Recent advances in boar semen cryopreservation

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Since 35 years ago boar semen has been frozen and used for artificial insemination (AI). However, fertility of cryopreserved porcine sperm has consistently been low as boar sperm are more sensitive to cellular stress imposed by changing osmotic balance, oxidative stress, low-temperature exposure, cryo-protectant intoxication etc. and are less able to compensate for these deficiencies at commercially applicable dosages. Additionally, differences in sperm freezability among individuals are well known. Here we review current advances on tests to screen sperm quality post-thaw, on ways of diminishing individual boar effects, on improvement of cryo-protection by novel extender components, on packaging and freezing protocols and freezing and thawing methods, and on the handling of sexed boar sperm. Major advances have been registered, which have improved cryo-survival and the capacity to process boar semen for commercial AI.

Introduction

The history of boar sperm freezing is characterized by three major events. About 60 years ago Chris Polge found by chance that glycerol was an effective cryo-protecting agent (CPA); Pursel & Johnson (1975) developed the Beltsville freezing and thawing procedure using a pellet method and Westendorf *et al.* (1975) started to freeze boar sperm in plastic straws. Since then important steps for freezing have been developed in laboratories around the world, but boar sperm cryo-survival has consistently been low. Throughout cryopreservation, sperm are exposed to cellular stress imposed by changing osmotic balance, oxidative stress, low-temperature exposure, and cryo-protectant intoxication. Apart from lethal changes due to cryo-injury, sub-lethal changes occur in sperm that take place at a molecular level, impacting on their cellular function and fertilizing capacity. Additionally, differences in sperm freezability among individuals are common. This review describes different perspectives and practical application of recent achievements in the improvement of the quality of frozen-thawed (FT-) boar semen.

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Integrity of frozen thawed boar sperm

Evaluation of frozen/thawed semen quality

Sperm kinematics assessed by computer-assisted sperm analysis (CASA) has great potential to give better insight into boar sperm function and heterogeneity. More than 30 different kinematic parameters related to linearity, velocity and lateral head displacement allow for detailed analysis of kinematic characteristics of single sperm. In addition to post thaw motility, the function of relevant sperm signal transduction systems, e.g. the activation of transduction systems (e.g. the activation of adenyl-cyclase) by short-term incubation with bicarbonate (Holt & Harrison 2002; Satake et *al.* 2006) can be measured with CASA systems. Despite this potential, CASA parameters were not found to be very sensitive at monitoring cooling-associated changes of sperm function (Saravia et *al.* 2007) or predicting fertility of FT- sperm (Rodriguez-Martinez 2007). One reason for this is related to the inability of some CASA systems to examine populations and at the same time focus on individual sperm. Means and standard deviation of sperm kinematics are inappropriate to detect small populations of rapidly and linearly moving sperm, which may be significant for fertilization (Holt et *al.* 2007).

The main damage to sperm due to cryo-injury occurs at the level of the plasma membrane and appears to be more closely related to fertility than sperm motility (Rodriguez-Martinez 2007). Following detection by fluorescence techniques, about 50% of boar sperm are commonly classified as "dead" after freezing/thawing. The additional detection of sub-lethal changes in plasma membrane function impairing sperm fertilizing capacity seems to be of high diagnostic relevance. Membrane characteristics of thawed sperm are similar in certain respects with sperm that are in advanced stages of capacitation. Although they do not correspond completely at the molecular level, this phenomenon led to the idea of "cryo-capacitation" (Watson 1995; Green & Watson 2001). Attempts to verify the occurrence of capacitating-like changes following cryo-preservation were made by detecting phospholipid disorders in plasma membranes using the lipophilic fluophore Merocyanine-540 (Harrison et *al.* 1996) and by induction of acrosome exocytosis using calcium ionophore A23187 in presence of bicarbonate as capacitation inducer (Guthrie & Welch 2005a). Compared to fresh semen, cryo-preserved sperm showed a reduced response to bicarbonate (Guthrie & Welch 2005a; Saravia et *al.* 2007). The presence of lipids and proteins of seminal plasma (SP) and/or egg yolk may alter the response to bicarbonate rather than the cooling process itself (Guthrie & Welch 2005b; Saravia et *al.* 2009b). Additionally, the sequential manner in which capacitational changes occur and contribute to a destabilization process leading to cell death (Petrunkina et *al.* 2007) may differ between fresh and FT- sperm. FITC-conjugated Annexin-V has been used to measure phospholipid scrambling by specifically binding to phosphatidylserine molecules that accumulate in the plasma membrane exoplasmic leaflet (Gadella & Harrison 2002). Cryo-preservation caused a significant increase in Annexin-V-positive staining in live sperm (Peña et *al.* 2003; Guthrie & Welch 2005b). It is suggested that increased Annexin-V-binding in thawed sperm results from plasma membrane damage incurred during freezing and thawing (Guthrie & Welch 2005b), rather than from expression of capacitation (Silva & Gadella 2006; Rodriguez-Martinez 2007).

