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Cryopreservation of boar semen

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Abstract: Sperm cryopreservation is the best technology for long-term storage of the semen. However, the damage of boar spermatozoa by cryopreservation is more severe than in other animal species and a standardized freezing protocol for efficient cryopreservation has not been established yet. Semen quality and freezability vary greatly between breeds as well as between individual boars and even the season. Boar spermatozoa are sensitive to low temperatures; they sustain damage and a high rate of mortality and freezing/thawing the boar semen may strongly impair the sperm function and decrease the semen quality. The freezability of boar semen can be influenced by a cryopreservation procedure, and also by using various additives to freezing and thawing extenders such as antioxidants. In order to obtain acceptable results after thawing the boar semen, it is necessary to combine an optimal amount of additives (glycerol, egg yolk, sugars, antioxidants), cooling and warming velocities.

Keywords: additives; antioxidants; extenders; freezability; sperm

Introduction

Sperm cryopreservation is the most efficient method for the long-term storage of semen in domestic animals (Yeste 2015). However, only 1% of the boar semen that is used for artificial insemination (AI) throughout the world is cryopreserved (Techakumphu et al. 2013). The remaining 99% is preserved in liquid form at 16–20 °C (Saravia et al. 2005).

Among the reasons for this restricted use of frozen boar semen are great variability of semen quality and freezability between breeds as well as individual boars and even the season. Frangez et al. (2005) found that it is the best to collect sperm from mature boars (20–24 months) two or three times per week. In their research Ratchamak et al. (2019) revealed no differences in sperm motility collected as sperm-rich fraction and bulk samples, and no differences in any sperm quality parameters after cryopreservation.

Moreover, in comparison with other mammalian species, boar spermatozoa have low survivability rates during the freezing/thawing process and the short lifespan of the surviving spermatozoa (Mazur et al. 2008; Techakumphu et al. 2013) due to their high cold shock sensitivity. Approximately 40% to 50% of boar spermatozoa do not survive cryopreservation. In comparison with insemination with fresh semen, insemination with frozen/thawed semen causes lower fertility rates, a decrease in farrowing rates by 20% to 30%, and smaller litter sizes (Saravia et al. 2005). Due to sublethal damage, fertility rates are still lower with frozen semen than they are with fresh semen, even when using similar numbers of motile sperm (Watson 2000).

The review is focused on using various additives to freezing and thawing extenders and freezing protocols for efficient cryopreservation.

Parameters of boar semen

Where analysing the quality and sperm fertilizing ability of boar semen, the following parameters are evaluated: semen volume, sperm concentration,

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Table 1. Parameters of boar semen quality

Paremeters		Values
Volume		150–300 ml
Concentration		$10 \times 10^9 – 100 \times 10^9$
Viability		> 75%
Motility	total motility	> 80%
	progressive motility	> 60%
Morphology	normal spermatozoa	> 80%
	mature spermatozoa	80–95%
	immature spermatozoa	5–15%
	abberant spermatozoa	5%
pH		7.2–7.5
Osmolality		~300 m Osm/kg

In Table 1 are written the values of parameters which good quality fresh boar semen should fulfil. It is written according to previous studies about the quality of boar semen

total number of spermatozoa per ejaculate, sperm viability, sperm motility and sperm morphology, percentage of mature, immature spermatozoa, sperm abnormalities or aberrant spermatozoa (Table 1).

However, for objective ejaculate evaluation several other markers such as measurement of sperm-produced reactive oxygen species, DNA fragmentation analysis and biomarkers (ubiquitin, aggresome) can be used (Sutovsky 2015).

Differences in sperm freezability

Differences exist between breeds, individual boars, within the same breed, and between ejaculates from the same boar (Techakumphu et al. 2013). Ejaculates from Duroc boars exhibit better freezability than those of Landrace boars; this is confirmed by the higher percentage of spermatozoa with intact plasma membranes and acrosomes in post-thawed samples taken from Duroc males (Waterhouse et al. 2006). According Roca et al. (2006), Landrace and Pietrain boars have higher post-thaw sperm motility, membrane integrity, mitochondrial membrane potential, and acrosomal integrity than do Large White, Duroc, and Yorkshire boars.

