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Cryopreservation of Porcine Gametes, Embryos and Genital Tissues: State of the Art

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1. Introduction

Preservation of germplasm (e.g. a term hereby applied to collectively gather spermatozoa, oocytes or early embryos whose use would –eventually– lead to offspring) for research, repository building and propagation of genetic material using Assisted Reproductive Techniques (ART) has been a long lasting priority (Mazur et al 2008). The first approaches, besides those historically anecdotic (see Flowers 1999) were directed to the application of artificial insemination (AI) of domestic species (Foote 1999) pertaining dissemination of genetics to a general population of, particularly, production animals. Positive effects for simple cryo-protectant agents (CPA) such as glycerol on animal sperm cryoprotection were demonstrated already by the end of the 1930's (Bernshtein & Petropavloski 1937) and a decade later it became apparent that spermatozoa could be cooled, frozen and thawed in solutions containing egg yolk and glycerol (Polge et al 1949). For some species, such as bovine, the fact that bull semen could be easily frozen with an acceptable sperm survival post-thaw and accompanied by acceptable fertility after intra-uterine AI led to the rapid development of such primary reproductive biotechnology (Rodriguez-Martinez & Barth 2007). Attempts in other species of domesticated animals followed, and it was soon realised that the success seen with bovine could not be reached, primarily due to low sperm survival, difficulties in attaining an optimal deposition or proper timing towards spontaneous ovulation. Differences in survival and fertility varied not only among species but also between individuals of a given species or even ejaculates within sires (Holt 2000).

Porcine male germplasm freezing started already by the 1950's (Polge 1956) but their post-thaw fertility was not reassured using cervical AI until a decade later (Crabo and Einarsson 1971, Graham et al 1971, Pursel & Johnson 1971), which revealed major constrains when applying cryopreservation on boar spermatozoa. Today, despite documented efforts to reach acceptable fertility and prolificacy after AI (Eriksson et al 2002, Roca et al 2011), the cryosurvival of boar spermatozoa is still consistently low in comparison to other species, owing to damage during a processing that is time-consuming, costly and yields few doses per ejaculate (see Rath et al 2009, Rodriguez-Martinez & Wallgren 2011). Number of piglets born is lower than for cooled or neat semen implying that sperm lifespan, deposition site and closeness to ovulation are yet significant hurdles to be overcome (Roca et al 2006b, Wongtawan et al 2006). Preservation of male genetics can also be performed by freezing of

epididymal spermatozoa (retrieved by biopsy of the cauda) or by tissue sampling through testicular biopsies (Keros et al 2005, Curaba et al 2011). However, these approaches are not relevant for porcine breeding. Cauda epididymal spermatozoa are easier to slow freeze than ejaculated spermatozoa (Rath & Niemann 1997) and testicular biopsies are not advisable in boars owing to their highly vascularized testicular capsule (Ohanian et al 1979).

Preservation of female genetics can be done either by freezing of germplasm (e.g. oocytes or embryos) or of ovarian tissue (slices or whole ovary), from which oocytes can thereafter be harvested for ART. Germplasm freezing in pigs has also followed a tortuous road, with deceiving results for decades, particularly related to the high sensitivity of pig oocytes and early embryos to chilling, similarly to other species containing large deposits of intracellular lipids (Zhou & Li 2009), in contrast to blastocysts where the lipid amounts were lower. Delipitation (or side-dislocation of lipid depots by ultracentrifugation) was soon shown to increase the survival of oocytes/embryos subjected to freezing (Nagashima et al 1995), survival that could be enhanced if the cytoskeleton could be preserved from damage using exogenous chemicals (Shi et al 2006). Use of alternative methods such as vitrification instead of slow cooling led to better survival (see Massip 2001) including the birth of offspring (Berthelot et al 2000). However, large variation was seen among methods, sources and laboratories (Holm et al 1999, Cuello et al 2007, Somfai et al 2008, Ogawa et al 2010), including the method used for intrauterine deposition (Rodriguez-Martinez 2007b, Roca et al 2011).

Cryopreservation of ovarian tissue (or even of whole ovaries) has been tested in several species including human (Isachenko et al 2009) pertaining the recovery of follicular oocytes for ART or ultimate autographs (Kim et al 2010). Procedures for porcine ovarian samples have followed methods tested in other species (Imhof et al 2004, Borges et al 2009) with promising results, albeit yet at an academic level, pertaining the advancement of xenografting (Moniruzzaman et al 2009). As well, experimental models using the porcine species have been developed for cryopreservation of genital tissues, particularly the uterus (Dittrich et al 2004, 2006) paving the way for human transplantation procedures (Diaz-Garcia et al 2011).

Thus, interest has been large to attempt routine cryopreservation of porcine gametes, embryos and genital tissues, yet with various degrees of success. Therefore, the present review aims to summarize the state-of-the-art regarding established and emerging methods for the cryopreservation of porcine gametes and embryos as germplasm, intending a critical revision of the underlying problems that still constrain their application for establishing repositories, their use in reproductive biotechnologies and, ultimately, for breeding. As well, it intends to describe our level of knowledge when attempting cryogenics of gonads and other genital tissues for comparative research, particularly on human regenerative medicine. The review is not exhaustive and focus on methodological aspects of procedures.

2. What happens during cryopreservation?

Independently of the cell or tissue above mentioned being considered, the current methods for their cryopreservation fall into one of the two following categories: (i) slow equilibrium freezing or, (ii) rapid non-equilibrium vitrification, and variations within. In either case, the

entire process basically concerns the way we handle the presence of water in and around the cells and whether its freezing is allowed (conventional cryopreservation, slow equilibrium freezing) or totally prevented (vitrification).

In the first method, which is the one traditionally used in biomedicine, particularly for sperm preservation, cells are subjected to slow cooling to temperatures below zero, with freezing rates of 0.5-100 °C/min). The method allows ice to form and solute to concentrate alongside the change in water phase. Both ice and high solute concentrations can cause direct (either initial or eutectic, Han & Bischof 2004a), respectively secondary damage, jeopardizing cell survival or handicapping vital cell functions post-thaw. At some moment during the process, water freezes to form ice, primarily extracellular, but even intracellular. Ice grows and becomes over time surrounded by an increasing amount of solutes which move to the areas where water did not yet changed phase. Cells balance ion concentrations at either side of the plasma membrane thus keeping proper osmotic pressure. Depending on the relative amounts of free and bound water, such a change of phase (either formation or dissolution of ice and de/rehydration phenomena) implies changes in ionic concentration caused by directional movement of water across the membrane, disturbing the homeostatic osmotic pressure of the cell/s (Pegg 2007). Cells respond by allowing water to leave the intra-cellular compartment, to compensate the increasing hyper-osmotic extra-cellular compartment caused by the progressive formation of ice. Those water movements lead both to cell dehydration and to a toxic hyper-concentration of solutes intracellular which, ultimately, affects cells viability (Watson & Fuller 2001). Freezing injury can then be related to high electrolyte concentration effects (solute effects), presence of intracellularly ice (formed direct or eutectic) and also the pressure of large extracellular crystals on the veins of concentrated (i.e. vitrified) extender and cells (Saragusty et al 2009). See **Figures 1 and 2** for an illustration of these events.

