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Evaluation of two prototype directional freezing methods and a 2ml flattened straw for cryopreservation of boar semen*

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Based on previous assessments on stallions, 40 ejaculates of 20 Duroc boars were split and evenly frozen with a conventional vapour freezing method and two directional, drum and directional, prototype methods using commercial extenders and relative standard procedures. The directional prototype was provided with a double internal setup that allowed the positioning of experimental 2ml flat straws with 1 billion sperm (Flat) in a fixed support, or both classical 0.5 ml paillettes with 250 million spermatozoa and flats in a rotating drum designed so as to ensure a more uniform heat exchange. Preliminary tests for individuation of the most appropriate thawing rate showed beneficial effects (P≤0.05) of thawing the sperm at 50°C for 13 s when compared to 42°C for 20 s, in terms of total motility (42.8±8.4% and 35.6±6.8%, respectively). With regard to freezing/packaging methods, major improvements (P≤0.05) were shown for the drum method with paillettes for total motility (38.6±14.2%) assessed immediately after thawing, when compared with the conventional (29.4±13.3%) and the directional methods with flats (30.2±12.8%), and for total motility (P≤0.01) assessed following incubation for 120 min at 37°C after thawing (24.8±11.6%) with respect to the conventional method (15.6±10.9%). Despite the statistical non-significance of results, both the prototype freezing approaches using the experimental flat straw showed some improvements in functional parameters assessed by cytofluorometry when compared to the conventional method.

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In swine, the constantly evolving insemination techniques have enabled the gradual reduction in the number of sperm per dose [Roca *et al*. 2011, Broekhuijse *et al*. 2015, Knox 2016], thus opening new possibilities for the use of cryopreserved semen [Roca *et al*. 2016]. However, cryopreserved boar semen is still used on a relatively limited scale because of the reduced fertility compared with the traditionally liquid stored [Knox 2015]. Encouraging reports indicate that 1000-500x10^6 frozen-thawed spermatozoa can ensure fertility levels comparable to liquid semen if combined with deep-uterine insemination [Roca *et al*. 2006].

The current standard freezing protocols for boar semen require primary dilution into extenders, centrifugation, final dilution into cryoprotecting media and relatively highly concentrated freezing. Beyond the time required, the sperm concentration of 200x10^6 spermatozoa/ml, which are classically packaged in 0.5ml medium straws, allow to obtain only a limited number of doses per ejaculate. Furthermore, a breeding dose for AI is still necessarily composed of multiple straws to provide sufficient numbers of spermatozoa. To restrict these disadvantages, a MiniFlatPack of small volume (approximately 0.7 ml) was designed for freezing sperm at high concentrations of 1-2 billion/ml [Bwanga *et al*. 1991, Wongtawan *et al*. 2006]. Still, these approaches collide with the need to have simple and standardized AI methods. Furthermore, the thawing procedures, and in particular the speed of thawing, prove to be of increasing importance for the vitality of frozen-thawed boar spermatozoa [Athurupana *et al*. 2015]. With regard to sperm damage, increasingly advanced analytical techniques have provided important opportunities for a more comprehensive assessment of the extensive cryodamage in boar semen alongside traditional kinetics evaluations [Torres *et al*. 2016, Wasilewska *et al*. 2016].

Recently, we evaluated a prototype directional freezing method by thermal gradient on equine species using a new 2ml flattened straw [Puglisi *et al*. 2016]. Although relatively limited improvements were obtained, this approach seems to be of potential interest for applications in other species, in which the development of simple methods for freezing larger volumes is of great utility. Therefore, the objective of the present work was to evaluate the performance of this prototype freezing method for cryopreserving boar sperm using commercial extenders and relative standard procedures.