In their research Waterhouse et al. (2006) found differences within breeds male-to-male in post-thaw percentages of live sperm, which may be related with the amount of long-chain and polyunsaturated fatty acids in the sperm plasma membranes. There are consistent and genetically determined variations in frozen-thawed sperm quality and fertility rates between boars. The farrowing rate is 20% higher in post-thawed spermatozoa from Large White boars than it is in that of Landrace boars; however, the farrowing rate of fresh semen from the same boars is higher for Landrace boars than for Large White boars (Johnson et al. 1981; Johnson et al. 1982; Thurston et al. 2001).

Differences in fertilizing ability between breeds, and even between individual boars, are related to differences in sperm head dimensions (Pena et al. 2006; Saravia et al. 2007). High-fertility boars produce spermatozoa with smaller and shorter heads than low-fertility boars (Hirai et al. 2001). Genetically determined characteristics of spermatozoa membrane structure are also important for a predisposition to survive under cryopreservation stress (Watson 2000).

Not all boar ejaculates possess the same ability to withstand freeze/thawing. For this reason, boars and their ejaculates are classified as 'good freezers' or 'bad freezers' (Hernandez et al. 2007a) and as 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE) (Yeste et al. 2013). Variability of semen to sustain sperm cryopreservation could be related to differences in the seminal plasma composition (Roca et al. 2006). Supplementing freezing extenders with seminal plasma from boars with good sperm freezability could eventually improve the ability of boar spermatozoa to sustain the freezing-thawing process, improve sperm motility, maintain acrosome integrity, delay capacitation-like changes, and increase resistance to cold shock or oxidative stress (Roca et al. 2005; Hernandez et al. 2007b). Sperm freezability in boars can be modulated through diet modifications, as well as through changes in feeding regimens and trial lengths (Yeste et al. 2010; Yeste 2015).

Overconsumption of high-energy diets has negative effects (Rato et al. 2014); on the other hand, consumption of some dietary fats (Safarinejad et al. 2010), microminerals and vitamins will maintain and improve sperm quality in humans (Vujkovic et al. 2009; Prasad 2013). In fresh boar sperm sample, sperm motility is appreciably slower in comparison with other mammalian species such as horses, dogs, bulls, or mice (Quintero-Moreno et al. 2003; Mogas et al. 2011), also boar sperm cryosurvival

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after freeze/thawing is consistently lower in comparison with other species (Rodrigues-Martines and Wallgren 2011).

Cryopreservation of boar semen

During cryopreservation, boar semen is exposed to physical and chemical stress, and less than 50% of the spermatozoa survive with their fertilizing ability intact (Waterhouse et al. 2006). Many researchers created protocols for the cryopreservation of boar semen, e.g. Pursel and Johnson (1975), Larsson et al. (1977), Bwanga (1991), Johnson et al. (2000), and Athurupana et al. (2015). The steps of cryopreservation are: 1) reducing (1 : 1) extension with buffer and a period of equilibration in the presence of homologous seminal plasma. The step promotes the capacity of spermatozoa to withstand thermal shock events usually done while cooling the semen to 15 °C. 2) Separation of the bulk of seminal plasma by centrifugation, thus re-concentrating the spermatozoa. 3) The inclusion of chilling protectants (lactose and egg yolk) during cooling to 5 °C. 4) The inclusion of penetrating cryoprotectants (the most commonly used glycerol at a 4% final concentration) immediately prior to freezing to enable rapid decreases of temperature, which are usually effected by programmable freezers.

When following the cryopreservation protocols, centrifugation is a necessary step taken prior to freezing because it facilitates the removal of seminal plasma and concentrates the spermatozoa that are rediluted with freezing extenders (Zhang et al. 2012). The optimal concentration of boar spermatozoa is about 1×10^9 spz/ml (Bolarin et al. 2006). Carvajal et al. (2004) demonstrated that high *g*-force (2 400 × *g*) and short centrifugation time (3 min) achieved high sperm recovery and yield, as well as a positive effect on the cryosurvival of boar sperm. However, other researchers recommend other values for centrifugation rates, i.e. 300 × *g* for 10 min (Pursel and Johnson 1975), 800 × *g* for 10 min (Westendorf et al. 1975), 1 400 × *g* for 5 min (Larsson et al. 1977), and 700 × *g* for 3 min (Athurupana et al. 2015).