Freezing injury during slow freezing can be minimized. Intracellular freezing is generally lethal but can be avoided by sufficiently slowing the rate of cooling. Solute-caused damage, which is the dominating feature under conventional slow freezing especially in cells in liquid suspension, can be minimized by the addition of CPA. Most CPA's (as glycerol, dimethyl sulfoxide (DMSO), ethylenglycol (EG), propyleneglycol (PG)) are highly soluble, permeating compounds of low-to-medium toxicity, whose primary role is to reduce the amount of ice formed at any given sub-zero temperature, by simply increasing the total concentration of all solutes in the system, thus defining the concept of slow equilibrium freezing (Pegg 2002 2007). Introduction of sufficient CPA would eventually avoid freezing and a glassy or vitreous state could be produced instead. Such concept is the theoretical rationale for the second method listed above: rapid non-equilibrium vitrification. Vitrification is the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures (often to -120 to -130 °C) to finally solidify into a metastable glass, without undergoing crystallization at a practical, high speed cooling rate (i.e. dipping onto LN₂). Use of ultra-high speed voids the need of penetrating CPA, open for using non-penetrating CPA (such as sucrose, fructose, glucose), but demands the use of small (5-50µL) suspension droplets. The glassy state is defined by its viscosity reaching 10-13 poises, sufficient for the aqueous material to behave as a solid, without any water crystallization. Once again, this waives the above listed sources for cell injury: ice crystals and increased/ill distributed solute

concentrations. The CPA used to vitrify cells include those used during conventional freezing but at very high concentrations (10-fold higher compared to slow freezing), near the maximum tolerated by the cells, thus becoming potentially harmful (Pegg 2005). Penetrating CPA-free vitrification was attempted already by the early 1940's using rabbit spermatozoa plunged into LN₂ (Hoagland & Pincus 1942). Use of non-penetrating "CPA" (CPA-free concept) such as sucrose has proven feasible for the spermatozoa of some species, including human (Isachenko et al 2004, 2005, 2008, Hossain & Osuamkpe 2007), primates (Dong et al 2009), or canine (Sanchez et al 2011), where sperm suspensions were vitrified (either drop-wise, Isachenko et al (2004, 2005) or contained in 50 μ L-plastic capillaries (Isachenko et al 2011)) by plunging in LN₂, with a cooling rate of \sim 10,000 $^{\circ}$ C/min. Basically, vitrification is therefore always determined by a relation between cooling rate, medium viscosity and sample size.

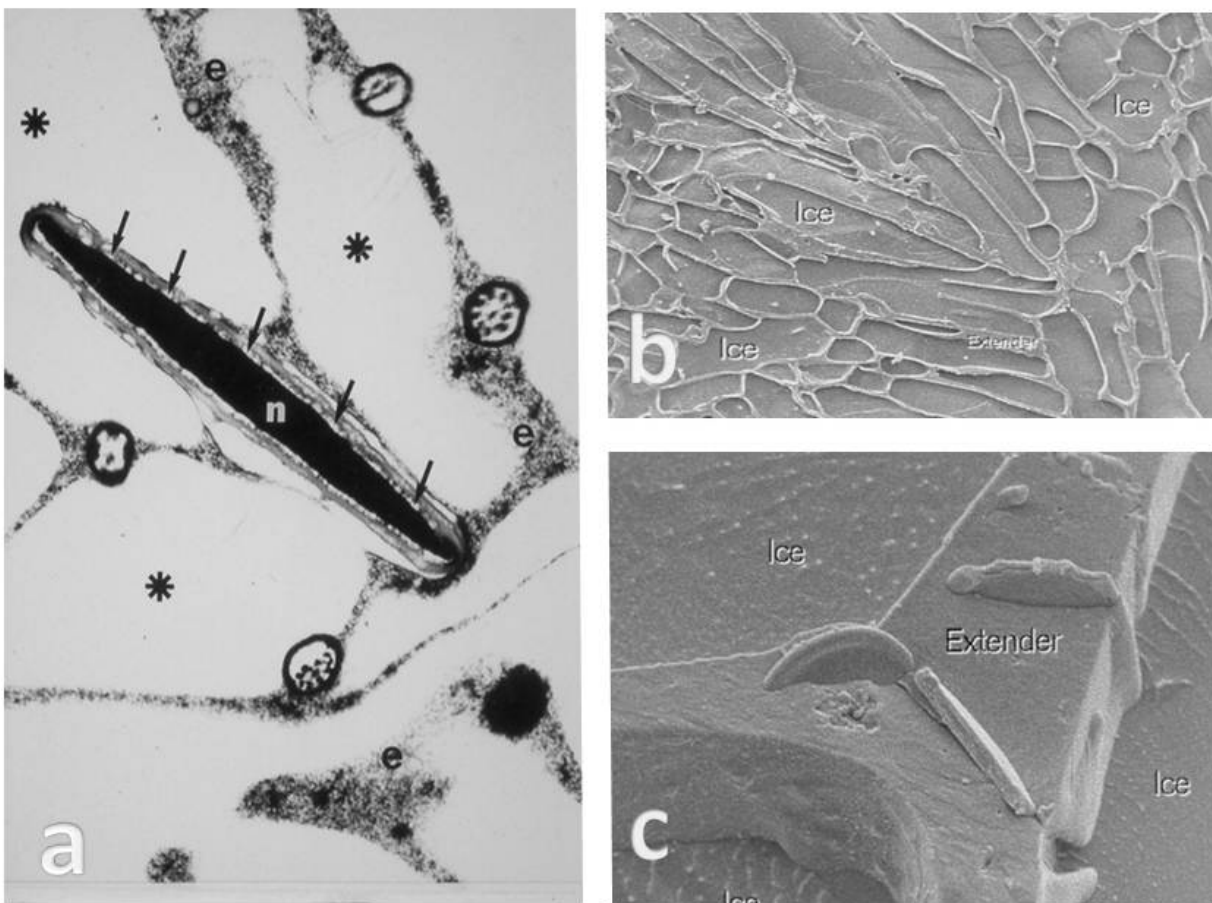


Fig. 1. Micrographs of frozen boar semen illustrated with (a) transmission electron microscopy or (b,c) Cryo-scanning electron microscopy. Spermatozoa were extended and conventionally frozen in maxi-straws (a, 5 mL) or FlatPack™ (b,c, 5 mL) and subjected to freeze-substitution (a) or partial sublimation (b,c) to depict extracellular ice lakes (* in a, marked with legend in b,c) and the veins of concentrated extender (e in a, legend in b,c). Note the presence of intracellular ice marks (arrows in a) and the dislocation of axoneme structures in the tails. Such marks are not seen in the FlatPack™ material (Photo: Dr Hans Ekwall, Uppsala, Sweden).