Material and methods

Forty ejaculates were collected from 20 Duroc boars of proven fertility housed in a semen production centre in Northern Italy. Ejaculates were collected using the gloved-hand technique, diluted (1:2, v/v) with SUS extender (Medi Nova sas, Reggio Emilia, Italy) and shipped to the laboratory within two hours at 15°C in a thermostatic chamber. Sperm was analysed for kinetic and quality parameters and frozen using

the following equipment and packaging: a programmable Microdigitcool freezer (IMV Technologies, L'Aigle, France) with sperm loaded into 0.5 ml French straw (paillettes) used in conventional freezing, while a prototype modified Digitcool (IMV Technologies) was used for two directional methods as previously described [Puglisi *et al*. 2016]. The prototype Digitcool may be equipped with a fixed support (directional method), which can accommodate an experimental 2ml flattened straw (flat), or with a rotating drum which supports both the paillettes and flat straws.

Sperm were processed at 15°C as follows: fresh ejaculates were stabilised for 60 min in a refrigerated cabinet and subsequently centrifuged for 25 min at 800xg. The pellets were extended with Boarciphos A (IMV Technologies) plus 20% (v/v) egg yolk to a concentration of 1 x 10^9 sperm/ml, and equilibrated for 90 min at 4°C. After equilibration, sperm were diluted in the freezing medium Boarciphos B (IMV Technologies) plus 10% glycerol, 20% egg yolk and 2% Equex (Nova Chemical Sales, Inc, Scituate, MA, U.S.A.), (v/v) respectively, to a concentration of 500 x 10^6 sperm/ml and loaded into 0.5ml paillettes and 2ml flats. Sperm were frozen following the manufacturer's recommendations, at the following rate: +4°C to -42°C at 3°C/min and -42°C to -140°C at 100°C/min. Frozen sperm were stored at -196°C in liquid nitrogen until analysis of kinetic and quality parameters. Kinetic parameters were also monitored after centrifugation and after equilibration at 4°C for 90 min prior to freezing. For frozen semen analysis, after thawing sperm were diluted (1:16, v/v) into prewarmed (37°C) Thawing and Insemination Extender (IMV Technologies, cat #: 006746).

As a preliminary assay, 3 boars were randomly selected and 10 paillettes or 5 flat straws prepared by each of the 4 freezing/packaging methods were used to evaluate two thawing rates: 13s at 50°C, and 20s at 42°C. The first aliquot was analysed for kinetic and quality parameters, and the remaining sperm were further incubated for 120 min at 37°C and analysed for motility.

Prior to analyses, semen were incubated for 14 min at 37°C. Total motility and mean velocity were assessed by the CASA system (HTM-IVOS vs. 14, Hamilton Thorne, Beverly, MA, USA), as described: two aliquots of one sample were layered on two pre-warmed Leja chambers (Leja Products B.V., GN Nieuw Vennep, the Netherlands) and a minimum of 100 sperm per chamber were detected using the following settings: frames per sec, 60 Hz; number of frames, 30; cell detection by minimum contrast 20 and minimum cell size of 10 pixels. Spermatozoa with an average path velocity >15 μm/s were defined as motile. Concentration and membrane integrity were determined using the NucleoCounter (SP-100™, ChemoMetec, Allerřd, Denmark).

High membrane fluidity, spermatozoa with polarised mitochondrial membranes and plasma/acrosomal membrane integrity were determined using the microcapillary flow cytometer (Guava EasyCyte Plus®, IMV Technologies), as described [Puglisi *et al*. 2016]. Briefly, sperm samples were diluted in PBS at 30 million sperm/ml and approximately 5000 spermatozoa were analysed in duplicates at flow rates of 200-300 cell/s. In order to assess membrane fluidity, samples were centrifuged for 5 min at

160xg and sperm pellets were suspended in PBS at 30 million sperm/ml. Two aliquots were incubated with 2.7 μM merocyanine 540 (Molecular Probes Inc., Eugene, OR, USA) for 5 min at 37°C in 96-well plates in PBS at approximately 500 sperm/μl. The two subpopulations with low and high membrane fluidity were discriminated by the red fluorescence histogram. Spermatozoa with polarised mitochondrial membranes and plasma/acrosomal membrane integrity were assessed using the dedicated Easykit 2 (mitochondrial activity; ref: 024864) and Easykit 5 (viability and acrosome integrity; ref: 025293), respectively, using the manufacturer's protocols and reagents (IMV Technologies).

