensated by inseminating a larger number of thawed spermatozoa cervically. The low fertility obtained following cervical AI relates to the difficulty of the FT-spermatozoa to pass

through the cervical barrier, progress into the uterus and reach the oviductal isthmus (see review by Salamon and Maxwell, 1995). A similar situation occurs in pigs, where in addition to the cervical folds, the extremely long uterine horns impair the ability of FT-spermatozoa to maintain their fertilising capacity when reaching the oviduct. Consequently, although as many as 5-6 $x10^9$ spermatozoa are cervically deposited, far fewer reach the oviductal reservoir. Certainly, the number of accessory spermatozoa in the zona pellucida of embryos can be as much as 10-fold lower in FT-spermatozoa compared to liquid preserved sperm, even when the AI-dose contained twice as many sperm (Waberski *et al.*, 1994; Wongtawan *et al.*, 2005).

With the aim of reducing the number of spermatozoa per AI-dose without impairing fertility, two different techniques and devices have been developed for trans-cervical semen deposition into different regions of the uterus, namely into the uterine body (Post-Cervical-AI, PC-AI) or deep into one uterine horn (Deep Uterine Insemination, DUI). Both have achieved high fertility levels with liquid semen using 1 x 10^9 (PC-AI, Watson and Behan, 2002) or only 150 x 10^6 (DUI, Martínez *et al.*, 2002) total spermatozoa per dose. In relation to their potential effectiveness using FT-semen, whereas at present no fertility data are available using the PC-AI procedure, promising results have been achieved using the DUI procedure.

The DUI technique involves the introduction of a flexible device through a conventional commercial AI spirette, previously inserted in the cervix folds as guide, into one uterine horn (for more details see Martínez *et al.*, 2001). The DUI device is able to progress along the uterine lumen facilitating the deposition of the semen at, at least, the proximal 1/3 of the horn, nearer to the utero-tubal junction, where the sperm reservoir is present in the pig (Martínez *et al.*, 2002). A recent study monitored the effectiveness of using DUI to routinely inseminate FT-semen in commercial farms (Roca *et al.*, 2003). Forty weaned sows were DUI twice at 30 and 42 h after onset of oestrus using low numbers of sperm (1 x 10^9 spermatozoa) suspended in a small volume (7ml) of fluid. The farrowing rate averaged 70% with a mean litter size of 9.25 piglets born. These fertility levels were comparable, or better, than some reported following conventional cervical AI with 5-fold more thawed spermatozoa (Hofmo and Grevle, 2000; Eriksson *et al.*, 2002). Moreover, the fertility of FT-spermatozoa after DUI can be considered as "acceptable" compared to that obtained in the same farm after conventional AI using fresh semen (84.2 % farrowing rate and 9.88 piglets born per litter). Thus, it seems clear that the DUI procedure has the potential to achieve high fertility levels using a total number of FT-spermatozoa as low as 1 x 10^9. This means a 5 to 6-fold decrease compared with current doses of FT-semen and a 2-to-3-fold decrease compared with current commercial doses of liquid semen. Thus, DUI could increase the effectiveness of FT-spermatozoa by reducing the number of thawed spermatozoa required in each dose. Therefore, DUI with FT-semen is currently used by some pig genetic companies in Spain. Although DUI performance is currently satisfactory, research is in progress in order to standardise the DUI procedure for routine application of FT-semen under commercial conditions, including the determination of the minimal number of spermatozoa needed to obtain maximal fertility levels. As an example, farrowing rates close to 50% with 9 piglets born per litter have been reported, using as few as 250 $x10^6$ frozen-thawed spermatozoa per dose (Bathgate *et al.*, 2005). The use of contemporary small packs to freeze semen in conjunction with DUI makes the deposition of small volumes of thawed, non re-extended semen deep intra-uterine possible. This approach avoids the deleterious effects of semen re-suspension post-thaw and of sperm losses associated with the reflux of semen after conventional AI, while retaining normal sperm transport (Wongtawan *et al.*, 2005). How sperm transport is elicited with these small volumes (0.5 to 7 mL of semen) deposited into one uterine horn appears more related to the inseminating device than to the insemination volume. The introduction of the long AI-catheter would trigger the spontaneous contractility of the female genital tract, thus contributing to the first phase of sperm transport (Rodriguez-Martinez *et al.*, 2005).