Detection of early changes in the sperm plasma membrane at any of the main three domains is very important for cryo-storage protocols. During cryo-preservation the major osmotic gradient across the sperm membranes essentially changes with sperm undergoing volumetric changes as water and solute leave and enter the cells. This osmotic response can be potentially lethal to the sperm if it causes them to swell or shrink beyond their osmotic tolerance limits. Cell death occurs mainly during the thawing process, when the dehydrated sperm are exposed to severely hypo-osmotic conditions (Gilmore et *al.* 1996). Electronic volume measurements by

cell counter display the response of large numbers of sperm to osmotic challenges including the identification of subpopulations with different osmotic responsiveness. A relationship of volumetric parameters to fertility was established for frozen bull sperm (Petrunkina *et al.* 2001) and sperm binding to oviductal explants in vitro seems to be related to fertility of frozen semen (De Pauw *et al.* 2002; Khalil *et al.* 2006). However, a prediction of fertility remains questionable due to difficulties in standardizing the assay and loss of tissue differentiation during culture. Microscopic assessment of cryo-preserved boar sperm using a simplified hypo-osmotic swelling test gave comparable results to flow cytometrically analyzed plasma membrane integrity, despite measuring different membrane domains (Saravia *et al.* 2005) and is significantly related to fertility (Perez-Llano *et al.* 2001).

Oxidative stress during the freezing-thawing cycle due to excessive generation of reactive oxygen species (ROS) or deficiencies in the antioxidant defense system can induce serious damage to many biological macromolecules, such as proteins, lipids and mitochondrial DNA (mtDNA) as well as nuclear DNA (nDNA), which could lead to impaired biological properties and eventually to cell death (Aitken & Baker 2004). Pro-apoptotic changes in cryo-preserved sperm are characterized by increased Ca^{2+} concentration, disturbance in mitochondrial membrane potential, reduced ATP levels and the release of pro-apoptotic factors in the cytoplasm (Martin *et al.* 2004, 2005; Peña *et al.* 2009). Different methods used to assess nuclear DNA damage after freezing and thawing have so far given controversial results. DNA fragmentation as detected with single cell gel electrophoresis (comet assay) was significantly increased in FT- compared to fresh sperm and was affected by the individual boar (Fraser & Strzezek 2005), the presence and type of the cryo-protectant, and by seminal plasma (SP) (Fraser & Strzezek 2007b; Hu *et al.* 2008). In contrast, although the sperm chromatin structure assay (SCSA) did not reveal biologically significant changes due to sperm freezing/thawing in unselected boars (Saravia *et al.* 2009b), sires classified as "good freezers" were less susceptible to sperm chromatin denaturation than "bad freezers" (Hernández *et al.* 2006). Varying results among different studies may depend on the innate variation among boars used but, since most stud boars have been selected for sperm quality, such differences are minor and only appear in a small number of males ($< 1/1{,}000$) it is more likely that the tests need further standardization. DNA fragmentation assays as determined by single cell electrophoresis may be less accurate as it is based on single cell determination and cannot distinguish between dead and live sperm.

Boar-to-boar variation on sperm freezability

As for many other mammals (Holt *et al.* 2005, Loomis & Graham 2008), male-to-male variability in post-thaw sperm quality has been extensively demonstrated in pigs (Larsson & Einarsson 1976, Thurston *et al.* 2001, Medrano *et al.* 2002a, Saravia *et al.* 2005, Roca *et al.* 2006b). This individual variability is the primary factor explaining differences in sperm cryo-survival between boars (Roca *et al.* 2006a). However, since ejaculates within a given male most often respond reproducibly to the same cryo-preservation protocol, grouping of sires as "good", "moderate" or "poor" sperm freezers can be done, based on post-thaw sperm quality. Between 20 to 33% of stud sires are considered "poor" sperm freezers (Thurston *et al.* 2002, Roca *et al.* 2006a, 2006b, Hernández *et al.* 2007a), most likely genetically driven. Thurston *et al.* (2002) compared the genomic DNA of boars using the Amplified Fragment Length Polymorphism (AFLP) technique and identified different AFLP profiles between "good" and "poor" sperm freezers. Some of these profiles were related to post-thaw sperm quality and were, therefore, considered to represent significant markers of sperm cryo-sensitivity. The underlying mechanism(s) for the genetic differences related to cryo-preservation-induced sperm injuries are yet unknown but may represent differences in