The effect of thawing rates on semen quality was statistically analysed using the non-parametric Kruskal-Wallis test. The effect of freezing/packaging modes was evaluated using the analysis of variance. The post hoc Bonferroni test was used to compare the treatment means. Statistical analyses were performed using the R software (R Foundation for Statistical Computing; Vienna, Austria. 2013. http://www.R-project.org).

Results and discussion

The descriptive statistics of sperm quality of ejaculates and kinetics monitored before freezing are listed in Table 1. Results of the preliminary test performed to individuate the most appropriate thawing rate showed general moderate beneficial effects for thawing at 50°C when compared to 42°C (Tab. 2). Therefore, experimental cryopreserved sperm were thawed at 50°C for 13 s. In this respect, testing of different cryopreservation conditions for individual ejaculates, especially for those having sub-optimal freezability, is a fundamental prerequisite for boar frozen semen production [Hernández *et al*. 2007]. In the cited work, although changes in the freezing curve were better tolerated by most ejaculates and did not show significant variations, fast thawing rates obtained in a very warm water bath at 70 °C for a short period improved sperm cryosurvival and were fundamental to some critical ejaculates.

With regard to freezing/packaging methods (Tab. 3), the drum method with paillettes improved ($P \leq 0.05$) total motility when compared with both the conventional method using paillettes and the directional technique with flat straws. The same method also improved ($P \leq 0.01$) total motility with respect to the conventional method after incubation for 120 min at 37°C. Results showed no detrimental effects of the prototype methods on the membranes status with respect to a standard vapour freezing in conventional 0.5 ml paillettes. Contrary to what we had previously reported for the stallion [Puglisi *et al*. 2016], both directional methods were feasible for cryopreserving high concentrated boar sperm in large volume flat straws without changes in procedural processing. These prototype methods are based on the approach of multi-thermal gradient (MTG), firstly devised by Arav [1999] and subsequently applied for cryopreserving sperm of various domestic and nondomestic species [Kumar *et al*. 2014]. However, in contrast to the MTG devices, in which biological samples are

Table 1. Sperm parameters (means and standard deviations) of 40 ejaculates of 20 boars, and kinetic parameters monitored before freezing. After stabilization for 60 min at 15°C, fresh spermatozoa were centrifuged for 25 min at 800xg (centrifugation), extended in egg yolk-containing medium and equilibrated for 90 min at 4°C (equilibration)

Step	VOL		CONC		MI		HF		POL		V+A+		TM		MV	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Ejaculates	79.5	28.3	169.5	77.5	89.3	3.0	36.3	8.0	76.2	12.2	80.2	6.3	90.3	6.1	100.7	15.6
Centrifugation													83.1	5.5	97.9	12.7
Equilibration													81.8	7.6	98.5	15.8

Vol (mL) – volume; CONC (million/mL) – concentration; MI (%) – membrane integrity; POL (%) – spermatozoa with polarised mitochondrial membranes; HF (%) – high membrane fluidity; V+A+ (%) – spermatozoa with intact plasma and acrosomal membranes; TM (%) – total motility; MV (μm/sec) – mean velocity.

Table 2. Quality parameters (mean and standard deviations) of boar (n=3) spermatozoa analysed immediately after thawing at two different temperatures and after incubation at 37°C for 120 min (I). Sperm from one ejaculate was frozen applying 4 freezing methods and packaged into paillettes and flat straws

T	TM		TM (I)		MV		MV (I)		MI		MI (I)		POL		HF		V+A+	
(C°)	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
42	35.6	6.8	19.4	8.5	52.1	9.0	44.5	4.7	42.6	8.2	27.5	5.0	52.6	9.8	48.2	5.7	49.8	5.2
50	42.8*	8.4	19.8	6.8	57.1	8.0	51.2	9.2	46.7	12.8	25.1	11.3	50.3	8.4	51.8	7.7	52.8	6.1

