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State-of-the-art of boar sperm preservation in liquid and frozen state

Marc Yeste[1]

Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain.

Abstract

Pig breeding is mainly conducted through Artificial Insemination (AI) in Western and developing countries. Apart from requiring specific catheters and trained staff, preserving boar semen in proper conditions is needed to ensure high reproductive performances. Although, at present, boar sperm may be preserved in liquid (15-17ºC) or frozen states, more than 95% of AIs are conducted using liquid semen. The present work reviews the state-the-art of these two preservation technologies. Thus, the composition and types of extenders for liquid-stored semen are discussed, together with the specific requirements for boar sperm, which are stored at 15-17ºC. Commercial extenders for liquid semen are compared and the effects of storage on sperm quality are also summarised. In the second part of the manuscript, the main features of boar sperm cryopreservation are described and reference to cryodamage is also made. These cryoinjuries mainly affect sperm motility, membrane permeability and chromatin integrity. Furthermore, the individual variability in the sperm resilience to withstand cryopreservation procedures is reviewed and a brief summary about freezability markers is also included. Final sections briefly discuss the improvement of freezing extenders with additives, such as seminal plasma and antioxidants, and highlight the relevance of using a proper AI technique to avoid dramatic drops in reproductive performance.

Keywords: boar semen, cryopreservation, liquid storage, preservation

Introduction

Pig breeding in Western and some developing countries is carried through Artificial Insemination (AI). This technique offers several advantages, such as the use of the genetic potential of the best boars with a large number of sows. In addition, not only does AI make genetic improvement easier, but reproductive performance is higher than that obtained with natural mating. Although the first AI protocols in swine were performed at the beginning of the XX century, it was not until the eighties when insemination protocols were standardised and AI started to become commercially generalised (Johnson *et al*., 2000; Rodríguez-Gil and Estrada, 2013).

Apart from requiring specific catheters for the type of AI that is to be performed, AI may be conducted with liquid/extended or frozen-thawed semen. In both cases, it is important to use the adequate media for maintaining the sperm in adequate conditions. This means that the medium and temperature at which sperm are stored are critical for preserving their quality and fertilizing ability. Given the relevance of AI in swine and the crucial role of sperm preservation when AI is used, the current work aims at reviewing the most crucial aspects for the conservation of boar semen in liquid and frozen states.

Preservation of boar semen in liquid storage at 15-17ºC

In addition to the high relevance of AI for pig breeding, it is worth noting that most AIs (99%) are performed using extended semen (Johnson *et al*., 2000). Extended semen usually consists of seminal doses of 50-100 ml (depending on the type of AI that is to be performed) and a concentration of 1-3 billion sperm per dose. Seminal doses result from the collection of ejaculate rich-fractions, further dilution with an extender and split of diluted semen in doses with the required volume and concentration. Such an extender or diluent is defined as the aqueous solution that allows increasing the volume of the ejaculate up to the required amount, contributes to reduce the sperm metabolic activity, preserves sperm function and maintains a suitable fertility level. Apart from the composition of the extender, the other crucial element to maintain the sperm metabolism at low level is the temperature of storage (Gadea, 2003; Estienne *et al*., 2007). These two elements will be discussed in further detail in the next paragraphs. On the other hand, as seminal doses are packed in plastic recipients, their composition is also critical. In effect, the presence of toxic compounds may have a strong impact upon reproductive performance (i.e. reduced fertility rates and litter sizes) without exhibiting a detrimental effect on routine sperm quality parameters (Nerin *et al*., 2014).

Composition of the extender

In order to carry out its function, the extender must provide the nutrients needed to maintain the sperm metabolism (monosaccharides such as glucose and fructose), protect cells from cold shock (bovine serum albumin, BSA), control the pH with buffers (Bicarbonate, Tris, HEPES) and the osmotic pressure (NaCl, KCl, sodium citrate), and inhibit microbiological growth (with antibiotics such as kanamycin or gentamycin; Estienne *et al*., 2007).

Nutrients

Spermatozoa produce the energy required to

[1]Corresponding author: marc.yeste@udg.edu
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maintain their metabolism and allow their intrinsic motility, principally through glycolytic pathways (Rodríguez-Gil, 2013). Therefore, any extender must contain an energy source, which is often glucose, as other sources such as galactose, ribose and threhalose have been reported to give worse results (Gadea, 2003). In addition to this, Medrano *et al*. (2005) evaluated the suitability of different combinations of sugars (glucose) and non-sugars energy substrates (citrate and lactate) during a 7-day period of storage at 17ºC. Interestingly, these authors observed that boar sperm quality was better preserved in a medium containing 150 mM glucose and 25 mM sodium lactate than standard BTS (205 mM glucose and 20 mM sodium citrate).

On the other hand, dilution of sperm involves a reduction in the levels of certain ions and molecules that are present in the seminal plasma and play a key role in sperm viability, such as potassium ion (K^+) and seminal plasma proteins (Harrison *et al*., 1978). Since the reduction of these substances may alter sperm function and survival, a proper formulation of the extender should also take this aspect into account. Thus, proteins such as BSA are included in the extenders as they maintain sperm motility, membrane integrity and reproductive performance (Waberski *et al*., 1994; Zhang *et al*., 2015).

Regulation of pH

Immediately after ejaculation, semen pH is approximately 7.4 ± 0.2. When pH decreases, there is a concomitant reduction in the energetic metabolism and sperm motility. As sperm glycolytic metabolism reduces the intracellular pH, and this ultimately decreases the cell metabolism, the presence of buffers in the extender is required to control pH variations.