Cryopreservation produces physical and chemical stress on sperm structures and reduces post-thaw sperm viability and fertilizing ability. There are many reasons for the negative effects of cooling, freezing and thawing. Boar spermatozoa are very susceptible to peroxidative damage because of the high content of polyunsaturated fatty acids (Esmaeili et al. 2015) and low level of cholesterol in the plasma membrane (Cerolini et al. 2000). The main causes are lipid phase transitions, ice crystallization and osmotic-induced water fluxes. It results in membrane reorganizations that can disrupt membrane integrity, structure and function. The successful freezing of boar semen is dependent on many factors that affect the capacity of spermatozoa to survive freezing and thawing. These factors may be classified into two categories: 1) internal or fixed factors such as the inherent characteristics of spermatozoa or differences between boars and ejaculates and 2) external factors such as composition of diluents, types and concentrations of cryoprotective agents, dilution and cooling rates, equilibration, and semen freezing and thawing methods (Johnson et al. 2000; Watson 2000).

While internal factors cannot be improved by modification of the freezing protocol, external factors have the ability to improve semen freezability. Sperm selection through density gradient washing has also been reported to increase ejaculate freezability prior to cryopreservation (Macias Garcia et al. 2009).

The freeze/thaw processes of boar semen are important, but some authors reported that the length of the holding time (total length of storage time at temperature of 15–17 °C from semen collection to the beginning of cryopreservation procedures) affects also the ability of the sperm to withstand freeze/thaw procedures (Juarez et al. 2011; Alkmin et al. 2014).

However, regardless of the protocol used, boar spermatozoa are very susceptible to cold shock, and rapid cooling from body temperature to temperatures below 15 °C irreversibly reduces their viability (Johnson et al. 2000). During slow freezing, boar spermatozoa are also sensitive to cellular injury and mechanical stress caused by ice formation around of the cells (Rodriguez-Martinez and Wallgren 2011; Di Santo et al. 2012).

The most critical variables that influence sperm cryosurvival and thus serve as prerequisites for an optimal sperm cryopreservation protocol are the cooling (Said et al. 2010) and thawing rates. The most appropriate freezing rate is the fastest one that allows extracellular water freezing without intracellular ice formation. The optimal cooling rate

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for freezing boar spermatozoa is –30 °C/min, while the most suitable rate for thawing is 1 200 °C/min. These cooling and thawing rates lead to acceptable post-thaw sperm survival rates (> 50%) and are thus appropriate to the majority of boars (Devireddy et al. 2004).

In their research Horvath et al. (2018) found that it is possible to improve post-frozen-thawed semen quality by an appropriate application of hydrostatic pressure to boar semen during cryopreservation. Li et al. (2018) concluded that the cryostorage time affected sperm kinetics while the sperm cryopreserved longer than 2 years can significantly decrease post-thaw motility.

In cryopreserved semen, fertilizing ability is lower than that of fresh or refrigerated semen (Rath et al. 2009). When using frozen-thawed boar semen instead of fresh semen for artificial insemination (AI), the farrowing rates are reduced by 10% to 25% and the litter sizes are reduced by 1 to 3 piglets (Johnson et al. 1981; Waterhouse et al. 2006). The boar semen has to be frozen in much larger quantities in comparison with the bull semen. The pellets of approximately 200 ml volume or 10–15 ml tubes are used provided that they contain sufficient spermatozoa for each insemination (Holt 2000).

During thawing, boar semen is exposed to all of the same damaging effects of the cooling and freezing phases, albeit in reverse order (Holt 2000). A high warming velocity is essential for the cryosurvival of spermatozoa (Mazur 1984), particularly within the critical temperature range (–15 °C and –60 °C) in which spermatozoa are most sensitive (Yeste 2015). Mazur (1984) found that the thawing rate effectiveness is dependent on the used freezing rate. The most effective thawing rate for boar semen is 1 200 °C/min to 1 800 °C/min (20 s in 50 °C water), which limits the exposure of the cell to such harsh conditions as well as regrowth of ice crystals (Fiser et al. 1993; Thurston et al. 2001). Athurupana et al. (2015) found that rapidly thawing boar semen at 70 °C for 8 s followed by a stabilizing procedure at 39 °C for 52 s also maintained all semen parameters. Thawing similar to freezing impaired boar spermatozoa motility, membrane, and acrosome integrity (Okazaki et al. 2009; Yeste et al. 2013) and the number of spermatozoa with damaged plasma membranes dramatically increased (Ortman and Rodriguez-Martinez 2010). Bamba and Cran (1985) reported that rapidly warming boar semen between 5 °C and 37 °C damaged acrosome membranes. However, only a small proportion of spermatozoa will survive proper thawing if semen is frozen at suboptimum rates (Johnson et al. 2000). Freezing at the optimum rate and fastest thawing rates is beneficial for all parameters of the semen quality, including the motility and acrosome integrity of spermatozoa (Salamon et al. 1973; Pursel and Johnson 1975). A fast thawing rate could not be achieved effectively when the ejaculate is packed in maxi-straws of large diameter (Salamon et al. 1973; Weitze et al. 1987). Fiser et al. (1993) suggested to use of 0.5 ml plastic straws cooled by a flow of liquid nitrogen vapour (using a programmable freezing system) to properly control the freezing rate, on the other hand in the research of Eriksson and Rodriguez-Martinez (2000) the ejaculate was cryopreserved in 5-ml plastic bags.