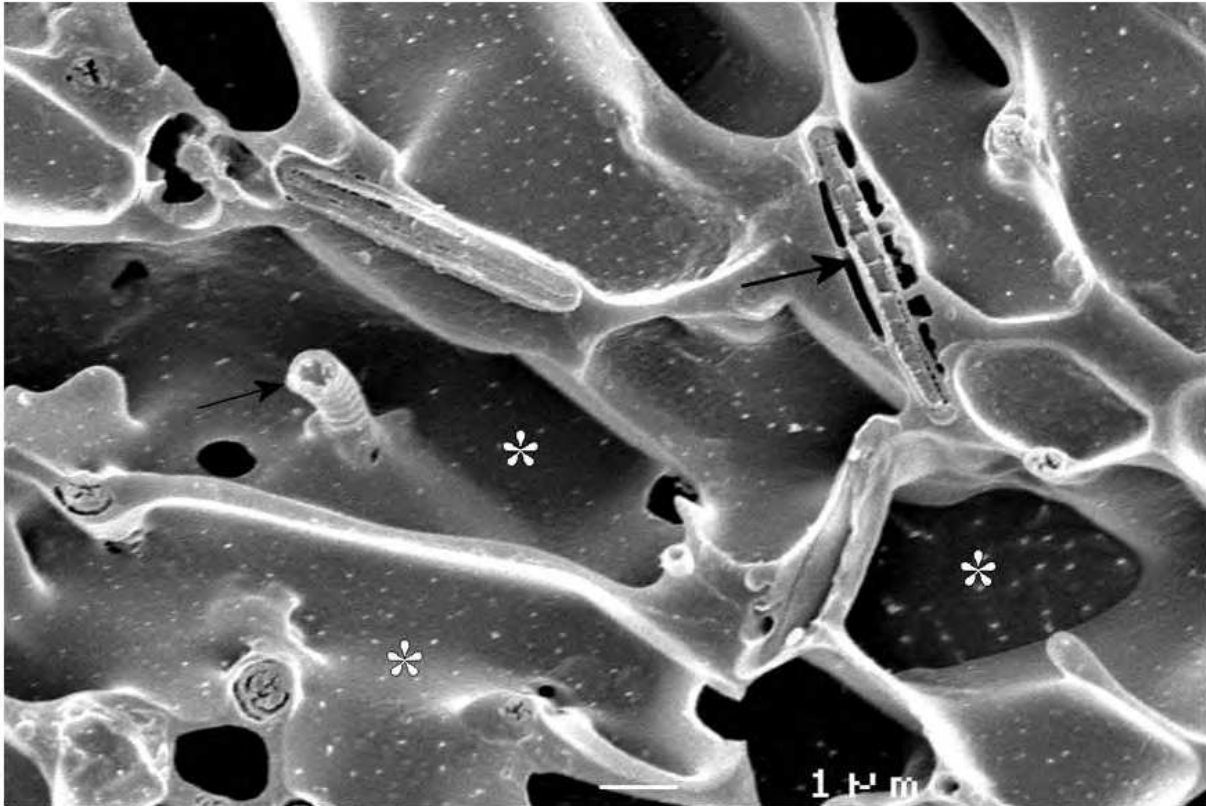


Fig. 2. Cryo-scanning electron microscopy (cryo-SEM) micrograph at higher magnification showing the contents of a maxi-straw frozen at the speed of 1,200°C/min (by direct plunging into LN₂ after initial cooling to +5°C). The ice lakes (*) are small, and surrounded by prominent veins. This fast cooling caused freezing of water both extra- and intracellularly, with clear evidence of sub-cellular distortion caused by the presence of intracellular ice crystals in the peri-nuclear and peri-axonemal areas, owing to a lack of sperm dehydration during the process. Note the fractured sperm head (large arrow) with marks of lethal intracellular ice, and the tail entrapped by extracellular ice (small arrow) with dislocation of the axoneme (Courtesy of Dr Hans Ekwall).

However, we should bear in mind that the physical phenomenon of vitrification (e.g. the process by which a liquid begins to behave as a solid during cooling without substantial changes in molecular arrangement or thermodynamic state variables) is as relevant to conventional freezing, where the cells survive in this glassy medium between ice crystals (see **Figure 1a-c**) as to vitrification *per se*, where the entire sample is vitrified (Wowk 2010). Therefore, seeding is quite relevant for supercooled vitrification solutions in conventional freezing, while it does not play any role during pure vitrification, provided that cooling rates are high. For instance, use of LN₂-slush (e.g. lowering the temperature to near the freezing point of LN₂, -205 to -210 °C by applying negative pressure to the LN₂, Yavin et al 2009) increases the cooling rate 2 to 7-fold compared to simple plunging in LN₂. Viscosity also plays a major role and must increase during cooling, until the glass transition (i.e. the change from liquid to solid) is reached. This concept opens for the freezing of highly concentrated semen samples, provided the size of the sample is small enough.

When thawing or re-warming occurs, the events above described basically reverse. Slow re-warming allows water to reflux to the areas where solutes are concentrated in cells treated

by slow freezing, but the time elapsing is not short enough to avoid the toxicity that the solute concentration exerts on the cells, either leading to cell death or dysfunction. If the re-warming is too slow, ice (intracellular in particular) can damage organelles and the cytoskeleton. Rapid re-warming diminishes these risks since the toxic solutes or CPA are only momentarily present.

For either method listed, the CPA has to gain access to all areas of the cell/tissue/organ. Traditional cooling and re-warming rates affect the fluidity of the membranes of the cell and the organelles through the rearrangement of structural proteins and the dislocation of constituent lipids. If these changes affect diffusion and/or osmosis, they can jeopardize -by causing changes in the viscosity of fluids or inducing osmotic imbalance- the proper distribution of the CPA, its introduction and removal and ultimately, the freezing and the thawing process (Morris 2006, Morris et al 2007). Cooling can disrupt the integrity of the cytoskeleton and of the chromatin structure, including DNA damage (Watson & Fuller 2001, Fraser et al 2011). In cells in suspension, such as spermatozoa, both the form and volume of the sample to be cooled/re-warmed, and the concentration of the contained cells play major roles during the most damaging interval in the process, i.e. during the changes in phase of the extra-cellular water, when heat is either dissipated (during cooling) or incorporated (during re-warming) (Mazur & Cole 1989, Morris et al 1999). It is therefore obvious that samples (cells, tissues, organs) have to pass cooling and re-warming under conditions where cell injury can be minimized (Morris 2006, Morris et al 2007).

3. Cryopreservation of boar semen: State-of-the-art

Porcine AI with liquid-stored semen where either the entire ejaculate or only the sperm-rich fraction (SRF) of the ejaculate is collected, the spermatozoa are re-suspended at low concentrations in chemically defined extenders and stored at 16–20°C for several days before use, most often for up to 3 days) has increased exponentially in the past 25 years. Globally, AI is practiced in 75% of sows (range 10-99%) using >160 million semen doses, with countries in Europe and the Americas having basically all sows under AI (Riesenbeck 2011). Fertility rates are similar to those obtained after natural mating (for a review, see Rodriguez-Martinez 2007a). Liquid semen is therefore used both for production breeding and for genetic improvement at national or regional level, with some export countries having a major international trade. However, the limited shelf-life of liquid semen, its decline in fertility over transit time, and risks of damage due to temperature, pressure or handling changes, all call for alternatives, with a focus on frozen-thawed semen.