The above results clearly show that DUI is an efficient procedure for the routine application of FT-semen in commercial pig AI programmes, providing good fertility levels with low sperm numbers. However, concern has been raised about potential damage that the DUI device may cause to the endometrium, compromising the subsequent fertility of the sow. To investigate this, we compared the reproductive performance of conventional AIs carried out with liquid semen before and after DUI-insemination on 159 weaned sows (A. Bolarín *et al.*, unpublished). No differences were found ($P > 0.05$) in pregnancy rates (87.4% *versus* 86.2%), farrowing rates (84.9% *versus* 84.3%) or litter size (11.08 ± 0.17 *versus* 11.50 ± 0.15 piglets born). Moreover, not-in-pig rates were also similar (2.9% *versus* 2.2%, $P > 0.05$). In addition, among those sows that were not pregnant after DUI, showing a regular or irregular return to oestrus, there were no significant differences ($P > 0.05$) in pregnancy losses post breeding before or after DUI (0.6% *versus* 2.5%). Neither did more intensive experimental studies of the use of DUI record uterine damage when using the procedure (Wongtawan *et al.*, 2005). Thus, it seems evident that the correct insertion of the DUI device into a uterine horn does not affect the subsequent fertility potential of the sows. Despite these reassuring data, it must be born in mind that DUI technology is still under development.

Timing of insemination

The fertility of FT-spermatozoa appears more influenced by the timing of AI relative to ovulation than by their post-thaw sperm quality. Thawed spermatozoa of all species studied have a limited life-span in the female genital tract, compared to fresh spermatozoa. Fertilisation of pig oocytes with FT spermatozoa has been successfully achieved only when inseminations were performed in a very short optimal period of 4-6 h before ovulation (Waberski *et al.*, 1994). Sub-optimum AI-times before or after ovulation lead to low conception rates and/or litter sizes (see Johnson *et al.*, 2000). Using DUI under experimental conditions, fertility of weaned sows inseminated with small doses of highly packed, frozen-thawed semen was related to the interval between DUI and spontaneous ovulation. The optimal interval was found to be between 8 and 4 hours before spontaneous ovulation (Wongtawan *et al.*, 2005). The reduced life span of the FT-spermatozoa was evident with a significantly lower fertility and replenishment of the sperm reservoir if the interval AI-to-ovulation was longer. Under commercial conditions, the reduced functional life span of thawed spermatozoa in the sow genital tract was again clearly demonstrated in a recent field trial (Roca *et al.*, 2002). Weaned, multiparous sows ($n = 80$) were DUI-inseminated at 30-32 and 40-42 h after detection of oestrus, with 1×10^9 thawed spermatozoa resuspended to 7 mL of BTS. A few minutes before each DUI, the ovaries were examined by trans-rectal ultrasonography and sows were allotted into 3 different categories: F sows: when follicles were visible during the two examinations, indicating pre-ovulatory DUI; O sows: when ovulation was visible during one examination, indicating peri-ovulatory DUI; and C sows: when corpora haemorragica were visible during the two examinations, indicating post-ovulatory DUI. The farrowing rate was significantly ($p < 0.01$) higher in O sows than in F or C sows (Table 1) and was similar ($P > 0.05$) to that achieved on the same farm using conventional AI with liquid semen (70/85, 82.3%). The data clearly demonstrate that good fertility can be achieved with FT-spermatozoa provided that AI is closely timed to ovulation.

As previously indicated, no comprehensive studies have been conducted to determine the number of FT-spermatozoa required per dose to achieve maximum fertility levels using the DUI procedure. It is clear that the number of spermatozoa per AI-dose influences fertility results (Flowers, 2001), especially when using FT-spermatozoa (Watson, 2000). In a recent field trial in which 228 weaned sows were DUI-inseminated with either 1×10^9 or 2×10^9 cells Bolarín *et al.* (2005), a 2-fold increase in sperm number improved farrowing rate (53.1% *versus* 70.0% respectively; $P < 0.05$). However, taking into account the ovarian status at the time of DUI, the improvement in farrowing rate was evident in F- (52.8% versus 73.2%) and C-sows (43.9% versus 60.6%), but not

in O-sows (81.1% versus 80.8%). These results clearly demonstrated that the interval between DUI and ovulation is a more important determinant of fertility than the number of sperm per dose. However, it is important to remember that the sperm numbers used might still be in excess for the achievement of maximal fertility. Further studies are still needed to elucidate this.