Previous research evaluated the suitability of different buffers, differentiating between simple systems, such as bicarbonate or sodium citrate, which both have only a limited ability to buffer pH, and more complex systems such as TES, HEPES, Mops or Tris, that are able to regulate pH within a wider range, even when changes in temperature occur (Gadea, 2003). Notwithstanding, while replacing bicarbonate by HEPES has been found to reduce the percentages of sperm exhibiting an exocytosed acrosome when exposed to calcium ionophore A23187, it also decreases sperm motility, as bicarbonate activates sperm motility (Murase *et al*., 2010). This indicates that it is important to achieve a proper balance between buffer systems.

The pH of commercial extenders usually ranges from 6.8 to 7.2 units, but two considerations must be taken into account. First, pH is not stabilised until 90 min after dilution of the powder in water; and second, different extenders present different patterns of pH values over time (Newth and Levis, 1999). For these reasons, care is needed when preparing the extender in order to avoid additional problems for sperm preservation. It is also worth noting that boar semen should be kept out of contact with air, as Vyt *et al*. (2007) demonstrated that high air volume is related to an increase in the pH and to a decrease in the proportions of motile spermatozoa. Therefore, it is important to decrease the air volume in the recipient as much as possible when packing seminal doses.

Osmotic pressure

Osmotic pressure varies between commercial extenders for liquid semen, ranging from 240 to 380 $mOsm \cdot Kg^{-1}$. While boar sperm have been found to be able to tolerate osmolalities ranging between 250 and 390 $mOsm \cdot Kg^{-1}$, isotonic (300 $mOsm \cdot Kg^{-1}$) or slightly hypertonic extenders have been reported as the best diluents for maintaining sperm quality (Schilling and Vengust, 1986; Fraser *et al*., 2001). In order to regulate osmotic pressure, halide salts such as sodium chloride (NaCl) and potassium chloride (KCl) are those most often used in semen extenders.

Antibiotics

Apart from the negative impact on the female, bacterial contamination results in different alterations on sperm integrity and function, including a decrease in sperm motility and viability, sperm agglutination, degenerative acrosome exocytosis, and changes in the pH (Althouse *et al*., 2000; Bussalleu *et al*., 2011; Sepúlveda *et al*., 2013, 2014, 2016; Prieto-Martínez *et al*., 2014). As a result, there is a reduction in the time that seminal doses can be stored at 15-17ºC. Therefore, antibiotics are added to avoid bacterial growth in the extender, as nutrients such as glucose and the storage temperature (15-18ºC) of refrigerated semen allow the development of gram-negative bacteria, such as *Escherichia coli*, *Salmonella sp.* and *Pseudomonas sp*.

The first studies about the effects of antibiotics on boar semen were conducted at the start of the 1980s. Sone *et al*. (1982) compared the ability of nine antibiotics to control the growth of eleven bacterial genera commonly present in boar semen and found that dibekacin, amikacin and gentamicin were the most effective with the lowest minimum inhibitory concentrations (Sone *et al*., 1982). Other works have aimed at elucidating how the presence of antibiotics in semen extenders affects the quality and fertilizing ability of boar sperm. A recent study by Bryła and Trzcińska (2015) combined gentamicin, one of the most common antibiotics used in boar semen, with others such as florfenicol and polymyxin B, for a period of 10 days and found that the combination of 100 µg/ml gentamicin and 100 µg/ml florfenicol was the one that yielded the best results.

Temperature

Mammalian sperm especially that from boars are very sensitive to cold-shock, due to their physico-chemical characteristics (Pursel *et al*., 1973). Specifically, the lipid composition of the plasma membrane explains this phenomenon, as the susceptibility of sperm to cold shock is related to low cholesterol content and high ratio of unsaturated:saturated fatty acids in phospholipids.

The lateral movements of plasma membrane phospholipids are reduced when the temperature

decreases to 5ºC and below, causing a lipid-phase separation and involving irreversible changes to membrane proteins. The destabilization of the plasma membrane induces premature capacitation-like changes, compromises the sperm viability and reduces the fertilizing ability (Buhr *et al*., 1989;White, 1993; Harrison, 1997). In addition, cold-shock by rapid cooling to near freezing point decreases sperm motility and damages acrosome and plasma membranes. The fact that boar spermatozoa present high content of unsaturated fatty acids also makes them susceptible to peroxidation of lipid membranes. Finally, if extracellular Ca^{2+} levels are high, there may be 'calcium intoxication' during cold-shock, as decreasing the temperature of the environment increases calcium uptake by sperm. All these aspects have to be taken into account when preserving boar semen in liquid and frozen storage (White, 1993; Vadnais and Althouse, 2011).

The aforementioned susceptibility of boar sperm to cold-shock makes necessary to keep sperm samples at temperatures ranging between 15 and 20ºC, as a more extreme reduction in storage temperature may strongly compromise the viability of seminal doses (Althouse *et al*., 1998; Zou and Yang, 2000; Huo *et al*., 2002a; Kommisrud *et al*., 2002; Purdy *et al*., 2010). However, storage at these temperatures puts a limit on the length of time sperm samples can be kept, because cell metabolism is not sufficiently reduced and the control of microbiological contamination is not as effective as it would be at lower temperatures, such as 5ºC. For this reason, and as discussed in the previous section, the presence of antibiotics is crucial to keep bacterial growth under control.