Boar spermatozoa are still being “best” cryopreserved (in terms of cryosurvival) using protocols originally devised in the mid 1970’s (Westendorf et al 1975) with modifications (most often empirically introduced). Most methods use standard lactose-egg yolk (or LDL)-based cooling and freezing media, following the removal of most of the seminal plasma by extension in chelate-containing (often EDTA) buffers and centrifugation. The freezing media most often include a surfactant (often laurylsulphate, Orvus es Paste-OEP) and glycerol as CPA (2-3% final concentration added at +5°C). Spermatozoa are further cooled beyond the eutectic temperature at 30 to 50°C/min. Thawing is done at 1,000-1,800 °C/min. The entire procedure takes most often 8-9 hours from semen collection to storage of the frozen doses in LN₂, being still tedious (many different steps) and, inconvenient, producing few AI-doses

per ejaculate (5-8). For examples of current protocols see Eriksson & Rodriguez-Martinez (2000), Saravia et al (2005), Parrilla et al (2009) or Rath et al (2009) and methods cited therein.

This general current protocol fits most boars but considering the large variation between ejaculates and -particularly- among boars for their capacity to sustain cryopreservation (Roca et al 2006a), the protocol has to be modified to accommodate those with sub-optimal sperm freezability (the so-called bad freezers), particularly regarding glycerol concentration and warming rates (Hernandez et al 2007a). Those changes usually allow for minimum acceptable cryosurvival (i.e. around 40%). However, it clearly shows that the methodology is still sub-optimal. Current semen cryopreservation techniques are technically demanding and expensive, both in terms of labour- and laboratory equipment costs, as well as time-consuming (rev by Roca et al 2006b, 2011). Last but not least, there is a lack of reliable laboratory tests for the accurate assessment of semen quality *in vitro*, that limits our capacity to properly monitor the methods used to freeze-thaw boar semen and, particularly, its relationship to AI-fertility (Rodriguez-Martinez, 2007b). This is critical, since despite having acceptable post-thaw survival (even above 60%) this cryosurvival is not reflected in fertility after AI. Thus, boar spermatozoa are considered one of the most demanding cell types with respect to sustaining viability during freezing and thawing, with a large proportion of the spermatozoa not surviving these procedures (Penfold & Watson 2001). Moreover, those surviving spermatozoa are usually a mixture of cells, some of which survive well while others show modified motility and a shortened lifespan, factors which compromise their fertilising ability. Insemination with such spermatozoa leads, ultimately, to lowered pregnancy rates and fewer piglets born, compared with AI using liquid-stored semen (Knox 2011). In sum, although freezing methods are nowadays rather stable in many laboratories and yield above 50% of sperm survival post-thaw, fertility after AI is extremely variable (Parrilla et al 2009). The major constrain is not only the inherent difficulties to freeze spermatozoa from this species (Holt, 2000a,b), but -within the species- the sire-dependent cryosurvivability to the current procedures (Eriksson et al 2002, Holt et al 2005, Gil et al 2005, Waterhouse et al 2006, Hernandez et al 2006, 2007a, Roca et al., 2006a, Parrilla et al 2009, Roca et al 2011).

This variation is usually compensated by the AI of excessive sperm numbers (at least 5×10^9 spermatozoa per AI-dose), i.e. double the numbers of total spermatozoa present in liquid semen doses. Fertility post-AI is nowadays substantially better, closer to AI with liquid semen (Eriksson et al 2002). See **Table 1** for an overview of fertility after conventional (cervical) AI with frozen-thawed boar semen. Fertility with lower sperm numbers is also becoming acceptable when deep intrauterine AI is practiced, although data are still restricted in numbers (Bathgate et al 2006, Roca et al 2006b, 2011). But, even with these huge sperm numbers, overall fertility (as farrowing rates) and prolificacy (as litter size) are still lower than for liquid semen (around 10-30 % lower farrowing rates, and 1-3 less piglets), indicating that other factors are limiting, such as the timing of insemination respective to spontaneous ovulation (Bolarín et al 2006, Wongtawan et al 2006). This implies that we are far from reaching the goals set up by the industry for the use of frozen-thawed semen: 85% of conception rates and a litter size of 11 piglets (Knox 2011). So, frozen-thawed boar semen is still basically limited to research, genetic banking or the export of semen for selected nuclei lines, constituting barely above 1% of all AIs.

Package	AIs	AI-dose (x10 ⁹)	Sows	Farrowed (%)	Litter size (n)	References
Pellet	2	6	334	40	6.4	Didion & Schoenbeck 1996
MS	2-4	2.5	392	48	10.4	Almlid & Hofmo 1996
MS	2-4	2.5	496	57	12.2	Almlid & Hofmo 1996
MS	2-4	2.5	350	50	9	Almlid & Hofmo 1996
MS	2	5	190	62.6	9	Hofmo & Grevle 2000
FlatPack™	2	5	352	72.2	10.7	Eriksson et al 2002

Table 1. Fertility after conventional (cervical) AI in field trials (>100 sows) with frozen-thawed boar semen (modified from Roca et al 2006).

Therefore, it seems -at first sight- unlikely that deep frozen semen will replace the use of fresh semen on an extensive basis even if the fertility levels were similar. It is too expensive considering that the current cryopreservation protocols barely yield half of the doses producible per ejaculate. Since the amount of spermatozoa per dose is minimum twice that of liquid-stored semen, such equation is simply undependable from a commercial point of view both in production costs for AI-doses and the sub-optimal boar use. However, having a reliable cryopreservation method for boar semen would (a) allow selection of genetics from all over the world, (b) enable planned, essential AIs' at the top of the breeding pyramid and so (c) facilitate preservation of top quality genetic lines for ongoing or future breeding programmes and/or (d) offer an extra health safeguard, by allowing completion of any health test specified by a country or breeding organization before use. The challenge is there, undoubtedly.

3.1 Improvements in boar semen freezing

Over the past decade, the cryobiology of boar semen has diminished its empiric approach towards a more experimental one. Major areas of research have involved: (i) the determination of *in vivo* features (particularly regarding seminal plasma (SP) and the presence of characteristic fractions of the ejaculate), (ii) the action of specific additives and different CPA, (iii) the use of automated freezers and of directional gradient freezing and, (iv) the use of novel containers adapted for the freezing of concentrated spermatozoa. Fertility post-AI is nowadays substantially better, closer to AI with liquid semen, even when using lower sperm numbers and alternative sites of sperm deposition, such as deeply intra-utero (Roca et al 2011).

Specific additives and different CPA's: Glycerol, a small, poly-hydroxylated solute highly soluble in water since it interacts with it by hydrogen bonding, and able to permeate across the