sperm biochemical composition and physiology (Holt *et al.* 2005). Whether the polymorphism of testis- and epididymis-expressed candidate genes affecting sperm quality traits in fresh boar semen (Lin *et al.* 2006) are linked to cryo-preservability remains to be proven. In this context, Waterhouse *et al.* (2006) showed a relationship between long-chain polyunsaturated fatty acids in the plasma membrane of thawed sperm and boar-to-boar differences in sperm cryo-sensitivity. These inter-boar differences might equally be, however, related to variations in SP composition. Rath & Niemann (1997) observed that boar differences in post-thaw motility were only significant for ejaculated sperm, whereas epididymal sperm had a consistently higher post-thaw motility. More recently, Saravia *et al.* (2007) showed a significant improvement of sperm cryo-survival together with a substantial reduction of boar-to-boar variability on post-thaw sperm quality when cryo-preserving just the first 10 ml of the sperm-rich fraction of the ejaculate, a portion characterized by the highest sperm concentration alongside with low presence of sex gland secretions. Altogether, these results seem to indicate that the exposure of sperm to the SP during the ejaculation process could modify their cryo-sensitivity.

Minimization of the negative effects of boar-to-boar variability on post-thaw sperm quality within the commercial semen freezing industry should first be addressed towards identification of potentially "good" or "poor" sperm freezers. In absence of a simple blood-based genetic test and given the fact that sperm freezability is highly consistent within stud boars (Roca *et al.* 2006a), the potential sperm freezability of a given boar can be identified by means of sperm freezability tests (SFT), consisting of the cryo-preservation of 5-10 ml of just one ejaculate per boar. Experience from Spain over the past 12 years indicates that implementing a SFT can predict its relative sperm freezability potential. After performing the SFT-test, 112 boars (24.6%) showed $\leq 35\%$ viable and motile sperm after thawing and were considered as "poor" sperm freezers. Additionally, action should be taken towards the improvement of sperm cryo-survival of ejaculates collected from boars classified as "poor" sperm freezers. The ability to cryo-preserve ejaculates from "poor" sperm freezers remains important in the context of genetic resource banking and international exchange of genetic material (Holt *et al.* 2005). Such action could be undertaken by customizing the cryo-preservation protocol. An example is by individually-tailoring the freezing-thawing protocol, a concept suggested by Watson (1995) and recently applied by Hernández *et al.* (2007b) using a so called "split-ejaculate freezing test". Individual ejaculates were divided and cryo-preserved using 12 different protocols that differed with regard to cooling rate, glycerol concentration or warming rate. Significant interaction was found between ejaculate and cryo-preservation protocol, with the influence of glycerol concentration and warming rate being particularly relevant for post-thaw sperm quality. Such handling reduced the percentage of "poor" freezers from 26.4 to 7.5%. An alternative has been recently presented by cryo-preserving the first 10 ml of the sperm-rich fraction of each ejaculate (Rodriguez-Martinez *et al.* 2008; Saravia *et al.* 2009a, b).

Cryo-injury in boar sperm

The sperm membrane is regionally differentiated and displays different behaviour and interactions depending on structural regions. The acrosome (especially the equatorial segment), the post-acrosomal and mid-piece segment and, the principal and end tail segments, are all structurally and functionally crucial to sperm function. Cryo/thawing-protocols cause loss of selective permeability and integrity of these domains, mostly during rewarming (Medrano *et al.* 2002b). Inadequate ice formation and the subsequent osmotic stress induced during freezing and re-warming are the two major factors responsible for sperm cryo-injury (Watson 2000) and compromise sperm function due to alterations in the membrane constituents, particularly lipids and proteins. These phenomena might be associated with pro-apoptotic changes in sperm caused by reactive oxygen species (ROS) produced under stress conditions.