TM (%), total motility; MV (μm/sec) mean velocity; MI (%) membrane integrity; POL (%), spermatozoa with polarised mitochondrial membranes; HF (%), high membrane fluidity; V+A+ (%) spermatozoa with intact plasma and acrosomal membranes.
*P≤0.05.

forced to advance through a linear temperature gradient generated by two warm and cold blocks, the prototype used in the present study generates the linear temperature gradient by current nitrogen vapour passing longitudinally through the biological samples that are not subjected to advancement. The prototype was provided with a

Table 3. Sperm quality parameters (means and standard deviations) of 40 ejaculates of 20 boars assessed at thawing and after incubation at 37°C for 120 min (I). Sperm packaged in paillettes or flat straws was frozen by Conventional and alternative directional (Drum and Directional) freezing methods

Variable	Conventional using a paillette		Drum using a paillette		Drum using a flat straw		Directional using a flat straw	
	mean	SD	mean	SD	mean	SD	mean	SD
TM	29.4^{a}	13.3	38.6^{b}	14.2	32.0^{ab}	15.2	30.2^{a}	12.8
TM (I)	15.6^{A}	10.9	24.8^{B}	11.6	21.0^{AB}	14.2	19.9^{AB}	11.7
MV	48.6	12.1	51.2	13.5	50.3	13.0	50.4	14.5
MV (I)	39.6	10.8	39.5	10.2	39.5	10.6	37.5	9.7
MI	37.9	10.6	44.0	12.8	43.8	12.8	38.8	12.4
MI (I)	24.8	11.9	30.3	11.8	32.3	14.5	29.6	13.3
HF	58.4	12.2	58.5	13.9	57.7	12.3	55.5	11.5
POL	46.3	11.4	47.9	13.8	48.5	11.3	50.1	10.7
V+A+	41.6	15.6	44.4	14.6	45.7	13.5	44.1	16.4

TM (%), total motility; MV (μm/s) mean velocity; MI (%) membrane integrity; HF (%), high membrane fluidity; POL (%), spermatozoa with polarised mitochondrial membranes; V+A+ (%) spermatozoa with intact plasma and acrosomal membranes.
$^{aA...}$In rows means bearing with different superscripts differ significantly at: small letters – $P \leq 0.05$; capitals – $P \leq 0.01$.

double internal setup facilitating positioning of either flat straws in a fixed support, or both paillettes and flats in a rotating drum to ensure a more uniform heat exchange. Overall, the great advantage of directional methods is that ice crystal propagation can be controlled using the linear temperature gradient so as to reduce mechanical damage during freezing. Despite this advantage, not only was the drum method using paillettes ineffective in freezing stallion sperm, but the use of 2ml flat straws was even detrimental to both kinetic and qualitative parameters. To evaluate some possible explanations for these differences, it is important to remind that sperm plasma membranes of boars are particularly rich in polyunsaturated phospholipids, which are primarily responsible for their fluidity, and poor in cholesterol, which is prevalently responsible for rigidity [Wasilewska *et al*. 2016]. Starting from the assumption that the specific lipid composition of boar sperm makes it extremely sensitive to cold shock [Watson 2000], the destabilisation of membranes measured by flow cytometry depicted different degrees of lipid disorder between the two species. While the percentage of stallion spermatozoa with high membrane fluidity almost doubled after thawing in comparison to fresh semen, the variation for boar sperm was limited to a more moderate 50% increase after freezing/thawing using all the methods. Given that the phospholipid composition influences fluidity of the membrane depending on the respective fluid- to gel- phase transition temperatures [Parks and Lynch 1992], and that cholesterol contrasts these lipid-phase changes, the particular low cholesterol : phospholipid ratio in swine suggests that the content of sterols should not be the main cause in determining the differences observed in the two species. Rather, it is more likely that the particular high content of long-chain polyunsaturated fatty acids