The exact temperature of storage is critical and separate extenders exhibit different properties according to the temperature of storage. Indeed, Schmid *et al*. (2013a) showed that Androstar Plus was able to keep sperm better than BTS, when semen was stored for four days at hypothermic conditions (5 and 10ºC). These differences were not only observed in sperm motility and membrane integrity but also in the sperm response to capacitation medium. In addition, these authors also found that after conducting an AI trial involving 778 sows with semen that was kept for two days, the reproductive performance of sperm stored in Androstar Plus at 10ºC did not differ from that preserved in BTS at 17ºC.

The decrease of the temperature of semen may be performed in one or two steps. However, in a study conducted by López Rodríguez *et al*. (2012), ejaculates were initially diluted 1:1 at 30°C and then re-diluted to a final concentration of 3×10^7 spermatozoa·ml^{-1} either at 29.3 or at 22.7°C. Semen was further stored, transported at 17ºC and evaluated, but there were no differences in the quality of liquid-stored semen between the two temperatures at the second dilution-step (López Rodríguez *et al*., 2012).

Long- and short-term refrigeration extenders

Refrigeration extenders are divided into two groups based on the period of storage. Accordingly, there are short-term extenders, able to preserve sperm for up to three days (Johnson *et al*., 1982); and long-term extenders, for keeping the semen for more than four days (Dubé *et al*., 2004; Vyt *et al*., 2004; Haugan *et al*., 2007). As 85% of the AIs conducted with extended semen are performed within the first two days after semen collection, the most rational decision in some cases may be the use of a short-term extender, which is less expensive and may be quite effective in preserving sperm quality.

As there are different commercial diluents available, separate studies have compared these media, including direct comparisons between short- and long-term diluents. An early comparative study between two short-term extenders (Kiev and Beltsville Thawing Solution, BTS) concluded that Kiev yielded better reproductive performance than BTS (Johnson *et al*., 1982). However, another work, which compared the preservation abilities of short- and long-term extenders (BTS, Androhep, Kiev and Zorlesco) at 17ºC for a 15-day period, found that Androhep and Zorlesco were the most suitable, ranking those media as follows: Androhep, Zorlesco, BTS and Kiev (Huo *et al*., 2002b). Therefore, in the study by Huo *et al*. (2002b), BTS reported better results than Kiev.

Dubé *et al*. (2004) conducted a study that compared a long-term (Androhep Plus) and a short-term extender (BTS) for a storage period of 12-days. Whereas no significant differences between extenders were observed for sperm viability, Androhep Plus presented significantly higher percentages of progressively motile spermatozoa. In addition, capacitation markers, such as chlortetracycline (CTC) and tyrosine-phosphorylation patterns of sperm proteins showed that capacitation-like changes occurred earlier in BTS than in Androhep Plus and these differences were already apparent at day 2.

Another study compared the sperm quality of semen stored in BTS for three days with semen stored for five days in three long-term extenders and found that membrane and acrosome integrities of sperm stored for five days in X-cell and Mulberry III were similar to those of sperm stored in BTS for three days (Waterhouse *et al*., 2004). This matches with the study of Vyt *et al*. (2004) which compared five different commercial extenders: three long-term (Mulberry III, Androhep and Acromax) and two short-term (Kobidil+ and BTS) following sperm storage for 7 days and found that Mulberry III was the one that better preserved the sperm motility. Membrane integrity did not significantly differ between extenders, even if the two short-term extenders supposedly preserved the sperm function over three days. Also, the increase in the pH over storage was lesser in samples stored in Mulberry III.

The work conducted by Estienne *et al*. (2007), compared the abilities of nine extenders (BTS, Merck-III, Androhep-lite, Sperm Aid, MR-A, Modena, X-Cell, VSP, and Vital) to preserve boar sperm motility for 7 days at 18ºC. They found that total sperm motility was better preserved by X-Cell, whereas progressive sperm motility and VAP were better preserved by Modena extender. In addition, VSL and LIN were better preserved in Androhep-lite. In spite of these differences, all the extenders except Sperm Aid presented a high

capability to preserve sperm motility during the 7-day period of storage (Estienne *et al*., 2007).

The study performed by De Ambrogi *et al*. (2006) compared a short-term extender (BTS+; IMV technologies) with two long-term extenders (MR-A and X-Cell) during storage at 17ºC for 96 h in their abilities to preserve sperm motility and viability, and chromatin integrity. The most interesting result of that study was that there were no changes in chromatin integrity at the end of the 4-day period. As this is a short period of time for long-term extenders, more research involving longer storage periods is warranted. Yet, one should note that basal levels of chromatin fragmentation in boar sperm are usually low (De Ambrogi *et al*., 2006).

Despite the fact that long-term extenders are widely used for sperm storage, AI with refrigerated semen is performed within the first three days of collection. In our lab, we compared a short- (BTS) and a long-term (Duragen) extenders and found that while the long-term extender preserved better the sperm quality than the short-term one, no differences between the two extenders were observed when AI was performed in the first two days after collection (Pinart *et al*., 2015a). This indicates that the use of long-term extenders is not required when AI is performed within the first two days post-collection.