Protein tyrosine phosphorylation, which plays a crucial role in the regulation of cell proliferation and differentiation, is associated with capacitation-like changes in sperm, sometimes termed "cryo-capacitation" when induced by cryo-preservation (Urner & Sakkas 2003, Bravo *et al.* 2005). The level of phosphorylation of tyrosyl groups is regulated by the balance of protein tyrosine kinases (PTKS) and protein tyrosine phosphatases (PTPases). Phosphorylation of tyrosine residues in sperm varies among boars and at different stages of the cryo-preservation procedure according to the "Kortowska" method (Strzezek *et al.* 1985). Recently it has been demonstrated that fresh semen from boars with good freezability was characterized by a low content of phosphotyrosine residues in the extracted sperm proteins, whereas semen from boars with poor freezability exhibited a high content of phosphotyrosine (Wysocki *et al.* 2009). Accordingly, phosphotyrosine proteins in sperm extracts could be dephosphorylated by the molecular form of acid phosphatase of boar SP (Wysocki & Strzezek 2003, 2006) and phosphotyrosine residues in sperm proteins could be completely dephosphorylated only by acid phosphatases isolated from the vesicular glands.

New approaches to improve the cryo-survival of boar sperm

Cryo-protectant agents

The biophysical changes brought about by the transition of water to ice during the relatively slow cooling most often used are the assumed main causes for sperm damage. If sperm are solely frozen in SP (neat semen) or extended with a buffer, such "unprotected freezing" is simply lethal, since ice is formed both extra- and intra-cellularly, damaging essential cell structures, particularly the membranes of organelles and the plasma membrane. Even when the extender contains a proper cryo-protectant agent (CPA), damage occurs, but many cells survive the process. Under these conditions, ice is formed in the aqueous extender medium surrounding the sperm and, as ice crystals grow in this extracellular milieu of free water, the amount of solvent decreases while the solute becomes more and more concentrated. Sperm lose intracellular water in order to compensate for this effective osmotic stress leading to a freeze-dehydration of the cells. Eventually, when temperatures pass ~ -80°C, the highly concentrated, viscous solution within and outside the sperm turns into a relatively stable glassy matrix, which is basically maintained when sperm are stored at -196°C (LN_2). The imaging of such concentrated medium, where sperm are embedded (the so-called veins) contrasts with the frozen free water (so-called lakes), when viewed using a cryo-scanning electron microscope (Cryo-SEM, Ekwall 2009). Interestingly, most sperm in the veins appear intact, i.e. they seem to survive the process of cooling. Even more interesting, intracellular ice is rarely formed, since the speed of cooling is usually low and the presence of the CPA increases viscosity, both of which add to the above process of cell dehydration (Bwanga *et al.* 1991a). Most cells are damaged during thawing, with membranes and axonemes deteriorating by the osmotic imbalance that has been created during cooling (Morris 2006; Morris *et al.* 2007).

As mentioned above, glycerol was the first recorded CPA added to a semen extender. This small, poly-hydroxylated solute is highly soluble in water interacting by hydrogen bonding and permeates across the plasma membrane at a low rate. Glycerol is, unfortunately, cell-toxic at body temperature and thus boar sperm are usually exposed to glycerol at ~ 5°C, which further slows permeation. Mixed with the other solutes of the extender in solution, it depresses the freezing point and ameliorates the rise in sodium chloride concentration during dehydration. Moreover, glycerol increases viscosity with lowering temperature to more than 100,000 cP at -55°C (Morris *et al.* 2006) retarding both ice crystal growth and dehydration speed on a kinetic basis. Glycerol also eliminates eutectic phase changes of the extender (Han & Bischof 2004),

thus becoming a very suitable CPA when added at 2-3%. It does not affect sperm cryo-survival in boars considered good freezers, and improves cryosurvival in those considered "moderate or bad freezers" (Hernández *et al.* 2007a).