Table 1. Farrowing rates and litter size of weaned sows according to their ovarian status at the time of deep uterine insemination (DUI). Sows were inseminated twice with 1 x 10^9 frozen-thawed spermatozoa at 30-32 and 40-42 h after oestrous detection (adapted from Roca *et al.*, 2002).

Ovarian status at DUI	Sows n (%)	Farrowing n (%)	Litter size (mean ± SEM)
Pre-ovulatory (F-sows)	13 (16.3)	5 (38.5)^b	9.60 ± 1.08
Peri-ovulatory (O-sows)	59 (73.7)	48 (81.4)^a	9.33 ± 0.30
Post-ovulatory (C-sows)	8 (10)	2 (25)^b	7.00 ± 1.00
TOTAL	80	55 (68.7)	9.27 ± 0.28

^{a,b}Values with different superscript in the same column are significantly different ($P < 0.05$)

Ultrasonography used as an ovulation diagnostic tool in herds is feasible, and although it is not a useful predictor for spontaneous ovulation (Soede *et al.*, 1998), it is probably the best means available at present. In the absence of a better predictive tool, the possibilities to forecast the moment of ovulation are reduced to the assessment of the average duration of oestrus, which varies according to the interval from weaning to oestrus. Overall, spontaneous ovulation takes place when two-thirds of the standing oestrus period has elapsed (see review by Soede and Kemp, 1997). Using the recorded onset of oestrus-to-ovulation interval (checked by frequent transrectal ultrasonography, a time-consuming procedure) inseminations in weaned sows can thus be performed in the following oestrus at the expected ovulation time (Mburu *et al.*, 1995; Wongtawan *et al.*, 2005). Following this reasoning, we have achieved promising farrowing rates (69.2%, 152/217) and litter sizes (9.22 ± 0.16 piglets born per litter) in several trials in a large commercial farm (more that 3,000 breeding sows) with an insemination strategy that included two DUI-inseminations with a total of 1 x 10^9 FT-spermatozoa per dose carried out at 30-31 and 36-37 h after onset of oestrus in sows weaned four days before. The short interval between DUIs was established taking account of the short functional life span of FT-spermatozoa in the genital tract.

Unfortunately, the duration of oestrus is very variable and it is influenced by many factors including genetics and on-farm breeding management of sows (see review by Soede and Kemp, 1997). From a practical viewpoint, it is important to note that the duration of oestrus varies considerably between farms, which could explain the fertility differences recorded between farms when FT-semen from the same frozen batch was used (Eriksson *et al.*, 2002). We evaluated this possibility recently (Bolarín *et al.*, 2005). The same batch of FT-semen from a pool of ejaculates of several mature fertile boars was used to DUI-inseminate weaned sows in two different farms with similar reproductive management. A total of 179 sows (97 from farm A and 82 from farm B) were DUI-inseminated at 30-31 and 36-37 h with 1 x 10^9 spermatozoa per dose. A few minutes before DUI, the ovaries were checked by trans-rectal ultrasonography and the sows allocated to category F, O or C as previously described. The overall farrowing rate differed ($P < 0.01$) between farms (70.1% versus 51.2%, farms A and B, respectively). Distribution of sows according to ovarian status also differed ($P < 0.05$) between farms (17.5%, 72.2% and 10.3% were classified as F-, O- and C-sows in farm A, versus 40.2%, 20.3% and 30.5% in farm B). Nevertheless, farrowing rates and litter size within the same ultrasonographic ovarian status did not vary between farms ($P > 0.05$). In either farm, similar high farrowing rates (82.9% and 83.3% for A and B farm, respectively) and litter sizes (9.38 ± 0.26 and 9.65 ± 0.51 for A and B farm, respectively) were achieved in O sows. Consequently, these results suggest that fertility differences of FT-semen between farms were due, to a large extent, to different insemination-to-ovulation intervals. The low fertility levels in farm B

were clearly due to a suboptimal AI-strategy. In conclusion, it is necessary to emphasise that before FT-semen is to be used for breeding, proper AI strategies must be established for each farm. A correct knowledge of the overall duration of oestrus, taking into account the weaning-to-oestrus interval (WOI), would be of great help in the establishment of the most appropriate AI strategy. Therefore, farmers should carry out a careful onset of oestrus detection and establish its duration taking into account the WOI.