In another comparative study involving four commercial extenders and a homemade medium, called swine fertilization medium (SFM) and intended to biotechnological applications, such as sperm-mediated gene transfer, Fantinati *et al*. (2009) found that such an extender presented a high capacity of preserving boar sperm for 15 days at 16.5ºC. This medium (which contained 11.25 g glucose, 10 g sodium citrate-$2H_2O$, 4.7 g ethylene diamine tetra-acetic acid (EDTA)-$2H_2O$, 3.25 g citric acid-H_2O, 6.5 g Trizma, 6 g BSA and 1 g ampicillin sodium salt per liter; pH adjusted to 6.8) yielded similar results to other commercial, long-term extenders, such as Androhep Enduraguard (long-term), BTS (short-term), MerkIII (short-term) and Androstar (BSA-free, long-term). Although the composition of commercial extenders may not be completely revealed due to intellectual property reasons, sodium bicarbonate, which as aforementioned is a capacitating agent, was not present in SFM and was replaced by Trizma as buffer. In addition, SFM contained high levels of BSA, which preserves sperm function and viability, and EDTA which is a chelating agent that sequesters divalent metal ions, such as Ca^{2+}. As Ca^{2+} influx plays a crucial role in sperm capacitation, the presence of EDTA prevents premature sperm capacitation.

Finally, while most of the works have compared the abilities of extenders to preserve sperm membrane integrity and motility during short- and long-term storage, only a few have compared their impact upon reproductive performance. Haugan *et al*. (2007) compared the reproductive performance of semen preserved for 4-5 days prior to AI with semen stored in BTS for 2-3 days. No significant differences were found when these two extenders were compared, and X-cell yielded good reproductive performances when used after 4-5 days. In a study that compared Androhep and X-Cell extenders and storage for 2, 3, 4, 5 and 6 days prior to use, Kutser and Althouse (1999) found that both extenders did not differ in terms of sperm aging and farrowing rates when semen was stored for 4 or 5 days. However, farrowing rates in gilts inseminated with Androhep were lower than those obtained with X-Cell when semen was stored for 6 days. In addition, whereas litter sizes did not differ between extenders at days 2 and 3, gilts inseminated with semen extended with Androhep showed lower litter size after storage for 4-5 days. The study recommended the use of Androhep extender within the first three days of collection and that of X-Cell if AI was to be performed after six days post-collection (Kuster and Althouse, 1999).

In summary, there is a consensus in that commercial long-term extenders preserve the sperm quality and fertilizing ability better than the short-term ones when the period of storage is longer than three days.

Effects of liquid-storage on sperm quality

Although extenders differ in the period in which they are able to keep the semen intact, storage affects sperm quality. Therefore, an important issue that deserves to be mentioned here is that apparent good preservation of boar semen in liquid storage may not correspond to good reproductive performance when this semen is used for AI. A main concern is the fact that conventional spermiogram parameters do not reflect the decline in the fertilizing ability during liquid storage at 15-17ºC. For this reason, different studies have tried to investigate whether the response of liquid-stored sperm to bicarbonate, the capacitation effector (Harrison, 1997; Yeste, 2013), varies over storage for separate time periods 12, 24, 72, 120, and 168 h (Henning *et al*., 2012). At each of these relevant time points, and even though the sperm quality evaluated through motility and membrane integrity was good, the response of sperm to bicarbonate after density gradient washing evaluated through intracellular calcium levels revealed that there was a binary response. In effect, while intact sperm were less able to respond to the presence of bicarbonate, the other sperm subpopulation was more unstable (Henning *et al*., 2012). That being said, another study conducted by the same research group and in which semen was stored in liquid storage at 17ºC for three days showed that despite the percentage of motile spermatozoa in response to bicarbonate decreasing over storage, the percentage of fast-linear sperm subpopulation remained unchanged. Again, this supports the idea of a binary response as there is loss of motility after three days of storage but the percentage of fast-linear spermatozoa does not change (Henning *et al*., 2014). Related to this, Schmid *et al*. (2013b) observed that the sperm response to capacitation stimuli in hypothermic conditions was related to the length and temperature of storage.

Semen storage in BTS at 15ºC for seven days has also been reported to increase oxidative stress (measured as photon emission), which is concomitant with a reduction in the intracellular ATP levels and sperm motility (Gogol *et al*., 2009). In spite of this, it is

worth mentioning that basal reactive oxygen species (ROS) and lipid peroxidation have been found to be low in viable, fresh and frozen-thawed semen, with levels being ≤4% (Guthrie *et al*., 2008). Liquid storage for three or more days has also been found to induce changes in the structure of sperm subpopulations based upon the status of plasma membrane (Pérez-Llano *et al*., 2009), increase chromatin fragmentation (Boe-Hansen *et al*., 2005), and decrease acrosin activity (Pinart *et al*., 2013) and penetration rates following IVF (Suzuki *et al*., 2005).

Finally, handling seminal doses may also affect sperm quality over liquid-storage. Indeed, Schulze *et al*. (2015) found that rotation of seminal doses impairs sperm quality (membrane integrity and motility) and alkalanizes the pH of BTS-extender, that is why rotation of doses is not recommended prior to AI.

Improvement of refrigeration extenders

A significant number of studies have tried to improve semen extenders by supplementation with specific additives. The nature and functions of these additives is very wide, as too are the results of that addition. For example, the addition of hyaluronic acid to BTS has been reported to delay premature capacitation induced by short-term storage at 15ºC (Yeste *et al*., 2008).