The protocol for the crucial steps of cryopreservation and thawing of boar semen according to listed authors is summarized in Table 2.

Table 2. Protocol for cryopreservation and thawing of boar semen according to listed authors

Processes	Values	References
Centrifugation	2 400 × g/3 min	Caravajal et al. (2004)
Concentration of semen	1×10^9 spz/ml	Bolarin et al. (2006)
Extenders	egg yolk based glycerol free extender contuining 100 mM trehalose	Athurupana et al. (2015)
Packaging	0.5 ml straws	Athurupana et al. (2015)
Optimal cooling rate	–30 °C/min	Devireddy et al. (2004)
Critical temperature range	–15 °C to –60 °C	Yeste (2015)
Thawing rate	1 200 °C – 1 800 °C/min or 20 s in 50 °C water or at 70 °C for 8 s	Fiser et al. (1993); Thurston et al. (2001); Athurupana et al. (2015)

In Table 2 a protocol is written – the main steps for cryopreservation and thawing of boar semen according to previous studies

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Extenders and antioxidants

Defensive strategies are useful for the cryopreservation of boar semen which comprise the addition of different cryoprotectants, antioxidants, animal serum, antifreeze proteins, fatty acids to freezing media to protect sperm cells against damage (Hezavehei et al. 2018). Extenders may be divided into the following categories: (1) extenders without buffers, such as egg yolk-glucose, egg yolk-lactose and egg yolk-saccharose, ethylenediamine-tetraacetic acid (EDTA), and Mg and Ca salts; (2) extenders with a buffering capacity, such as glycine-phosphate and glucose-phosphate, egg yolk-glucose-citrate, egg yolk-glucose-citrate-EDTA-potassium-unitol-urea, Beltsville F3, Beltsville F5, Tes-tris-fructose-citrate-egg yolk, TEST, Tes-NaK-glucose-egg yolk, Tris-fructose-EDTA-egg yolk, and Tris-glucose-EDTA-egg yolk (Jian-Hong et al. 2006). Many researchers have used different extenders for the cryopreservation of boar semen.

Freezing extenders for boar semen usually contain egg yolk in addition to other agents, such as buffers, additives and cryoprotectants (Gutierez-Perez et al. 2009). Egg yolk is widely used as a cryoprotective agent in semen freezing extenders to protect the spermatozoa from cold shock during cryopreservation (Bathgate et al. 2006) Consequently, low density lipoproteins (LDL) are commonly used in the extenders at concentrations of 9% (Jian-Hong et al. 2006). The most common cryoprotectant for boar sperm is glycerol at concentrations of 2% to 4% (Corcuera et al. 2007). Higher concentrations of glycerol decrease survivability post-thaw and compromise acrosomes, resulting in reduced fertility (Holt 2000). Amides, especially DMA (dimethylformamide) and DMF (dimethylacetamide), can successfully replace glycerol as penetrating cryoprotectants used in freezing the boar semen. Although glycerol and amides can protect sperm cells during cryopreservation, their cryoprotectant properties are achieved through different mechanisms (Bianchi et al. 2008).

The most commonly used extender additives include sugars, proteins and lipoproteins, detergents, antioxidants. The combination of sugar and glycerol has been found to be indispensable in providing protection during freezing. This is so because the osmotic properties of sugar provide extracellular protection (Salamon et al. 1973). Sugars do not penetrate into plasma membranes; they increase the percentage of unfrozen water at a given temperature or reduce the concentration of salts in the unfrozen extracellular water (Mazur 1984). The use of cryoprotectants like trehalose, sucrose, lactose, glucose, and fructose that are added to the extenders results in enhancing spermatozoa viability and fertilization parameters after thawing the boar spermatozoa (Malo et al. 2010; Athurupana et al. 2015). Gomez-Fernandez et al. (2012) tested the effects of different monosaccharides (glucose, galactose, fructose) and disaccharides (lactose, sucrose, melabiose, trehalose) on boar sperm quality after cryopreservation and found that the freezing extenders supplemented with monosaccharides provided a lower cryoprotective effect than the extenders supplemented with lactose.