(PUFA) in boar sperm membranes [Mandal *et al*. 2014] is the principal characteristic conferring the greater resistance to freezing when compared to that of stallion sperm. Furthermore, the level of selection for fertility in the horse is not comparable to that observed in pig breeding, in which the removal of individuals with poor semen quality is a rule [Robinson and Buhr 2005, Schulze *et al*. 2014]. Given this assumption, in the swine industry increasing genetic progress of the most valuable boars using frozen-thawed semen, supported by increasingly efficient insemination techniques, must become more and more realistic for the development of the sector [Roca *et al*. 2016]. To achieve this goal, it is mandatory to reduce the number of sperm per dose, ideally with spermatozoa of only one boar, and not pooled doses from multiple boars as it is the general practice. However, only a limited number of studies have introduced practical solutions for freezing of boar semen with such characteristics. Among these, various types of packaging systems, such as paillettes of volumes ranging from 0.25 to 5 mL, flat paillettes and plastic bags of 5 mL (FlatPacks), have not found practical commercial applications [Rodriguez-Martinez and Wallgren 2011]. The main reasons for the lack of practical use of these advantages are that large volumes of cryopreserved sperm allow the use of classical cervical insemination, but do not meet the requirements of optimising the use of the ejaculates. In turn, smaller containers with very high sperm numbers, such as the so-called MiniFlatPacks, imply the use of intrauterine AI and are potentially inferior in terms of their manageability and storage. From this point of view, the flat straws tested in the present study have the advantage of requiring no modification of the standard procedures for sperm processing, facilitating an acceptable compromise between handling, volumes and sperm concentration, thus representing a further alternative for combinations with other solutions available. In this regard, a recently proposed approach for sperm freezing is of great interest, as it provides for the use of the first sperm rich fraction of the ejaculate [Rodriguez-Martinez and Wallgren 2011]. The sperm of this fraction showed greater resistance to cold shock compared to other portions of the ejaculate and could be frozen without centrifugation as the enrichment process. This would preserve the exploitation of the remaining part of the ejaculate for the production of refrigerated semen.

In order to meet industry standards, post thaw swine sperm motility should preferably exceed 50%, while our results were on average below this threshold. It has been estimated that the percentage of ejaculates problematic to freeze fall within the range of 25 to 35% [Roca *et al*. 2006]. It is unrealistic that these individuals, referred to as "bad" freezers, will ever be used as prospective donors of frozen semen. However, if deserving of high genetic value, these subjects should be individually approached using specific freezing procedures [Hernández *et al*. 2007]. Unfortunately, a majority of pre-freezing sperm characteristics are limited for predicting sperm freezability [Casas *et al*. 2009, Yeste 2016]. Therefore, although the boars used in our present work were of proven fertility and had good seminal quality, it is very likely that their distribution in bad, medium and good freezers fell in the percentages reported in the literature, thus lowering mean results.

In conclusion, freezing boar sperm at one billion in a single 2ml dose using directional freezing approaches had no detrimental effects in comparison to standard vapour freezing. Potential damage in the prototype directional methods was not detected either immediately after thawing or after incubation for 120 min at 37°C. Rather, in particular the drum variant applying conventional paillettes improved kinetics and was even tended to improve quality parameters. Furthermore, based on previous indications stressing the importance of thawing rates on boar sperm viability after freezing under the influence of multiple variables, such as cryoprotectant, sperm concentration and packaging systems [Bamba and Cran 1985, Eriksson and Rodriguez-Martinez 2000], we preliminarily evaluated two simple thawing conditions in four different experimental variants. Although this type of assessment was outside the scope of the present work, the largest possible evaluation of the working conditions is essential, especially when dealing with problematic species such as the pig, where even minor improvements are relevant. Future research needs to be conducted in order to enhance the potential offered by this technology, in particular for bad to moderate boar freezers, by applying ad hoc refinements to conventional extenders and procedures applied in this study.

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