Another substance that has been tested is BSA. Zhang *et al*. (2015) supplemented Modena extender with different concentrations of BSA (1 to 6 g/L) and found that the addition of 3-5 g/L increased sperm motility and plasma membrane integrity and decreased malondialdehyde (MDA) levels during liquid storage. The best effect was obtained when 4 g/L was added, as that supplementation extended the sperm survival up to 7.5 days. These results reveal that this protein is a main component of long-term extenders.

Reduced glutathione has also been tested as an additive in liquid-storage extenders. In a study performed by Zhang *et al*. (2016), this antioxidant was reported to increase the sperm motility and membrane integrity and decrease MDA levels of boar semen diluted in Modena extender and stored at 17ºC, the optimal concentration being 1 mM.

The effects of adding 10 ng/ml and 100 ng/ml indole-3-acetic acid (IAA, plant auxin) on the ability of BTS to preserve sperm function and survival at 15ºC for 13 days were tested by Toniolli *et al*. (1996). Whereas they found no effect on sperm motility, they observed an increase in the proportion of viable sperm exhibiting an intact acrosome at the end of the storing period when 10 ng/ml IAA was present.

The effects of supplementing the liquid-storage extender with melatonin (1 μM), which has antioxidant properties, are less clear as it increases the percentages of viable spermatozoa with an intact acrosome but reduces the proportions of progressively motile spermatozoa after seven days of storage at 17ºC. These results do not encourage the use of melatonin in long-term storage (Martín-Hidalgo *et al*., 2011).

Polymyxin B is known to neutralise the activity of lipopolysaccharide (LPS), which is released by gram negative bacteria and negatively affects sperm quality. Okazaki *et al*. (2010) found that adding the semen extender with 100 μg/ml Polymyxin B combined with penicillin G increased the sperm motility after ten days of liquid storage. In addition, the presence of Polymyxin B in that extender also resulted in an increase of farrowing rates (Okazaki *et al*., 2010).

Finally, some other substances have been tested when added after storage as it is the case of relaxin. Following incubation of stored-semen at 37ºC for 1h, relaxin increases sperm motility without affecting membrane integrity or cAMP levels (Feugang *et al*., 2015).

Apart from improving the extenders through additives, a recent approach has consisted of magnetizing the semen extender, as magnetized water has higher electron donor ability which could allow free radicals to be better removed. Lee and Park (2015) evaluated whether a previous, short (5 min) exposure of the extender to different magnetic fields (2000, 4000 and 6000 G) improved their preservation characteristics over a 7-day storage period. These authors found that plasma membrane integrity after seven days of storage was better preserved when the extender had been exposed to a magnetic field of 4000 G. In addition, *in vitro* fertilization outcomes using semen preserved for seven days were also higher when the extender had been previously magnetized at 6000 G.

Individual response to liquid storage

There is a high individual variability in terms of sperm processing through different techniques, including liquid storage at 15-17ºC, cryopreservation, and sex-sorting of liquid and frozen-thawed sperm, which indicates that the response to semen-processing techniques heavily relies upon each individual boar/ejaculate (Parrilla *et al*., 2012). In this context, an interesting issue regards to whether there is any interaction between breeds and extenders. Martín-Hidalgo *et al*. (2013) investigated the use of MR-A (Kubus) and X-Cell (IMV Technologies) extenders in Duroc and Iberian breeds over a storage period of 7 days, and found that sperm viability did not differ between extenders or breeds. Instead, sperm motility differed between breeds, and while the extender that yielded the best results for Iberian breed was X-Cell, both extenders gave similar results in Duroc breed. In contrast, the effects of storing boar sperm at 18ºC on chromatin integrity have been found to be independent from the breed (Duroc, Landrace, Hampshire, and Danish Large White; Boe-Hansen *et al*., 2005). Therefore, while more research is required to address the potential breed-effect, the current consensus is that individual rather than breed differences exist.

Cryopreservation of boar sperm

Introduction

As aforementioned, pig breeding is mainly performed through AI using extended boar semen. Only in 1% of cases, frozen-thawed semen is utilised and this

is not only explained by the high costs, necessity of equipment and trained staff, but also because of the reduced fertilizing ability which results from sperm cryodamage (See Yeste, 2015, 2016 for reviews). However, cryopreserved boar sperm may be advised in some cases, as that of germplasm banks, and the advances made in the last years have allowed to reach acceptable reproductive performances.

The discovery of glycerol was an important forward step for the cryopreservation of mammalian sperm. In addition, boar sperm cryopreservation also benefited from the use of controlled-rate freezers, as damages inflicted upon the cell are much greater otherwise. In effect, whereas one may cryopreserve mammalian sperm through the conventional method (i.e. nitrogen vapours and immersion into liquid nitrogen), the use of controlled-rate freezers significantly increases boar sperm survival at post-thawing.

Detrimental effects of cryopreservation

Although significant advances in the optimization of boar sperm cryopreservation have been made in the last two decades, freeze-thawing protocols negatively impact on sperm function and survival, which also underlies a further reduction in the reproductive performance (Johnson *et al*., 2000; Yeste, 2015, 2016). Figure 1 and the following subsections are intended to summarise the main cryoinjuries resulting from freeze-thawing procedures.