plasma membrane, at a low rate, is by far the mostly used CPA for boar semen conventional freezing. Since glycerol disturbs cell metabolism at body temperature, boar spermatozoa are usually exposed to this CPA at $\sim 5^{\circ}\text{C}$, which –unfortunately– further slows its low rate permeation. Mixed with the other solutes of the extender in solution, it depresses their freezing point and ameliorates the rise in sodium chloride concentration during dehydration. Moreover, glycerol increases viscosity with lowering temperatures to more than 100,000 cP by -55°C (Morris et al 2006), leading to a retardation of both ice crystal growth and of dehydration speed on a kinetic basis. Moreover, glycerol eliminates eutectic phase changes of the extender (Han & Bischof 2004b), 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 (Hernandez et al 2007a). A broad range of other solutes (mostly alcohols, sugars, diols and amides) have also been tested for CPA capacity (Fuller 2004, Buranaamnuay et al 2011), but boar spermatozoa react variably. Alcohols and diols can induce membrane blebbing. Sugars (such as the disaccharides sucrose, raffinose or trehalose which both increase viscosity and stabilise the membrane by interacting with phospholipids) are not better than glycerol, regarding cryosurvival (Hu et al 2008), but shows synergistic effects (Gutierrez-Perez et al 2009, Hu et al 2009). On the other hand, replacing glycerol with amides (formamide; methyl- or dimethylformamide, MF- DMF; acetamide; methyl- or dimethylacetamide (MA- DMA) at $\sim 5\%$ 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 (Bianchi et al 2008). Other additives enhance cryosurvival of boar spermatozoa, such as L-glutamine (de Mercado et al 2009) or low rates ($<0.1\%$) of N-acetyl-D-glucosamine (Yi et al 2002a), the latter possibly interacting with the surfactant OEP (Yi et al 2002b). Laurylsulphate, albeit its mode of action is yet unexplained in detail regarding interaction with egg yolk and the sperm plasma membrane, has repeatedly proven valuable (Karosas & Rodriguez-Martinez 1993, Buranaamnuay et al 2009). Use of low-density lipoproteins (LDLs), isolated from egg-yolk from different species (Jiang et al 2007), has proven beneficial for sperm function post-thaw, particularly for DNA-integrity. Similarly, sperm cryosurvival has been enhanced by the addition of antioxidants (Peña et al 2003, 2004a, Roca et al 2005, Jeong et al 2009, Kaeoket et al 2010), hyaluronan (Peña et al 2004b), or platelet-activating factor (PAF, Bathgate et al 2007), although the beneficial effects vary, particularly when different sperm sub-populations are used. Cryosurvival of several cold-shock susceptible species, of which the porcine is one, has been found to improve when cholesterol-loaded cyclodextrins (CLC) are used as additives before cooling (Zeng & Terada 2001, Mocé et al 2010). Cyclodextrins can encapsulate hydrophobic compounds, such as cholesterol, and transfer the cholesterol into membranes down a concentration gradient (Zidovetzki & Levitan 2007). However, it is yet to determine if the effects are substantial and not only individually-related (Waterhouse et al 2006).

Automated freezers and directional gradient freezing: Controlled freezing using programmed freezers improves cryosurvival by use of “optimal” cooling (and thawing) rates e.g. those that substantially diminish the period during which heat is released/absorbed in the sample when water changed phase (i.e. ice was formed/melt). Interestingly enough, experimentally-determined optimal rates of the range $30\text{-}50^{\circ}\text{C}/\text{min}$ (Thurston et al 2003, Medrano et al 2009, Juarez et al 2011) have been theoretically predicted (Devireddy et al 2004, Woelders & Chaveiro 2004) and confirmed by use of novel procedures such as directional freezing where the thermal gradient is monitored by modifying the velocity at which the liquid-ice interface grows so that the size and shape of the ice crystals is maintained within optimal limits. In this methodology,

derived from the principle of seeding, the biological material is moved through a linear temperature gradient, so that both the freezing rate and the ice front propagation are controlled (Arav et al 2002, Woelders et al 2005, Saragusty et al 2007). The method can be advantageously applied both to large samples, frozen in large containers moving along a rim of seeding or to highly concentrated samples, in smaller (i.e. mini-straws) containers.

Cryobiologically best-suited packaging containers: use of cryobiologically adequate packaging systems for the extended spermatozoa, showed a direct improvement of cryosurvival. Boar spermatozoa has been 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 metal (Fraser & Strzezek 2007) or in plastic bags of various types and constitution (Bwanga et al 1991, Mwanza & Rodriguez-Martinez 1993, Ortman & Rodriguez-Martinez 1994; Eriksson & Rodriguez-Martinez 1994; Eriksson & Rodriguez-Martinez 2000, Saravia et al 2005). The latter developed, denominated "FlatPacks™" proven equally good or better than 0.25 mL straws in terms of sperm cryosurvival despite of 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 when thawing. Fertility after conventional cervical AI of FlatPack™ frozen-thawed semen yield acceptable farrowing rates and litter sizes (Eriksson et al 2002). See **Figure 3** for a schematic description of the differences between containers. The FlatPack™ was considered as cryobiologically convenient (very thin and with a large surface) to dissipate heat during cooling and warm rapidly, as those small containers tested. It is important to remember that the freezing in these containers, with high heat dissipation, inflicts less damage to the cells by intra-container mechanical pressure (Saragusty et al 2009). **Figure 4** shows cryological differences in shape and size of frozen water lakes between mini-straws and FlatPack™.

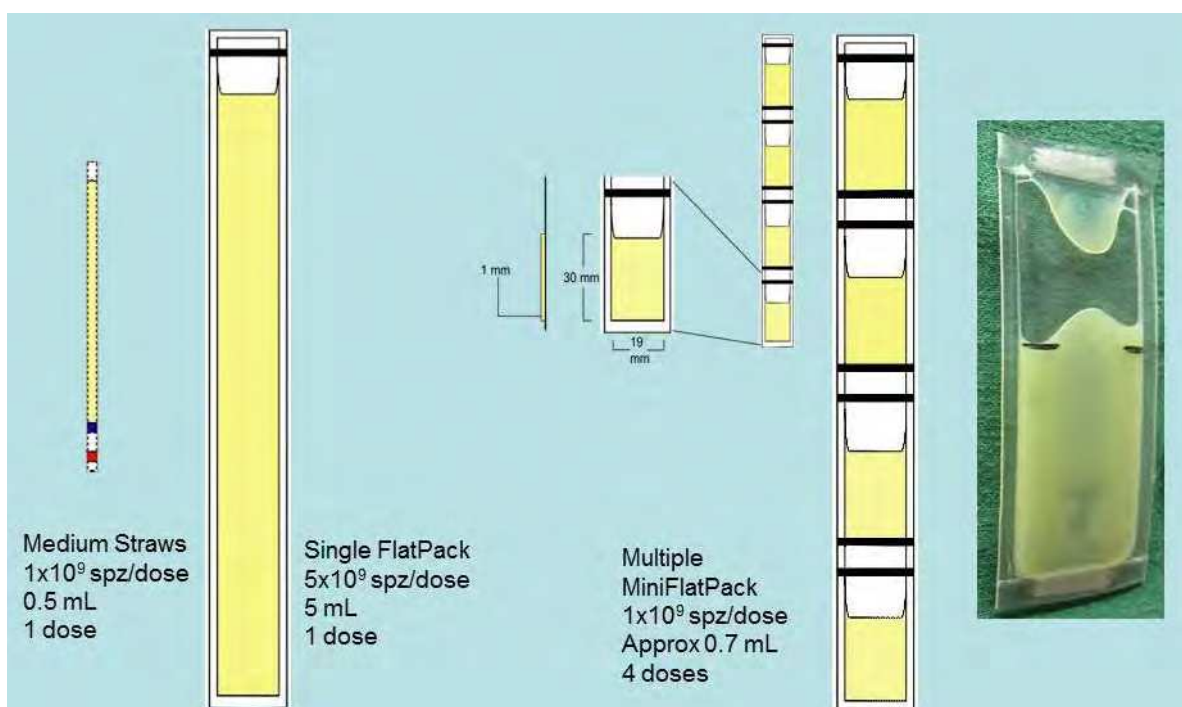


Fig. 3. Schematic representation of the major differences between plastic 0.25 mL mini-straws, with single and Multiple FlatPack™ (the latter also named "MiniFlatPack™, see adjacent photograph of a filled and sealed MiniFlatPack™)(Diagramme/photograph: courtesy of Dr Fernando Saravia).