A broad range of other solutes (mostly alcohols, sugars, diols and amides) has also been tested for CPA capacity (Fuller 2004). CPA capacity differed greatly in tests using boar sperm compared to the sperm of other species. Alcohols and diols can induce the formation of blebs, which are spherical cellular protrusions in the membrane. Sugars, which both increase viscosity and stabilize the membrane by interacting with phospholipids, have not led to a higher cryo-survival compared to glycerol (Hu *et al.* 2008). Replacing glycerol with amides (formamide; methyl- or dimethylformamide, MF- DMF; acetamide; methyl- or dimethylacetamide (MA- DMA) at ~ 5% concentration, has proven acceptable (Bianchi *et al.* 2008). Although cryo-survival was not dramatically enhanced in most boars, cryo-susceptible boars benefited somewhat from use of DMA, probably because the amide permeates the plasma membrane more effectively than glycerol, thus causing less osmotic damage during thawing. Additives other than CPAs increase cryo-survival too. At very low rates (< 0.1%) N-acetyl-D-glucosamine has been able to enhance cryo-survival of boar sperm (Yi *et al.* 2002a) possibly interacting with the surfactant Orvus es Paste (OEP) (Yi *et al.* 2002b). The value of OEP or other surfactants has been confirmed (Karosas & Rodriguez-Martinez 1993, Pettitt & Buhr 1998) when used with egg yolk (Buranaamnuay *et al.* 2009). Use of low-density lipoproteins (LDL), most often isolated from egg-yolk from different species (Fraser & Strzezek 2007a; Jiang *et al.* 2007) has proven beneficial for sperm function post-thaw, particularly for DNA-integrity (see below).

Lipoproteins

Typically, boar sperm cryo-storage media contain up to 20% chicken yolk, whose active component (i.e. the low-density lipoprotein (LDL)-fraction), has been suggested to be largely responsible for protecting sperm against cold shock damage (Demianowicz & Strzezek 1996; Moussa *et al.* 2002; Jiang *et al.* 2007, Hu *et al.* 2008). Despite some success in species such as the horse (Clulow *et al.* 2007), yolks from duck and quail failed to improve the post-thaw quality of frozen boar sperm (Bathgate *et al.* 2006). In contrast lyophilized lipoprotein fractions isolated from ostrich egg yolk (LPFo) provided good protection of sperm cells by preventing physical damage and alteration of membrane fluidity during cooling at variable temperatures (i.e. from 5oC to 16°C or from 16°C to 5°C) and cryo-preservation (Strzezek *et al.* 2004; Strzezek *et al.* 2005a). Besides its protective action, LPFo has been shown to possess specific antioxidant properties (Strzezek *et al.* 2004). The overwhelming positive effects of LPFo on sperm quality characteristics have led to the development of a new semen preservation technology for liquid storage at different temperatures and cryo-preservation (Fraser & Strzezek 2005; Fraser & Strzezek 2007a,b). Moreover, AI of sows with liquid-stored or FT-semen supplemented with LPFo gave acceptable results (Fraser *et al.* 2007a). Investigations on the interaction between yolk lipoproteins and SP-proteins indicated that together they either enhance (Manjunath *et al.* 2002) or diminish (Vishwanath *et al.* 1992) sperm cryo-protection, suggesting that revisiting storage media without egg yolk could provide a more biosecure means of transporting and storing boar sperm by avoiding (contaminated) materials from other species.

Seminal plasma

The SP has a chequered history as a supplement for sperm storage media and current opinion is still divided over its benefits. The binding of SP-proteins to sperm stabilizes the plasma membrane components, masks the antigens exposed to the cell surface and prevents premature acrosomal

reaction (Muiño-Blanco *et al.* 2008). The boar SP contains a wide range of components that differ between species, males and ejaculates (Maxwell *et al.* 2007). This may explain the disparate results reported by different groups. It is now clear that mammalian SP contains components that can both inhibit and induce sperm capacitation, stimulate sperm motility and even provide protection against damage incurred during manipulation and storage, such as extension and cold-shock (Maxwell *et al.* 2007). The major proteins identified to date in boar SP are the non-heparin-binding proteins PSPI/PSPII heterodimer (PSPI/PSPII), the heparin binding spermadhesins AQN-1, AQN-3, AWN and the lipid-binding protein pB1. PSPI/PSPII, more specifically the PSPII subunit, inhibits zona penetration and increases sperm longevity, suggesting that this subunit can be used to improve the fertility achieved with stored sperm (Caballero *et al.* 2008). Incubation of FT-sperm with PSPI/PSPII prior to *in vitro* insemination resulted in an increased proportion of viable sperm but a reduced number of penetrated oocytes, compared with sperm incubated without PSPI/PSPII. The same treatment applied to fresh sperm had no effect on oocyte penetration (Caballero *et al.* 2004). Fertility was also improved after laparoscopic insemination of low doses of sex-sorted sperm pre-incubated in the presence of PSPI/PSPII (Garcia *et al.* 2007). The PSPI/PSPII heterodimer has also been shown to modulate the uterine immune response to the introduction of semen in both an inhibitory (Veselský *et al.* 1992) as well as a stimulatory manner (Rodriguez-Martinez *et al.* 2005), suggesting a direct effect on spermatozoa as well as a preparatory role for embryo attachment. Thus, addition of PSPI/PSPII to the sperm suspension medium prior to insemination may be influencing fertility by the modulation of both sperm function and the secretory and/or physical environment of the female reproductive tract.