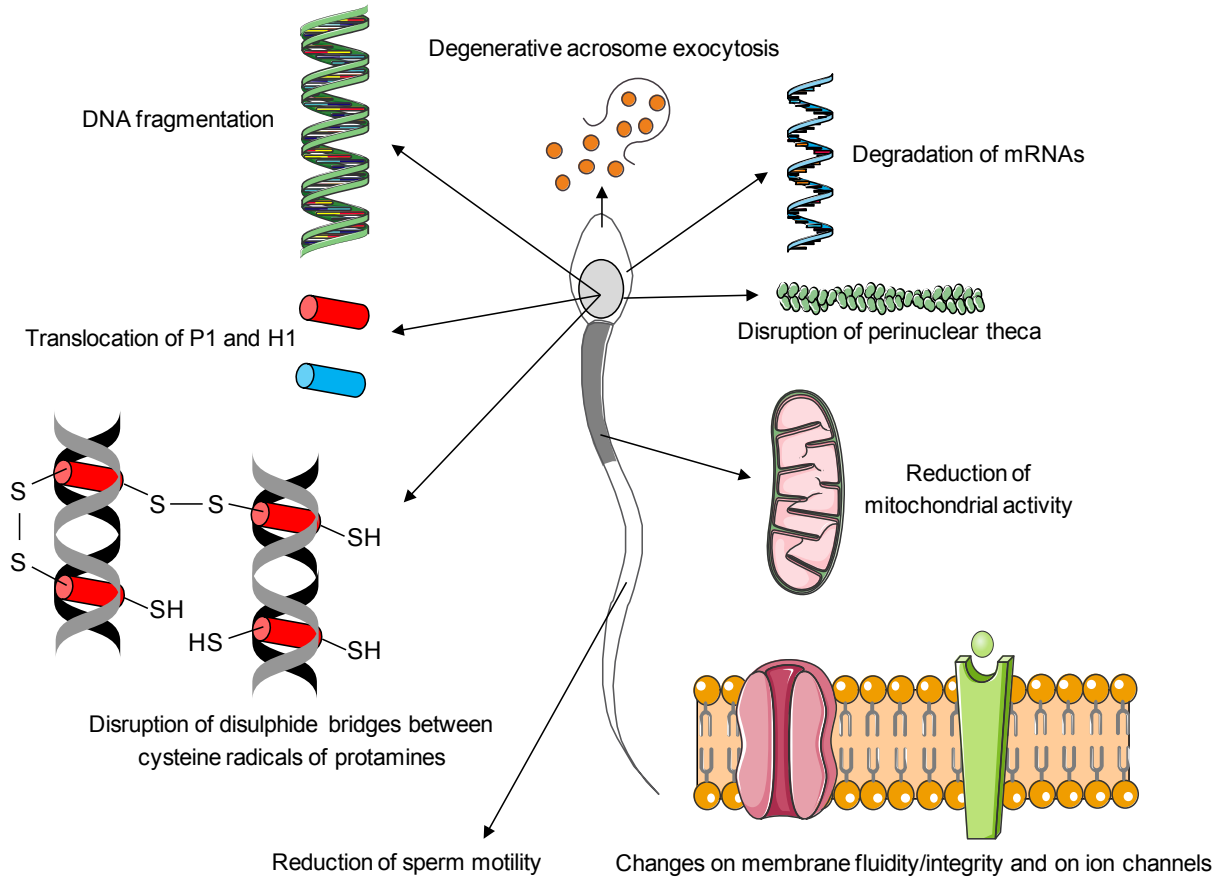

Figure 1. Main cryoinjuries in boar sperm. From Yeste (2016) with permission.

Plasma membrane integrity

As aforementioned, plasma membrane of boar spermatozoa contains high levels of unsaturated phospholipids and low amounts of cholesterol, which makes these cells very sensitive to cold shock (Casas and Flores, 2013). Therefore, temperatures equal to or lower than 5ºC lead the plasma membrane to destabilize, and this affects Ca^{2+} homeostasis, acrosome integrity and membrane lipid packaging (Watson, 2000; Johnson *et al*., 2000; Cerolini *et al*., 2001; Bailey *et al*., 2008). During cold shock, there are lipid-phase separations which cluster integral membrane proteins, release cholesterol molecules and disrupt lipid and protein interactions (Harrison and Miller, 2000; Vadnais and Althouse, 2011). All these changes affect the function of some proteins, such as ions channels, and the selective permeability of plasma membrane, which leads to an increase in the influx of some ions (e.g. Ca^{2+}) from the extracellular space (Johnson *et al*., 2000; Watson, 2000; Bailey *et al*., 2008).

Sperm motility

Significant decreases in the percentages of total and progressive motile spermatozoa and in the values of kinetic parameters are very apparent following freeze-thawing (Flores *et al*., 2009; Yeste *et al*., 2013a, b, 2014; Estrada *et al*., 2014; Fig. 1). However, the reduction of all these sperm motility parameters is

greatly influenced by ejaculate freezability, as ejaculates with poor freezability present significantly lower motility values than those with good freezability (Yeste *et al*., 2013a, 2014).

Chromatin integrity

The integrity of sperm chromatin is also affected by freeze-thawing procedures, and this damage regards to nucleoproteins (mainly protamines), DNA and the interaction between protamines and DNA. On the one hand, cryopreservation of boar spermatozoa has been reported to induce changes in the localization of protamine 1 (P1) and histone 1 (H1; Flores *et al*., 2008, 2011; Fig. 1). In addition, there is an increase in DNA fragmentation, although this damage may not be apparent upon thawing (Flores *et al*., 2011) but rather it may be latent and only evident after incubating frozen-thawed sperm at 37°C for at least 2 h (Alkmin *et al*., 2013; Yeste *et al*., 2013a, b). Cryopreservation of boar sperm also results in an increase in the disrupted disulphide bridges between cysteine radicals of protamines (Flores *et al*., 2011; Yeste *et al*., 2013a). This effect has not only been observed in porcine spermatozoa but also in other mammalian species, such as equine (Yeste *et al*., 2015).

