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Review Article

Advances in Boar Semen Cryopreservation

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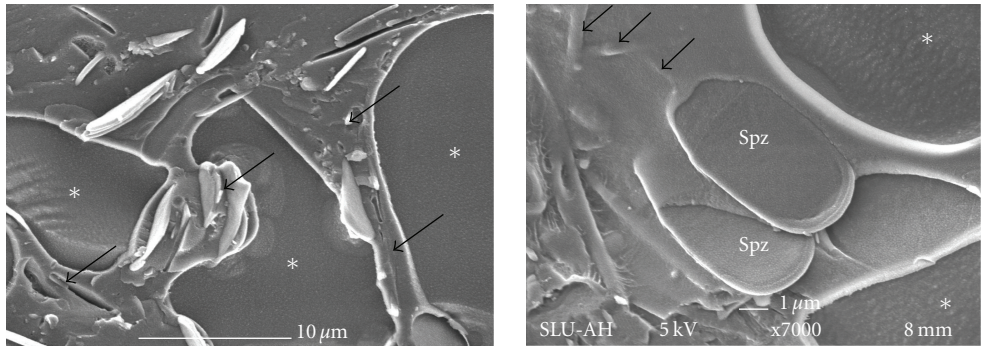
The present paper highlights aspects of the cryopreservation of boar semen, a species with particular large, fractionated ejaculates, and a cumbersome cryotechnology that had prevented its commercial application. With the dramatic increase of use of liquid pig semen for artificial breeding over the past decade, developments on cryopreservation alongside the routine use of stud boar semen for AI had been promoted. Recent advances in our laboratory, accommodating the best use of portions of the sperm-rich fraction of the ejaculate for cryopreservation of the sperm-peak portion (P1) and parallel use of the rest of the collected ejaculated spermatozoa, appears as a suitable commercial alternative.

1. Introduction

Freezing of boar spermatozoa started already by the 1960's and their fertility was reassured using cervical artificial insemination (AI) by 1971 [1].We made the hltd change in the second address. Still, despite documented efforts to reach acceptable fertility and prolificacy after AI, overall boar sperm cryosurvival is consistently low in comparison to other species, owing to damage during a processing that is time consuming, costly and yields few doses per ejaculate. Here we succinctly describe major advancements of boar semen cryopreservation, relevant for research but, particularly, for pig breeding. Selected results had been presented elsewhere (APVS, Japan, 2009).

2. What Happens during Cryopreservation of Boar Spermatozoa?

The biophysical changes brought about by the transition of liquid water to ice during the relatively slow cooling most often used, are the assumed main causes for sperm damage. If spermatozoa are solely frozen in seminal plasma (neat semen) or simply extended with a buffer, such "unprotected freezing" is basically lethal, since ice is formed both outside and inside the cell, damaging essential structures, particularly the plasma membrane and the mitochondria. 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 spermatozoa and, as ice crystals grow in the free water that builds the bulk of this extracellular milieu, the amount of solvent decreases while the solute becomes more and more concentrated. Spermatozoa loose intracellular water in order to compensate for this effective osmotic stress, leading to freeze dehydration of the cells. Eventually, when temperatures are below $\sim -80^{\circ}$C, the highly concentrated, highly viscous solution within and outside the spermatozoa turns into a metastable glassy matrix, which is basically maintained when spermatozoa are stored at -196°C (LN_2). The imaging (using a Cryo-Scanning Electron Microscope, Cryo-SEM [2], Figure 1) of the concentrated medium where spermatozoa are embedded (the so-called veins, arrows in Figures 1(a) and 1(b)) contrasts with the frozen free water (so-called lakes, *in Figures 1(a) and 1(b)). Most spermatozoa in the veins appear intact (Figure 1(b)), that is, they seem to survive the process of cooling. Interesting enough, intracellular ice is rarely formed here, since the speed of cooling is usually low and the presence of the CPA increases viscosity,



(a) (b)

Figure 1: Cryo-Scanning Electron microscopy (Cryo-SEM) micrographs of boar semen conventionally frozen in Flat-Pack, showing the frozen extender (arrows), containing spermatozoa (spz and small arrows) and the areas of frozen free water (* = lakes), Bars: (a) 10 μm, (b)1 μm (Photo: H Ekwall, Uppsala).

enhancing the phenomenon of cell dehydration [3]. So, when are cells damaged? Most of them during thawing, with membranes and axonemes deteriorating by the osmotic imbalance created during cooling [4, 5].