AQN-1, a major heparin-binding protein prevalent in boar SP, may have a role in the congregation of sperm in the isthmic reservoir (Ekhlasi-Hundrieser *et al.* 2005) and both AQN-1 and -3 may regulate the initiation of capacitation (Dostalova *et al.* 1994). AWN appears to modulate sperm-oocyte interaction (Rodriguez-Martinez *et al.* 1998), suggesting that addition of these proteins to sperm prior to artificial insemination may improve fertility outcome. The lipid-binding protein pB1 has been implicated in the capacitation of epididymal boar sperm (Plucienniczak *et al.* 1999; Lusignan *et al.* 2007). Unlike homologous proteins found in the SP of other species, pB1 forms a complex with AQN-1 (Calvete *et al.* 1997). Manipulation of boar sperm storage media to remove this complex might prolong the shelf-life of sperm, or it could be exploited as a capacitation factor prior to IVF.

SP-vesicles (prostasomes) have been regarded as influencing fertility in humans (Kravets *et al.* 2000). Although probably of prostate origin, such has not yet been identified in the boar. Prostasomes consist of lipid and protein conglomerates that are concentrated in the sperm-rich fraction of the boar ejaculate but are of unknown function in this species (El-Hajj Ghaoui *et al.* 2007). Similar structures in other species have been identified as fusing with sperm membranes, possibly stabilizing the lipids and postponing the acrosome reaction (Frenette *et al.* 2002).

Recently, it has been shown that a 5-h period of dialysis of boar ejaculate in semi-permeable dialysis bags (12 to 14 kDa cut-off) prior to freezing had a significant effect on the polypeptide profiles of the SP (Strzezek *et al.* 2005b) as well as a significant improvement in post-thaw sperm quality characteristics, such as caffeine-stimulated sperm motility, plasma membrane integrity and mitochondrial status (Fraser *et al.* 2007b). Dialysis of ejaculates prior to freezing may be a useful technique to eliminate certain seminal plasma proteins with sperm toxic effects during cryo-preservation (Strzezek *et al.* 2005b).

Other additives

Boar sperm are susceptible to peroxidative damage induced by the cryo-preservation process (Hernández *et al.* 2007c) due to the high proportion of unsaturated fatty acids present in their

membranes (Cerolini *et al.* 2000). Hence, attempts have been made to add various antioxidants to different fractions of boar ejaculate to improve post-thaw sperm survival (Peña *et al.* 2003, Gadea *et al.* 2005), including butylated hydroxytoluene (BHT; Roca *et al.* 2004), catalase, superoxide dismutase (SOD; Roca *et al.* 2005), reduced glutathione (GSH; Woelders *et al.* 1996), and α-tocopherol (Breininger *et al.* 2005). The latter reduced cryo-induced oxidative damage to sperm membranes, protein tyrosine phosphorylation and the capacitation-like events (Satorre *et al.* 2007).

Additional additives that have shown some potential for improving quality of boar sperm post thaw are hyaluronan (Peña *et al.* 2004), platelet-activating factor (PAF; Kordan & Strzeżek 2002; Bathgate *et al.* 2007a) and platelet-activating factor: acetylhydrolase (Pafase; Bathgate *et al.* 2007a). PAF, a member of the family of the acetylated glycerophospholipids, is a component of plasmalemma lipids of boar sperm. PAF is involved in several sperm functions, including capacitation, the acrosome reaction and the regulation of motility of sperm, particularly during storage at 5° to 16°C (Kordan & Strzezek 2002). Supplementation of the cryo-preservation medium with PAF or the recombinant PAF acetylhydrolase appeared to have beneficial effects on the *in-vitro* quality of FT- sperm (Bathgate *et al.* 2007a).