Mitochondrial activity and ROS generation

While cryopreservation is known to reduce mitochondrial activity in boar sperm (Flores *et al*., 2010; Fig. 1), the increase of intracellular ROS levels in response to cryopreservation, which has been reported in other mammalian species, is less clear (Guthrie and Welch, 2006; Awda *et al*., 2009; Kim *et al*., 2011; Yeste *et al*., 2013b). In effect, basal levels of peroxides and superoxides in fresh and frozen-thawed boar sperm are low (Guthrie and Welch, 2006, 2012). Flores *et al*. (2010) found that cryopreservation decreased the capacity of mitochondria to produce ROS. With regard to differences between ejaculates of different freezability, Gómez-Fernández *et al*. (2013) and Yeste *et al*. (2013a) found that intracellular levels of ROS and proportions of sperm exhibiting membrane lipid peroxidation did not differ between ejaculates with different resilience to withstand freeze-thawing procedures.

Other impact: proteins and mRNA

As aforementioned, the function of some relevant proteins, such as ion channels, may be compromised as a result of cryopreservation (Kim *et al*., 2008). It is also worth noting that freeze-thawing induces changes in the localization of proteins, such as actin and mitofusin 2 (Flores *et al*., 2010) and glucose transporter GLUT3 (Sancho *et al*., 2007). Moreover, cryopreservation may also lead to premature phosphorylation of specific sperm proteins, as that of p32 (which probably corresponds to acrosin binding protein) following cooling and freeze-thawing (Kumaresan *et al*., 2011).

Cryopreservation has also been reported to reduce the levels of certain mRNAs, probably because of their degradation or as a result of the loss of the selective permeability of plasma membrane (Zeng *et al*., 2014; Fig. 1).

Ejaculate resilience to withstand cryopreservation

As aforementioned, there is a high variability in the response of ejaculates to sperm processing techniques, including the preservation of liquid and cryopreserved boar sperm (Parrilla *et al*., 2012). In the latter case, this high variability has led to classify boar ejaculates as good or poor freezability ejaculates (Watson, 1995; Casas *et al*., 2009), where freezability is defined as the ability to sustain freeze-thawing procedures (Yeste, 2016). The high individual variability is not only restricted to boars, but has also been observed between ejaculates coming from the same boar and even between fractions of the same ejaculate (Holt, 2000; Holt *et al*., 2005; Peña *et al*., 2006; Waterhouse *et al*., 2006; Yeste *et al*., 2013a).

Good (GFE) and poor freezability ejaculates (PFE) exhibit different motility and sperm membrane integrity following freeze-thawing (Casas *et al*., 2009; Yeste *et al*., 2013a). Apart from more research still being required to address the mechanisms that underlie the different response of GFE and PFE cryopreservation, a main inconvenient is that conventional spermiogram parameters evaluated in fresh semen do not give an accurate prediction of ejaculate freezability (reviewed in Yeste, 2015, 2016). In this context, it is worth mentioning that the levels of certain proteins in fresh/extended sperm appear to confer higher resilience to withstand freeze-thawing procedures. These proteins, which have been identified in sperm and seminal plasma, are known as freezability markers and their relative levels evaluated in fresh sperm should allow the prediction of sperm cryotolerance prior to conducting freeze-thawing procedures.

In the case of sperm, freezability markers are membrane channels and other proteins that appear to be involved in the response to osmotic and thermal stresses. Thus far, the following sperm freezability markers have been identified: heat shock protein 90 (HSP90AA1), acrosin binding protein (ACRBP), triosephosphate isomerase (TPI) and voltage-dependent anion channel 2 (VDAC2; Casas *et al*., 2010a; Vilagran *et al*., 2013, 2014). Acrosin activity in fresh sperm is also related to ejaculate freezability, the ejaculates presenting higher cryotolerance being those with higher acrosin activity (Estrada *et al*., 2015; Pinart *et al*., 2015b).

With regard to seminal plasma, relative amounts of fibronectin 1 (FN1) in fresh seminal plasma have been reported to predict ejaculate freezability (Vilagran *et al*., 2015). In addition, the activity of N-acetyl-β-hexosaminidase in fresh seminal plasma has been found to be negatively correlated with sperm motility, survival and lipid peroxidation, which indicates that high levels of β-HEX activity are linked to low resilience to withstand freeze-thawing procedures

(Wysocki *et al*., 2015).

Cryopreservation extenders

LEY and LEYGO

In pigs, sperm cryopreservation takes place in two steps. A first step consists of gradually decreasing the temperature from 15-17ºC to 5ºC (this process lasts between 30 and 240 min, depending on the protocol). In the second step, the temperature is decreased from 5 to -196ºC (Eriksson and Rodríguez-Martínez, 2000; Casas *et al.*, 2009). The extenders used in both steps (LEY and LEYGO) contain buffer systems (like Tris), detergent agents and non-permeating cryoprotectant agents (CPAs), such as egg yolk and sugars (Hu *et al.*, 2012; Benson *et al*., 2012). Non-permeating CPAs do not pass through plasma membrane and develop its role extracellularly. The combination of egg yolk with the surfactant Orvus ES Paste (Equex) facilitates the interaction of egg yolk proteins with the sperm plasma membrane (Holt, 2000; Rodríguez-Martinez and Wallgren, 2011). In addition, LEYGO, which is the freezing extender used for the second step contains glycerol, a permeating CPA(Gao and Critser, 2000; Gadea, 2003). Therefore, the inclusion of CPAs is critical as slow freezing may lead to form ice crystals within the cell and the presence of CPAs may help prevent the formation of such crystals. That being said, CPAs are usually harmful for the cell upon thawing and it is thus critical to use the proper concentration and proceed to a quick removal.