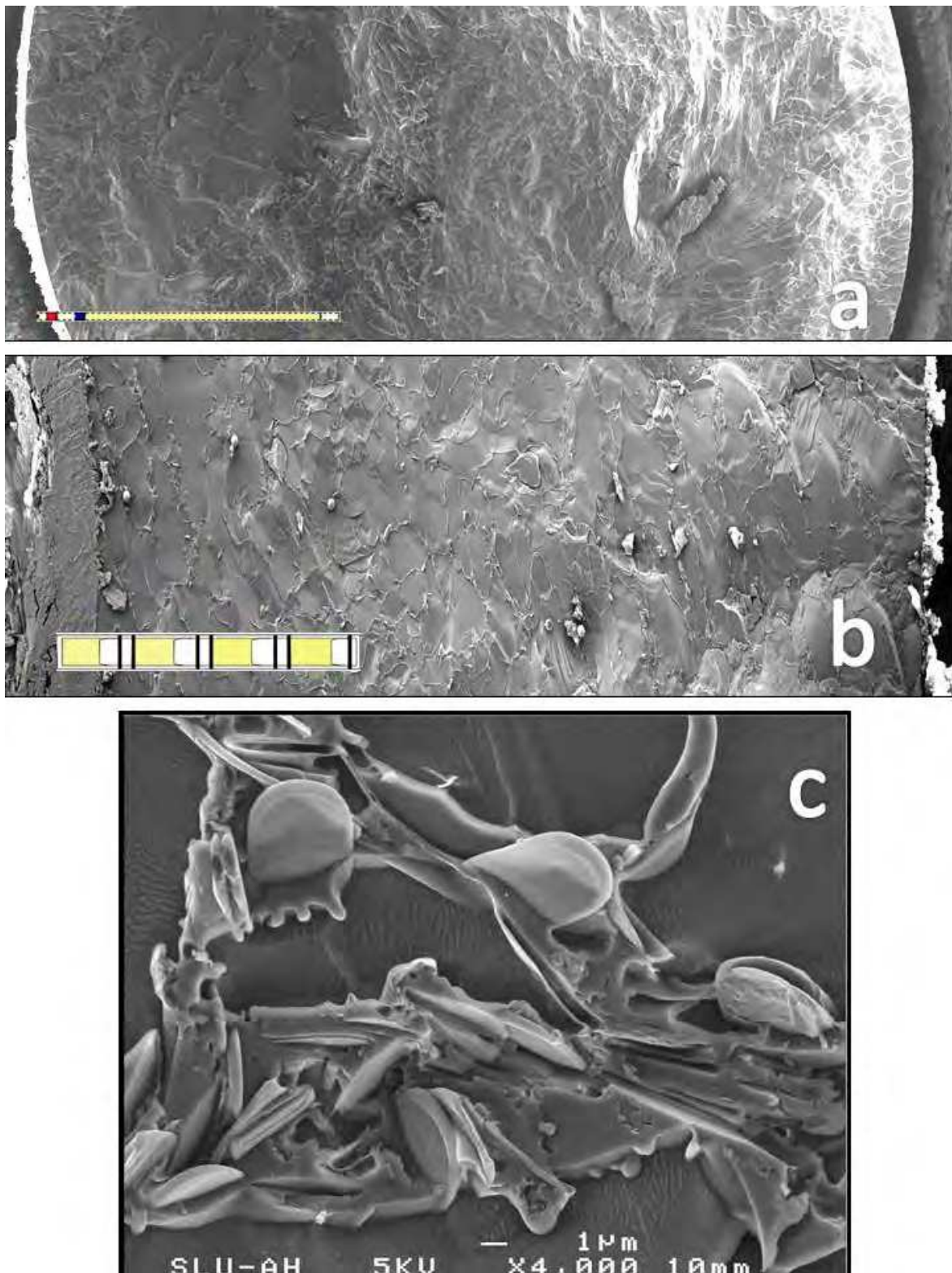


Fig. 4. Cryo-SEM micrographs at low magnification of cross sectioned frozen mini-straw (a) and a MiniFlatPack™ (b) depicting major differences in the orientation and size frozen water lakes/extender veins. In (c) a higher magnification of (b) showing morphologically well preserved boar spermatozoa from the sperm-peak portion of the ejaculate (Courtesy of Dr Hans Ekwall).

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 have been frozen, highly concentrated, in small volumes (0.5-0.7 mL) in novel containers, the so-called "MiniFlatPack™" (Saravia et al 2005, 2010, Pimenta-Siqueira et al 2011), as 1-2 billion spermatozoa/mL. Interestingly, cryosurvival (see **Table 2**) was equal or higher than for 0.5 mL plastic straws, suggesting the shape maintained the cryobiological advantages of the FlatPack™ (Ekwall et al 2007), including fertility when using deep-intrauterine AI (Wongtawan et al 2006).

Sperm vitrification: Boar spermatozoa packed in 0.12 mm thick film plastic bags were frozen ultra-rapidly at various stages of conventional freezing-thawing and besides survival, samples were explored ultrastructurally, for presence of ice damage. Survival was minimal and ice presence was detected, indicating that cooling rates, although high, were not enough to handle the volumes assayed (Bwanga et al 1991b). Non-penetrating sugars have either been used for vitrification (Meng et al 2010) and also for empirical improvement of slow freezing (Malo et al 2010). There is, *a priori*, nothing against the use of vitrification for freezing small suspensions of boar spermatozoa (for instance for intracytoplasmic sperm injection, ICSI, Meng et al 2010) but there is no practical use for breeding, since the amounts needed are too large to achieve ultra-rapid cooling and thawing rates.

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

Table 2. Cryosurvival (Computer Assisted Sperm Analysis, CASA, mean±SEM), as percentages of motile spermatozoa, 30 min post-thaw at 38 °C) of ejaculated boar spermatozoa from the sperm-peak portion (P1, first 10mL of the sperm-rich fraction, SRF) or the entire SRF subjected to a simplified (SF, 3.5h) or a conventional freezing (CF, 8h) and an equal thawing (35°C for 20 seconds) (Modified from Saravia et al 2010).

Learning from the ejaculate: Boar SP is a composite, heterogeneous fluid composed by fractionated secretions of the epididymal caudae and the accessory sexual glands. *In vivo*, spermatozoa contact some of these fractions but not necessarily all, and different effects (sometimes deleterious, sometimes advantageous) have been recorded *in vitro* when removing (Fraser et al 2007) alternatively keeping boar spermatozoa in its own SP, depending on the fraction used (Guthrie & Welch 2005, Rodriguez-Martinez et al 2009, 2011). The SP or the sperm-rich fraction (SRF) might not be necessary for cryosurvival or fertility, since spermatozoa from boars that were semino-vesiculectomised were able to sustain freezing and thawing equally well as spermatozoa bathing in seminal vesicular proteins (Moore et al 1977). However, we have recently determined that boar spermatozoa

contained in the first 10 mL of the SRF (also called sperm-peak portion or P1, where about $\frac{1}{4}$ of all spermatozoa in the SRF are) were more resilient to handling (from extension to cooling) and cryopreservation than the spermatozoa contained in the rest of the ejaculate (Peña et al 2003, Rodriguez-Martinez et al 2009, Saravia et al 2007, 2010, Rodriguez-Martinez et al 2008). It appeared that it was actually the SP in this sperm-peak P1 portion 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 (Rodriguez-Martinez et al 2011), compared to other fractions of the ejaculate (Saravia et al 2010).