Controlled freezing

Rates of cooling (and of thawing) can be controlled by use of programmed freezers, and "optimal" cooling rates are those that substantially diminished the period during which heat was released in the sample when water changed phases (i.e. ice was formed). Interestingly, experimentally determined optimal rates of the range 30-50°C/min (Thurston *et al.* 2003) have been theoretically predicted (Devireddy *et al.* 2004; Woelders & Chaveiro 2004) and confirmed by use of novel procedures, such as equilibrium freezing (Woelders *et al.* 2005). Boar sperm are still "best" (in terms of cryo-survival) cryo-preserved in standard lactose-egg yolk (or LDL)-based cooling and freezing media, the latter including a surfactant (often OEP) and glycerol (2-3% final concentration); cooled at 30 to 50°C/min and rapidly (1,000-1,800 °C/min) thawed. This protocol would serve most boars while for those with sub-optimal sperm freezability, the protocol must be modified, particularly regarding glycerol concentration and warming rates (Hernández *et al.* 2007b). The entire procedure takes most often 8-9 hours from collection to storage of the frozen doses in LN_2, is still tedious and inconvenient, and produces few AI-doses.

Packaging systems

The use of different packaging for extended sperm resulted in differences in cryo-survival. Boar sperm were processed in plastic straws of different volumes (0.25 to 5 ml) (Johnson *et al.* 2000), in flattened 5 ml straws (Weitze *et al.* 1987), in aluminium tubes (Fraser & Strzezek 2007b) or plastic bags of various types and constitution (Bwanga *et al.* 1991b; Karosas & Rodriguez-Martinez 1993; Mwanza & Rodriguez-Martinez 1993; Ortman & Rodriguez-Martinez 1994; Eriksson & Rodriguez-Martinez 2000a, b). The latter, denominated "FlatPack™", proved equally good or better than 0.25 ml straws in terms of sperm cryo-survival, despite the fact that they held 5 ml of semen, which could accommodate an entire dose for cervical AI with 5 billion sperm, thus pooling innumerable straws after thawing was not necessary. The use of FlatPack™ resulted in acceptable farrowing rates and litter sizes (Eriksson *et al.* 2002). FlatPack™ was considered as cryo-biologically convenient to dissipate heat during rapid cooling and warming as those smaller containers tested.

However, doses with such large sperm numbers are not the best use of the ejaculates. Since the introduction of intrauterine deposition of semen, small numbers of sperm are sufficient for successful insemination and the reduced volume of the dosage allowed the use of smaller containers. Recently, boar sperm have been frozen in highly concentrated (1-2 billion sperm/ml) small volumes (0.5-0.7 ml) in novel containers, the so-called "MiniFlatPack™" (Saravia *et al.* 2005). Interestingly, cryo-survival in MiniFlatPacks was equal or higher than survival in 0.5 ml plastic straws, suggesting the shape maintained the cryo-biological advantages of the FlatPack™ (Ekwall *et al.* 2007), including improvements in fertility (Wongtawan *et al.* 2006).

New simplified freezing of boar semen

Processing semen in the current manner is impractical and, therefore, unattractive for routine, commercial use. Sperm from boars that were semino-vesiculectomised sustained freezing and thawing equally well compared to sperm exposed to seminal vesicular proteins (Moore & Hibbitt 1977), indicating that the SP of the sperm-rich fraction (SRF) might not be necessary for cryo-survival or even fertility. Recently, it was determined that boar sperm contained in the first 10 ml of the SRF (also called Portion 1 or P1, containing about ¼ of all sperm in the SRF) were more resilient to handling (from extension to cooling) and cryo-preservation than the sperm contained in the rest of the ejaculate (Peña *et al.* 2003; Saravia *et al.* 2007; Rodriguez-Martinez *et al.* 2008). It appeared that it was actually the SP in this Portion 1 that was beneficial for sperm, either because of its higher contents of cauda epididymal fluid, its lower amounts of SP-spermadhesins or its lower bicarbonate levels. An attempt was very recently made to simplify the cryo-preservation protocol by freezing solely the P1-sperm, in concentrated form for eventual use with intrauterine AI. These sperm were packed into MiniFlatPacks™ for customary freezing using 50°C/min cooling rate. This "simplified" entire procedure, lasted 3.5 h compared to the "conventional freezing", which lasted 8 h. As controls, sperm from the SRF were compared to P1-sperm. Cryo-survival was equally good (above 60% of the processed cells, Saravia *et al.* 2009a).

There are several advantages to using this simplified, shorter protocol, namely the exclusion of primary extension and of recovering this conspicuously beneficial SP-aliquot by centrifugation; as well as waiving the need of an expensive refrigerated centrifuge. Moreover, inter-boar variation was minimized by use of P1-sperm, which, not only were the "best" sperm to be cryo-preserved, but also left the rest of the collected sperm for liquid semen processing. This simpler protocol ought to be an interesting alternative for AI-studs to freeze boar semen along with production of conventional semen doses for AI with liquid semen.