3. Old and New CPA's: Any Improvement?

Accidentally, the apparently first recorded occasion when a CPA was added to a semen extender was the addition of glycerol, a small, polyhydroxylated solute. Glycerol is highly soluble in water through interaction by hydrogen bonding, and can permeate across the plasma membrane, but at a low rate. Since glycerol disturbs cell metabolism at body temperature, boar spermatozoa are usually exposed to this CPA at ~5°C, which; unfortunately; further slows membrane permeation. Mixed with the other solutes of the extender in solution, glycerol depresses their freezing point and ameliorates the rise in sodium chloride concentration during dehydration. Moreover, this simple CPA increases viscosity with cooling (>100,000 cP by −55°C) [6], leading to a retardation of both ice crystal growth and on dehydration speed on a kinetic basis. Moreover, glycerol eliminates eutectic phase changes of the extender [7], making it a very suitable CPA when added at 2%-3% rates. While such interval does not affect cryosurvival in "good-freezer" boars, those considered moderate or bad freezers benefit from a minimum of 3% glycerol [8]. A broad range of other solutes (mostly alcohols, sugars, diols, and amides) have also been tested for CPA capacity [9], but boar spermatozoa react variably. While alcohols and diols can induce membrane blebbing, sugars (such as the disacharides sucrose, raffinose or trehalose which both increase viscosity and stabilise the membrane by interacting with phospholipids) are not better than glycerol, regarding cryosurvival [10]. On the other hand, replacing glycerol with amides (formamide; methyl- or dimethylformamide, MF-DMF; acetamide; methyl- or dimethylacetamide (MA-DMA) at ~5% concentration has proven beneficial for cryo-susceptible boars, probably because the amide permeates the plasma membrane more effectively than glycerol, thus causing less osmotic damage during thawing [11].

4. Do Other Additives than CPA's Increase Cryosurvival?

Some do, such as the use of N-acetyl-D-glucosamine which, at very low rates (<0.1%) has enhanced cryosurvival of boar spermatozoa [12], possibly by interaction with the surfactant Orvus Es Paste (OEP) [13]. Use of OEP or other surfactants [14] are of value when interacting with egg yolk and the plasma membrane [15] (albeit details are still unexplained). Use of low-density lipoproteins (LDLs), most often isolated from egg-yolk from different species [16], has proven beneficial for sperm function post-thaw, particularly for DNA-integrity. Similarly, sperm cryosurvival has been enhanced by the addition of antioxidants [17–19], hyaluronan [20], or platelet-activating factor (PAF) [21], although the beneficial effects vary, particularly when different sperm sub-populations are used.

5. Does Controlled Freezing Improve Cryosurvival?

Yes, it does. This matter has been tested when rates of cooling (and of thawing) could be controlled by use of programmable freezers, and where "optimal" cooling rates were those that substantially diminished the period during which heat was released in the sample during the change of phases of water (i.e., ice was formed). Interestingly enough, experimentally established (yet often empirically) optimal rates of the range 30–50°C/min [22] have been theoretically predicted [23, 24] and confirmed by use of novel procedures, such as equilibrium freezing [25]. In sum, boar spermatozoa are still being "best" cryopreserved (in terms of cryosurvival) 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)

Table 1: Cryosurvival (examined using Computer-Assisted Sperm Analysis, CASA) mean ± SEM percentages of total motile spermatozoa, 30 min post-thaw at 38°C) of boar spermatozoa from P1 (1st 10 mL of the sperm-rich fraction, SRF) or the entire SRF when subjected to a simplified (SF, 3.5 h) or a conventional freezing (CF, 8 h) and an equal thawing (35°C for 20 s).

Simplified freezing (SF, 3.5 h)		Conventional freezing (CF, 8-9 h)	
P1-spermatozoa	SRF-spermatozoa	P1-spermatozoa	SRF-spermatozoa
62.9 ± 3.13[a]	54.2 ± 3.50[a]	70.0 ± 4.40[a]	64.0 ± 2.60[a]

[a]$P > .05$.

thawed. As cited earlier, this protocol would serve most boars while for those with suboptimal sperm freezability, it must be modified, particularly regarding glycerol concentration and warming rates [8]. The entire procedure usually takes between 8 and 9 hours from collection to storage of the frozen doses in liquid nitrogen (LN_2), being still tedious (many different steps) and, inconvenient, producing few AI-doses per ejaculate. Moreover, there is a large variation between ejaculates and —particularly— among boars, for their capacity to sustain cryopreservation [26].