Previous works have evaluated the impact of replacing one of the non-permeable CPAs (egg yolk) by low-density lipoproteins (LDL), which are part of the egg yolk mixture and are thought to exert the protective effect. These reports concur that replacing the egg yolk by LDL increases boar sperm quality and chromatin integrity as the assessment of sperm at post-thawing indicates (Fraser and Strzezek, 2007; Fraser *et al*., 2007; Hu *et al*., 2008).

With regard to permeating CPAs, glycerol is the most commonly used CPA, despite other agents such as dimethylsulfoxide (DMSO) being used for cryopreserving sperm in other mammalian species. Permeating CPAs decrease the concentration of cytoplasm electrolytes and reduce the extent of osmotic shrinkage at low temperatures (Mazur, 1984; Gao and Critser, 2000). In the case of boar sperm, no permeating CPA has yielded better results than glycerol (Buranaamnuay *et al*., 2011; Malo *et al*., 2012) and its optimal concentrations have been reported to range between 2 and 3% (Buhr *et al.*, 2001; Okazaki *et al*., 2009; Zeng *et al*., 2014).

Improvement of cryopreservation extenders

Different attempts have aimed at improving the sperm function and survival at post-thawing by supplementing freezing and thawing extenders with different additives. The present section discusses the additives that have given better results, such as seminal plasma, cholesterol-loaded cyclodextrins and antioxidants.

The addition of seminal plasma (5%) obtained from GFE to freezing extenders increases the survival, motility, and in vitro fertilization outcomes of frozen-thawed sperm of poor freezability ejaculates (Hernández *et al*., 2007). The results of adding thawing medium with seminal plasma are less clear as whereas some authors indicate that it is beneficial for sperm motility and membrane integrity (Gómez-Fernández *et al*., 2012; Okazaki and Shimada, 2012), others report that it leads to a decrease in the proportions of sperm exhibiting an intact plasma membrane (Fernández-Gago *et al*., 2013). Since conditions between studies differ, more research is required to address this inconsistency.

The addition of cholesterol-loaded cyclodextrins (CLC) to freezing extenders has been reported to increase the levels of cholesterol in the plasma membrane, which results in an augment in the stability of that membrane and thus in the post-thaw sperm survival and fertilizing ability (Tomás *et al*., 2011, 2013; Blanch *et al*., 2012).

Supplementing freezing and thawing extenders with different antioxidants has also been tested as a way to improve the sperm quality at post-thawing. There is quite a long list of antioxidants tested that have been reported to provide good results, including L-cysteine, α-tocopherol, lutein, butylated hydroxytoluene, Trolox, reduced glutathione and ascorbic acid (Roca *et al*., 2004; Chanapiwat *et al*., 2009; Jeong *et al*., 2009; Kaeoket *et al*., 2010; Satorre *et al*., 2012; Giaretta *et al*., 2015; Varo-Ghiuru *et al*., 2015). Among them, reduced glutathione is one of those that have been more extensively studied and has been found to increase sperm quality at post-thawing and augment the fertilizing ability both in vivo and in vitro (Gadea *et al*., 2005; Yeste *et al*., 2013a, 2014; Estrada *et al*., 2014, 2015; Giaretta *et al*., 2015).

Fertility of frozen-thawed boar sperm

It is generally understood that the reproductive performance of cryopreserved boar sperm is lower than that of fresh/extended semen (Knox, 2015). In spite of this, the research conducted in the last decades has allowed to increase the fertilizing ability of frozen-thawed sperm. In effect, recent reports indicate that litter sizes equal to or higher than ten piglets born per litter may be obtained (Didion *et al*., 2013; Knox, 2015). In addition to this, both farrowing rates and litter sizes may be increased if additives such as reduced glutathione are used (Estrada *et al*., 2014; Knox, 2015).

A very important point to be kept in mind is the AI technique used for insemination, with post-cervical and deep intrauterine insemination being more suitable than conventional AI (Martínez *et al*., 2001; Casas *et al*., 2010b). Other aspects that must be taken into account are the interval of insemination-to-ovulation and the fact that AI with frozen-thawed sperm yields better results when double insemination is performed (Spencer *et al*., 2010). If all these points are not considered, the risk of a dramatic drop in reproductive

performance is very high.

Conclusions

Artificial insemination is widely used for pig breeding. Most of AIs are conducted with extended semen, as the capacity of extenders to preserve sperm quality and fertilizing ability allows obtaining high farrowing rates and litter sizes. In contrast, boar sperm cryopreservation is not routinely used as, despite being the most effective method for long-term preservation of mammalian sperm, it is costly and may reduce sperm quality and fertilizing ability. In spite of this, boar sperm cryopreservation may be advised in some cases, as that of germplasm banks, and the advances made in the last years have led to reach acceptable reproductive performances.

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