In an attempt to simplify the freezing protocol, only the P1-spermatozoa were frozen in concentrated form for eventual use with deep-intrauterine AI. These spermatozoa were firstly kept in their SP for 30 min, and thereafter, without centrifugation (i.e. without removal of the SP) they were 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™'s 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 2**, equally good (above 60% of the processed cells (Saravia et al 2010, Pimenta-Siqueira et al 2011). Moreover, the spermatozoa in the sperm-peak-fraction of the boar ejaculate showed a maintained plasmalemmal intactness and fluidity and a lower flow of Ca²⁺ under capacitation conditions post-thaw, which might account for their higher membrane stability after cryopreservation (Hossain et al 2011).

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, inter-boar variation was minimised by use of P1-spermatozoa which, not only were the "best" spermatozoa to be cryopreserved, but uses a portion of the sperm-rich fraction where the documented "fertility-associated" proteins are present (Rodriguez-Martinez et al 2011). Finally, the procedure frees the rest of the collected spermatozoa (75% of the total sperm count) for additional processing of liquid semen AI-doses. This simpler protocol ought thus to be an interesting alternative for AI-studs to -using the one and the same ejaculate- freeze boar semen (P1) for gene banking or for repopulation or commercial distribution, along with production of conventional semen doses for AI with liquid semen, using the rest of the ejaculate. Such procedures would not disturb routine handling of boars or their ejaculates. Inseminations in the field (deep intra-utero) have shown acceptable figures for farrowing and litter size (Wallgren, personal communication).

4. Cryopreservation of oocytes and embryos

The slow freezing technique developed for oocytes and embryos in the 1970's (Willadsen et al 1978) has been thoroughly established by the increasing repertoire of CPA where they were gradually exposed to. Cultured cells/embryos are exposed to relatively low concentration of permeating CPA's (glycerol, DMSO, EG or PG at 1-1.5 M (oocytes) or 1.3-1.5 M (embryos) alternatively non-permeating CPA in the culture medium, loaded into mini-straws and cooled at -5 to -7°C, equilibrated for some minutes followed by seeding of extracellular ice nucleation, to be thereafter slowly cooled at ~0.3-0.5°C/minute to -40/-65°C and final plunge in LN₂ for

storage of the now carefully dehydrated and vitrified germplasm (for a comprehensive review see Saragusty & Arav 2011). However, pig oocytes, zygotes and cleavage embryos are rich in cytoplasmic lipids, and very sensitive to temperatures below 15°C (Wilmot 1972), a sensitivity that decreases -along with the amount of lipids- with development, towards peri-hatching blastocysts (Niimura and Ishida 1980). Offspring has been obtained after embryo transfer (ET) of slow-frozen and thawed 2-4 cell pig embryos where these cytoplasmic lipids were removed *in vitro* (de-lipitation) before cooling (Hayashi et al 1989, Nagashima et al 1994, 1995, 1996) and thereafter the technique, albeit cumbersome, has been thoroughly applied (Yoneda et al 2004). The results enhanced when the cytoskeleton was preserved from damage using exogenous chemicals (Shi et al 2006).

Over the past years, vitrification (Rall and Fahy 1985) appeared as a better alternative for long-term storage of pig oocytes and embryos. On one hand, the small size of the material to process provided another dimension: vitrification could be modulated via size of sample (10 µL in most cases) so that neither cooling rate nor CPA-amounts ruled so that the method was more practical and less risky. Samples could be handled and carried/stored through either "surface" methods (e.g on liquid loops, mesh of different materials etc) or "tubing" carriers (thin straws, cyopipettes, ultrathin tubing etc). Both yield high cooling rates but while the surface type has the highest warming rates, the other is much easier to handle and, safer (Saragusty & Arav 2011).

On the other hand, vitrification of oocytes and embryos differ in degree of difficulty. As already mentioned, oocytes are more sensitive than embryos, particularly morulas or blastocysts since oocytes have a high cytoplasmic lipid contents (chilling sensitive). Moreover, oocytes have easily disrupted submembranous actin microtubules (which decreases plasmalemma robustness) and fragile meiotic spindle and cytoskeleton, which complicates the resumption of development. Lastly, the process of freezing and thawing can increase the risk for ROS-attack and the premature emptying of cortical granules, thus changing the structure of the zona pellucida (ZP) (Gajda 2009). Therefore, chemical stabilization of the cytoskeleton (Esaki et al 2004) and the use of increased pressure following vitrification (Du et al 2008) had been successfully applied, obtaining development post-rewarming towards the fetal stage (Ogawa et al 2010). Other measures, such as induction of osmotic stress (by exposure to NaCl) has shown to improve developmental competence after vitrification (Lin et al 2008). Centrifugation (lipid depot relocation) for vitrification appears detrimental for *in vitro*-matured oocytes, but not in zygotes or later stages (Somfai et al 2008)

Vitrification of *in vivo*-developed, ZP-intact pig embryos, where lipids were polarized by centrifugation of the blastomeres, by delipitation and/or treatment with cytochalasin for cytoskeleton stabilization, has resulted after rewarming and ET, in piglets (Dobrinsky 1997, Dobrinsky et al 2000, 2001, Kobayashi et al 1998, Berthelot et al 2000, 2003, Cameron et al 2000). Blastocysts were also developed by *in vitro* fertilization (IVF) of follicular oocytes vitrified as cumulus-oocyte complexes from offal porcine follicles (Somfai et al 2010).

Recently, piglets were even obtained following vitrification of delipitated 4-8 cell stages of *in vitro* produced (IVP), parthenogenetic embryos and ET (Nagashima et al 2007). Vitrification, usually done within 0.25 mL plastics-straws, yield better embryo survival post-warming when Open Pulled Straws (OPS; Vajta et al 1997), which increases the cooling rate achievable in 0.25 mL straws (2,500°C/min) by almost 8-fold (Cuello et al 2004a-b), were used, again resulting in piglets born (Berthelot et al 2000; 2001). Higher cooling-rates

(>20,000°C/min) can nowadays be reached using using cryo-loops (Lane et al 1999) or with straws with a smaller inner diameter and wall thickness (the Superfine Open Pulled Straws: SOPS; Isachenko et al 2003), and by applying immersion in LN₂-slush, which allowed for the use of lower concentrations of toxic cryoprotectant.

Vitrification of untreated morulae and blastocysts has resulted in high survival rates after warming (Berthelot et al 2003), especially when re-warming after SOPS is done in one stage (direct warming, a very practical solution for ET, Cuello et al 2004b), yielding live litters (Cuello et al 2005). For blastocysts (See **Figure 5**), use of the SOPS waived the need for centrifugation (dislocation of lipids) or microtubule stabilization, thus making the method a very practical one and indicating the procedure is now reaching maturity for commercial application (Cameron et al 2004, Beebe et al 2005, Martinat-Botté et al 2006, Cuello et al 2008, 2010, Sanchez-Osorio et al 2009, 2010). Cryopreservation of *in vitro*-pig embryos -owing to differences in the cytoskeleton and the distribution of the lipid deposits- has been, until recently (Esaki et al 2004), considered as more difficult than for *in vivo*-developed, but the birth of piglets resulting from ET of IVP, transgenic pig embryos, has modified this view opening for the commercialization of highly valuable, modified genetic material (Li et al 2006, Kawagami et al 2008). Despite peri-hatching blastocyst stage embryos are the ones best sustaining vitrification and warming with continued *in vitro* development (Dobrynsky 2001), this particular embryo stage can not be commercially used since there is no ZP.