Advanced insemination strategies with FT- boar semen

Recently, farrowing rates ranging from 72 to 85% and 11-12 piglets born per litter have been achieved in FT-inseminated sows in Taiwan and Canada with semen cryo-preserved in Europe (Eriksson *et al.* 2002; Roca *et al.* unpublished observations). These results indicate that FT-semen has the potential for sufficient fertilization rates and can be used to improve international semen trade. However, the high sperm number currently required per AI-dose (5-6 x 10^9sperm) together with the sometimes inconsistent fertility, limit FT-semen usage to the introduction of genetics.

Therefore, it is of utmost importance to reduce the number of sperm per AI-dose without impairing fertility. At present, this objective can be achieved by means of the deep intrauterine insemination (DUI) procedure, which allows deposition of the sperm dose deep into a

uterine horn. Recently, the performance of DUI for swine AI and its suitability for an efficient application of FT-semen have been extensively evaluated (Bathgate *et al.* 2005; Roca *et al.* 2006c; Grossfeld *et al.* 2008; Vazquez *et al.* 2008). Overall, DUI appeared as a safe procedure leading to satisfactory fertility with as few as 1-2 x 10^9 FT-sperm per AI-dose. In this manner, in two more representative studies, farrowing rates above 70% with more than 9 piglets born per litter were achieved after insemination of 500 weaned sows under commercial conditions (Roca *et al.* 2003; Bolarin *et al.* 2005). Transcervical insemination could be another suitable AI-procedure for FT-semen, to allow deposition of sperm into the uterine body (Roca *et al.* 2006c). Unfortunately, it has been scarcely used with FT-semen, despite the promising fertility results achieved with liquid semen (Watson & Behan 2002; García *et al.* 2007). In the only report found using FT-semen, Abad *et al.* (2007) reported farrowing rates below 50% in weaned sows inseminated with 3 x10^9 live FT-sperm.

Fertility using commercial AI of FT-semen varies among trials, depending more on the interval between AI and ovulation than either post-thaw sperm quality or the sperm number inseminated. FT-sperm have a very short functional life span in the female genital tract, such that their fertilizing ability is dramatically impaired when inseminations are performed outside an interval 4-8 h before expected ovulation time (Wongtawan *et al.* 2006). Bolarin *et al.* (2005) achieved high farrowing rates above 80% in weaned sows using DUI with either 1-2 x 10^9 sperm per AI-dose, provided that the 4-8 h interval from AI to ovulation was respected. However, in contrast to these excellent results, the same authors and others (Wongtawan *et al.* 2006) achieved very low farrowing rates in sows using DUI outside of this "safe" pre-ovulatory interval, independent of how many living sperm were inseminated. Therefore, it is imperative that an accurate prediction of ovulation time is made to define an appropriate insemination timetable when FT-semen is used. As spontaneous ovulation takes place when two-thirds of the standing oestrus period has elapsed (Soede & Kemp 1997), appropriate insemination timetables can be established when the duration of standing oestrus is known. Finally, because seasonal differences in farrowing rates are greater for FT-semen than for liquid semen (Bolarin *et al.* 2008), the influence of season on the elapsed time between onset of oestrus and ovulation should also be considered.

Special freezing protocols for sex sorted boar sperm

A special application for FT-semen is related to sex sorted sperm. Currently, freshly collected boar sperm have been sexed successfully by flow cytometry and offspring were produced by surgical insemination (Johnson 1991), IVF (Rath *et al.* 1997, 1999; Abeydeera *et al.* 1998), ICSI (Probst & Rath 2003), and DUI (Rath *et al.* 2003; Grossfeld *et al.* 2005) under laboratory conditions. Due to the limited throughput of the sorting technology, a broader commercial application is not possible. Cryo-preservation might be a method of storage between sorting and insemination. Only a few studies investigated the freezing and thawing of sexed boar sperm. Sexed, frozen/thawed sperm have been subjected to IVF and DUI (Bathgate *et al.* 2007b, 2008). Whereas in-vitro fertilization with FT sperm was successful and pregnancies were initiated (Bathgate *et al.* 2007b), all sows returned to oestrus within 57 days. A combination of low sperm numbers and potentially compromised developmental capability of embryos derived from sex-sorted sperm may have resulted in this early stage loss of pregnancy based on fertilization with spermatozoa that are affected by non compensable defects (Bathgate *et al.* 2008).

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