In addition to farm differences, WOI affects the duration of oestrus (Mburu *et al.*, 1995), which makes decisions on appropriate strategies to inseminate most of the sows within the short optimal period before ovulation more difficult. Moreover, seasonal influences are not to be disregarded. As an example, a recent experiment carried out in the southeast of Spain (Bolarín *et al.*, unpublished observations) showed that the above reproductive parameters can vary largely depending on season on the same farm. The occurrence of ovulation was monitored, by use of trans-rectal ultrasonography, on 122 sows (58 and 68 during winter and summer, respectively). Data presented in Table 2, clearly indicate that some sows showed longer WOI and oestrous duration during summer, which implies a more heterogeneous ovulation time. These seasonal differences could be explained by the adverse effect of high environmental temperature (Peña *et al.*, 1998). It is tempting to suggest that specific AI strategies should be designed for summer months in the warm regions to achieve consistent high fertility levels throughout the year when FT-semen is used. At present, to the best of our knowledge, no results are yet available. In particular, alternative AI schedules including a third insemination may be beneficial.

Table 2. Distribution of weaned sows according to weaning-to-oestrus and the onset of oestrus-to-ovulation intervals during winter (a) and summer (b) seasons within a single farm. Ovulation was checked by transrectal ultrasonography. (Bolarín *et al.*, unpublished observations)

(a) Onset of oestrus	Weaning to onset of oestrus (d)					
to ovulation (h)	3	4	5	6	7	Mean (%)
0 to <12		1	1	1		3 (5.2)
≤12 to <24		5	8	2		15 (25.9)
≤24 to <36	1	12	4	1		18 (31)
≤36 to <48	4	11	2			17 (29.3)
≤48 to <60	1	2	1			4 (6.9)
≤60 to <72	1					1 (1.7)
≤72 to <84						
≤84 to <96						
≥96						
Total (%)	7 (12.1)	31 (53.4)	16 (27.6)	4 (6.9)		58 (100)

(b) Onset of oestrus	Weaning to onset of oestrus (d)					
to ovulation (h)	3	4	5	6	7	Mean (%)
0 to <12						
≤12 to <24		5	1	1	1	8 (11.8)
≤24 to <36	1	9	2	3	2	17 (25)
≤36 to <48	2	10	2	2		16 (23.5)
≤48 to <60	7	6	2			15 (22.1)
≤60 to <72		3	2			5 (7.3)
≤72 to <84	3		2			5 (7.3)
≤84 to <96		1				1 (1.5)
≥96		1				1 (1.5)
Total (%)	13 (19.1)	35 (51.5)	11 (16.2)	6 (8.8)	3 (4.4)	68 (100)

Fixed insemination time

Unfortunately, inadequate or incorrect oestrous detection strategies are not unusual in farms, due to a lack of time and skilled labour. An alternative strategy is to use exogenous hormonal treatment (eCG followed by either hCG or GnRH analogues) to improve estimation of the time of ovulation in sows and thus improve the results of AI with FT-boar semen, particularly during the summer months or in those farms with inappropriate breeding management. A common protocol of synchronisation of ovulation time in weaned sows consists in the exogenous application of eCG 24 h after weaning, to promote ovarian follicular development, followed by hCG 60-72 h later, to induce ovulation. Overall, ovulation should occur 42-44 h after hCG application. The correct synchronisation of ovulation has an additional advantage since only a single AI at a fixed time is required, reducing expense in semen costs without fertility losses. Using the above synchronisation protocol, we have achieved high fertility levels (77.5% of farrowing rate with 9.31 ± 0.41 piglets born per litter) in 49 weaned sows DUI-inseminated with 1×10^9 FT-spermatozoa per dose at 40 h after hCG application (Roca *et al.*, 2003). Despite these promising results, fixed-time AI should be carefully evaluated and its implementation balanced against cost, benefits, animal welfare issues and eventual pharmaceutical residues.