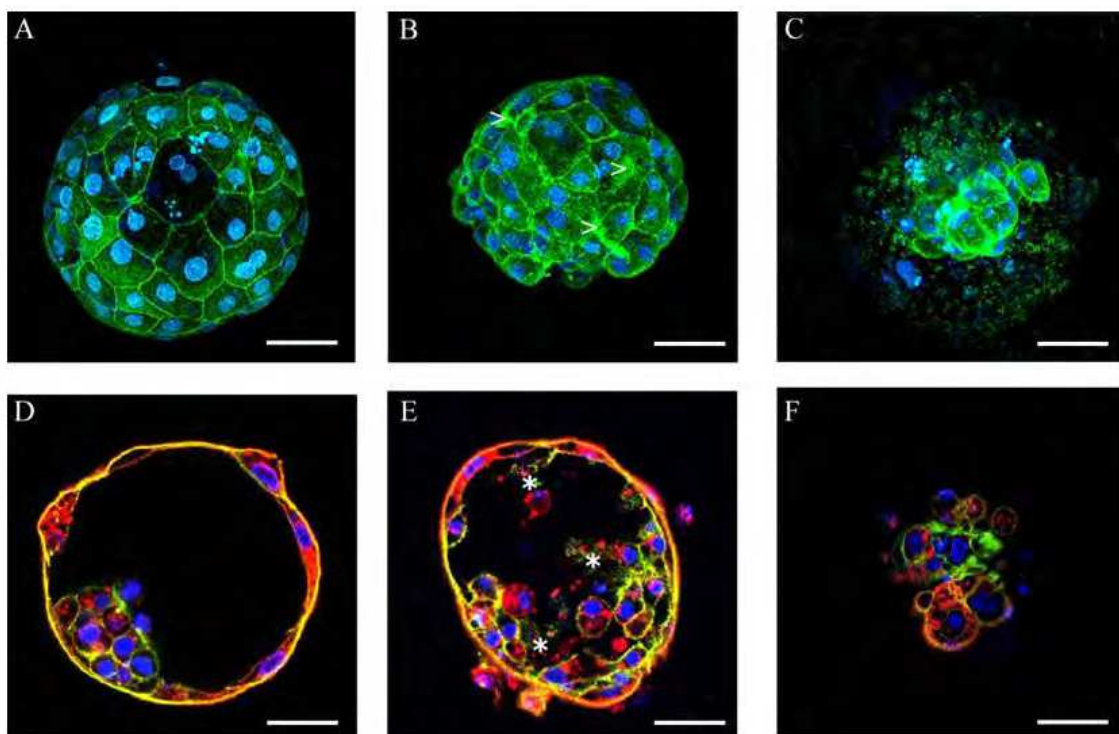


Fig. 5. Laser scanning confocal microphotographs of grade I (A and D), grade II (B and E) or grade III (C and F) *in vivo*-derived fresh (A-C) and superfine open pulled straws (SOPS)-vitrified (D-F) porcine blastocysts, following uploading of Hoescht H-33342 (blue, cell nuclei), phalloidin-Alexa Fluor 488 (green, actin filaments) and wheat germ agglutinin-Alexa fluor 594 (red, lectin reactive membrane elements). Note the high degree of morphological intactness even after rewarming compared to fresh controls (Reprinted from Cuello et al 2010, with permission).

5. Cryopreservation of genital tissues

Freezing of ovarian tissue in humans relate primarily, but not only, to a dramatic measure to warrant availability of oocytes in cases of oncotherapy, when sterility is foreseen, similar to the ongoing sperm banking prior to onco- or hormonal therapy. Rescue of oocytes from frozen samples of ovarian cortex is then feasible for ART (Shaw & Trounson 2002). Both slow freezing and directional freezing had been assayed with acceptable results (Arav & Natan 2009), opening possibilities for the cryopreservation of large samples and even of whole ovary for autografting purposes and possibly evolving in oocyte banking as an insurance against childlessness. Adult testicular samples (aspiration or biopsy) are mainly issued during biopsy for recovery of spermatids for ICSI (Keros et al 2005, Curaba et al 2011). However, the strongly ongoing research in adult stem cells shall be based on the absolute need of properly cryopreserving pre-pubertal testicular tissues. Transplantation of other organs or tissues (uterine in particular) is also within the scope of not-far, albeit discussable, scenarios (Bredkjaer & Grudzinskas 2001).

Regarding the porcine species, although there is no obvious rationale for most of the above considerations in human, it provides an excellent animal model for experimental reproductive medicine, particularly considering transplantation surgery. Porcine whole uteri were arterially perfused with CPA (DMSO) prior to slow controlled freezing. Rewarmed tissues were able to present live cells 7 h post rewarming (Dittrich et al 2006) and even to demonstrate contractility *in vitro* 60 min post-rewarming (Dittrich et al 2010). As such, comparative analyses of equilibrium freezing and vitrification procedures have involved pig ovarian fragments (Gandolfi et al 2006, Borges et al 2009), or whole ovaries (Imhof et al 2004). These attempts were all done using slow freezing, but evidence is now provided that vitrification of thin slices of ovarian cortex is feasible and that rewarmed primordial follicles from these samples were able to develop (albeit slower than controls) in murine xenografts (Moniruzzaman et al 2009). Further development in this area is expected.

6. Conclusions

Vitrification as a method for cryopreservation in porcine applies thus far to small samples that can be managed at high cooling and rewarming rates without need of applying permeating CPA of potential toxicity. Therefore, the technique has developmental potential for oocytes, COCs and embryos for IVF and ET. Boar spermatozoa are yet to follow this path, and although there is a potential breach for vitrifying limited volumes of sperm suspensions, such approach is yet solely academic in nature. Semen for breeding ought to be frozen conventionally, albeit with a focus on increased cell lifespan, and managing concentrated semen doses for deep intrauterine AI. There is much yet to be learned from the ejaculate and the relationships between specific components of the seminal plasma and sperm function.

7. Acknowledgements

The studies of the author have been made possible by grants from the Swedish Research Council Formas, the Swedish Farmer's Foundation for Agricultural Research (SLF), and The Swedish Research Council (VR), Stockholm, Sweden.

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Current Frontiers in Cryobiology

Edited by Prof. Igor Katkov

ISBN 978-953-51-0191-8

Hard cover, 574 pages

Publisher InTech

Published online 09, March, 2012

Published in print edition March, 2012

Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

How to reference

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Heriberto Rodriguez-Martinez (2012). Cryopreservation of Porcine Gametes, Embryos and Genital Tissues: State of the Art, *Current Frontiers in Cryobiology*, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0191-8, InTech, Available from: <http://www.intechopen.com/books/current-frontiers-in-cryobiology/cryopreservation-of-pig-spermatozoa-oocytes-and-embryos-state-of-the-art>

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