Gadea et al. (2004) found that adding glutathione to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability. In contrast, the addition of glutathione to the thawing extender resulted in a significant increase in sperm fertilizing ability. Jeong et al. (2009) found that the freezing extender supplemented with α-tocopherol (200 M) prior to cryopreservation had a positive effect on post-thaw boar spermatozoa survival. The addition of the antioxidant vitamin E to boar semen prior to cryopreservation positively affected motility after thawing (Pena et al. 2003). The seminal plasma (SP) contains abundant antioxidants (Makker et al. 2009), and evidence suggests that as long as spermatozoa are suspended in SP, they are protected from oxidative damage (Saleh and Agarwal 2002). In their research Barranco et al. (2019) tested the effect of seminal plasma cytokines (Barranco et al. 2015) on the cryopreservation of boar semen and they proved that seminal plasma cytokines are capable to sustain the preservation of sperm in frozen state. Varo-Ghiuru et al. (2015) found that adding lutein to the semen extender before freezing improved motility, acrosome integrity, plasma membrane function and DNA integrity of boar semen after thawing.

L-glutamine has the ability to cryoprotect the boar sperm by enhancing post-thaw sperm motility, and it can be used as a partial glycerol substitute in the freezing extenders (de Mercado et al. 2009). The addition of sodium dodecyl sulphate (SDS) to semen extenders increases the survival of spermatozoa (Axner et al. 2004). The addition of superoxide dismutase (SOD) and catalase (CAT) to a semen extender before cryopreservation re-

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Table 3. Extender additives used for the cryopreservation of boar semen

Additives		References
Egg yolk		Bathgate et al. (2008)
Low density lipoproteins (LDL)		Jian-Hong et al. (2006)
Glycerol		Corcuera et al. (2000)
L-glutamine		de Mercado et al. (2009)
Sodium dodecyl sulphate (SDS)		Axner et al. (2004)
Sugars	trehalose, sucrose, lactose, glucose, fructose	Malo et al. (2010); Fernandez et al. (2012); Athurupana et al. (2015)
Antioxidants	glutathione	Gadea et al. (2004)
	α-tocopherol	Jeong et al. (2009)
	seminal plasma	Barranco et al. (2015)
	vitamin E	Pena et al. (2003)
	ascorbic acid	Breininger and Beconi (2014); Varo-Ghiuru et al. (2015)
	lutein	Varo-Ghiuru et al. (2015)
	superoxide dismutase (SOD)	Roca et al. (2005)
	catalase (CAT)	Roca et al. (2005)

In Table 3 are listed all extender-additives used for the cryopreservation of boar semen in previous studies

sulted in better post-thaw sperm survival (Roca et al. 2005). Adding ascorbic acid to the thawing extender decreased lipid peroxidation in a dose-dependent manner and increased post-thaw motility in the boar sperm (Breininger and Beconi 2014; Varo-Ghiuru et al. 2015).

Extender additives used for the cryopreservation of boar sperm are summarized in Table 3.

Conclusion

Although the boar semen is sensitive to low temperatures and only the good quality semen is suitable for cryopreservation, development of new cryopreservation protocols is important, because the semen in liquid form can be stored for 7 to 8 days, but the cryopreserved semen can be stored for a much longer period of time and used when necessary. An optimal protocol for freezing and thawing of individual boar sperm as well as supplementation of extenders with buffers, additives and cryoprotectants is crucial for viability and fertility of spermatozoa. One solution is to add the antioxidant to the boar semen before cryopreservation to avoid oxidative damage of sperm cells. The other solution is to add the seminal plasma semen before cryopreservation and to store the cryopreserved sperm in liquid nitrogen no longer than 2 years. This paper will encourage researchers to continue work on the cryopreservation of boar semen for better understanding of the composition of sperm cells, and to create a protocol which will contribute to improving results in practice. This will result in better utilization of boar semen and greater economic profits.

Conflict of interest

The authors declare no conflict of interest.

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