Concluding remarks

The efficient inclusion of FT-boar semen on routine AI programmes in commercial farms is now feasible. This is possible because both current cryopreservation protocols and accurate selection of "good" freezers enable acceptable cryosurvival rates to be achieved. In addition, high fertility levels can be achieved with a reduced number of FT-spermatozoa following DUI. However, to obtain consistent high fertility levels, the semen must be inseminated close to ovulation. Fair estimation, but not accurate prediction, of spontaneous ovulation is only possible under very good management practice, making the development of prediction markers for ovulation a priority.

Future directions

The present and future of the commercial application of FT-boar semen should be not negatively influenced by the excellent fertility levels achieved with fresh or cooled semen. Irrespective of current limitations, FT-spermatozoa plays an important role in certain sectors of the pig industry, where good fertility results can already be achieved, even under farm conditions. Despite these promising signs, there is considerable scope to achieve a wider utilisation of FT-boar spermatozoa in commercial pig breeding.

Priorities for research need to be directed towards:

1. The standardisation of an optimal cryopreservation procedure. In spite of recent methodological improvements, further advances are necessary in order to both improve post-thaw sperm survival and prolong the functional life span of FT-spermatozoa. Research tools such as cryomicroscopy (see recent review of Holt *et al.*, 2005) and the incorporation of new freezing devices such as "multi-thermal gradient" freezing (Arav *et al.*, 2002) can help to achieve this goal. In addition, the development of genetic probes to identify "good" and "bad" freezer boars is expected.

2. Freezing extenders need improvement. Current freezing extenders are basically the same as those used 30 years ago. The development of more effective extenders to both stabilise the sperm membranes and extend the functional life span of thawed spermatozoa is a major goal. Boar spermatozoa undergo lipid peroxidation during the freezing and thawing cycle. Addition of antioxidants or chelating agents such as vitamin E (Peña *et al.*, 2003), glutathione (Gadea *et al.*, 2004), butylated hydroxytoluene (Roca *et al.*, 2004) or catalase and superoxide dismutase (Roca *et al.*, 2005) in the freezing extender have proven effective.

3. Efforts should be geared towards the identification of subpopulations of FT-spermatozoa that maintain an intact fertilising capability. The overall percentage (usually 50%) of surviving spermatozoa, normally assessed according to objective motility or/and intact plasma and acrosomal membranes, are the criteria currently used to define cryosurvival success. However, a definition of "good" freezer is not always a warranty for fertilising ability, particularly when doses with a low number of FT-spermatozoa are used (Rath, 2002). Not all boars considered "good" freezers achieve high rates of *in vitro* fertility (Gil *et al.*, 2005), pointing out the limitations of current sperm evaluation tests to identify potentially fertile spermatozoa post-thaw. Based on the number of spermatozoa attached to the zone pellucida of oocytes after AI (Waberski *et al.*, 1994), it seems that the fully functional sperm population in thawed samples is not higher than 10%.

4. Development of techniques for accurate prediction (or at least estimation) of the time of ovulation is a priority. The fertility success for FT-semen in AI programmes is highly dependent of the interval between AI and ovulation. A sensor system using basic principles of immunological detection is currently under development for the automatic analysis of steroid hormone profiles in samples of saliva in sows (TT Mottram, Silsoe Research Institute, England, personal communication). For instance, a fall in oestradiol-17ß concentrations could be used as an estimator of ovulation time.

5. Sperm transport through the sow genital tract should be also improved, especially when FT-spermatozoa are cervically deposited. Proper uterine myometrial contractility plays a major role during sperm transport. Although strategies to ensure this can be as simple as the use of the "boar effect" during AI; the exogenous application of compounds (including hormones such as oxytocin and prostaglandins) to stimulate myometrial contractions is also discussed (Langendijk *et al.*, 2005). To the best of our knowledge, there are no results yet available of the effectiveness of the above mentioned approaches to improve fertility levels when using FT-semen for AI.

6. New insemination procedures that allow the deposition of semen intra-uterus offer interesting possibilities for a more effective use of FT-semen in commercial breeding. At present, DUI with as few as 1 x10^9 total spermatozoa per dose yields acceptable fertility. Evaluation of the minimum sperm number per dose, of best insemination-timing and the number of inseminations per cycle are still needed to standardise DUI insemination strategies.

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