6. Are There Any Other Procedures That Improve Cryosurvival?

Yes, the use of cryobiologically adequate packaging systems for the extended spermatozoa has proven successful. Boar semen has been processed in plastic straws of different volumes (0.25 to 5 mL) [27], flattened 5 mL straws [28], metal [29], or in plastic bags of various types and constitution [14, 30–34]. The latter, denominated "FlatPacks" proven equally good or better than 0.25 mL straws in terms of sperm cryosurvival despite the fact that they held 5 mL of semen (an entire dose for cervical AI, 5 billion spermatozoa), thus waiving the need of pooling innumerable straws at thawing. FlatPacks are considered as cryobiologically convenient (very thin and with a large surface to dissipate heat during cooling and warm rapidly during thawing), as those small containers tested. Consequently, the FlatPack proved successful when fertility was tested, with acceptable farrowing rates and litter sizes [35]. However, doses with such large sperm numbers conspire against the best use of the ejaculates and, with the introduction of intrauterine deposition of semen, it opened for the use of smaller containers with high numbers of spermatozoa to contain a single AI-dose. Recently, boar spermatozoa was frozen, highly concentrated, in small volumes (0.5–0.7 mL) in novel containers, the so-called "MiniFlatPack" (MFP), as 1-2 billion spermatozoa/mL [3]. Interestingly, not only the freezing was more homogeneous in MFP than in medium straws [36], also cryosurvival [37] and fertility when using deep-intrauterine AI [38], were equal or higher than for 0.5 mL plastic straws. However, processing semen in the current manner is still unpractical and, therefore, unattractive for routine, commercial use.

7. New Handling of Boar Semen for Freezing

Boar seminal plasma (SP) is a composite, heterogeneous fluid built up by fractionated secretions of the epididymal caudae and the accessory sexual glands. In vivo, spermatozoa contact some of these fractions but not necessarily others, and different effects (sometimes deleterious, sometimes advantageous) have been recorded in vitro when keeping boar spermatozoa in its own SP, depending on the fraction used [39]. The SP or the sperm-rich fraction (SRF) might not be necessary for cryosurvival or fertility, since spermatozoa from boars that were seminal-vesiculectomised were able to sustain freezing and thawing equally well as spermatozoa bathing in seminal vesicular proteins [40]. We have recently determined that boar spermatozoa contained in the first 10 mL of the SRF (also called Portion 1 or P1, where about 25% of all spermatozoa in the SRF are) were more resilient to handling (from extension to cooling) and to cryopreservation than those spermatozoa contained in the rest of the ejaculate [17, 39, 41–43]. It appeared that it was actually the SP in this sperm-peak P1 that was beneficial for spermatozoa, either because of its higher contents of cauda epididymal fluid and specific proteins, or its lower amounts of seminal plasma spermadhesins, bicarbonate, or zinc levels [39], compared to other fractions of the ejaculate [44]. In any case, an attempt was very recently done to simplify the cryopreservation protocol by freezing only the P1-spermatozoa, in concentrated form, for eventual use with deep-intrauterine AI. These spermatozoa were firstly kept in their own SP for 30 min, and thereafter, without centrifugation (i.e., without SP-removal) mixed with lactose-egg yolk (LEY) extender and cooled down to +5°C within 1.5 h, before being mixed with LEY + glycerol (3%) and OEP and packed into MiniFlatPack for customary freezing, using 50°C/min cooling rate. This "simplified" entire procedure (SF), lasted 3.5 h compared to the "conventional freezing" (CF) that was used as control procedure, which lasted 8 h. As controls, spermatozoa from the SRF were compared to P1-spermatozoa. Cryosurvival was, as seen in Table 1, equally good (above 60% of the processed cells) [44]. Moreover, recent studies (unpublished) have shown that there are no major differences when "quickly" freezing the P1 compared to the rest of the SRF-spermatozoa, indicating that the simplified freezing could be routinely applicable, using MFPs.

There are several advantages of using this simplified, shorter protocol, namely, the exclusion of the customary primary extension and the following removal of this conspicuously beneficial SP-aliquot by centrifugation. As well, it waives the need of expensive refrigerated centrifuges. Moreover, interboar variation was minimised by use of P1-spermatozoa which, not only were the "best" spermatozoa to be cryopreserved, but uses a portion of the SRF where

the documented "fertility-associated" proteins are present [39]. Moreover, the procedure frees the rest of the collected spermatozoa (75% of the total) for additional processing of liquid semen AI-doses. This simpler protocol ought thus to be an interesting alternative for AI-studs to—using one and the same ejaculate-freeze boar semen (P1) for gene banking, for repopulation or for commercial distribution, along with the routine production of conventional liquid semen doses for AI, using the rest of the ejaculate. Such procedures would not disturb current handling of boars or their ejaculates. We are at present awaiting the performance of a field trial using such procedures.

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