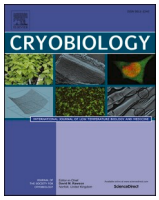
Cryobiology 113 (2023) 104583

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo



# Successful recovery of motile and viable boar sperm after vitrification with different methods (pearls and mini straws) using sucrose as a cryoprotectant

María Serrano Albal [a,*], Marie Claire Aquilina [a], Louisa J. Zak [b], Peter J. Ellis [a], Darren K. Griffin [a]

[a] School of Biosciences, University of Kent, Canterbury, United Kingdom
[b] Topigs Norsvin Research Center, Beuningen, the Netherlands

ARTICLE INFO

*Keywords:*
Cryopreservation
Slow freezing
Vitrification
Motility
Boar
Spermatozoa

ABSTRACT

Vitrification of sperm by direct contact with liquid nitrogen is increasing in popularity as an alternative to conventional (slow) freezing. Although slow freezing is very challenging in boar sperm cryopreservation, this is currently the standard method used. We compared vitrification in "pearls" and in "mini straws" using the in vitro fertilization media Porcine Gamete Media with 0.3 M sucrose with the standard (slow) method used to preserve boar sperm. Both vitrification methods reduced the viability of the sperm sample more than slow freezing (42.2 ± 4.3% total motility and 71.4 ± 2.3% alive), however, both protocols allowed for the successful recovery of the sperm samples. By comparing two different methods of vitrification and two different methods of post-thaw preparation we were able to determine the optimal vitrification-thaw protocol for boar sperm. When comparing pearls and mini-straws, the smaller liquid volume associated with pearls had a positive effect on the survivability of the samples, reducing sperm DNA damage (1.2 ± 0.2% vs. 5.1 ± 0.1.7%) and preserving motility (26.15 ± 2.8% vs 9.39 ± 0.9%) after thawing. In conclusion, the pearl method was the most suitable of the vitrification techniques for use with boar sperm.

## 1. Introduction

The global increase of pork consumption and the growth in biomedical research have compelled the pig industry to increase their production in a more efficient and sustainable manner [27]. Cryopreservation is the best technology to store boar sperm for a long period of time and facilitate sample transport for commercial purposes [45,58]. It is an important tool for the conservation of important productive lines and as a bank of genetic resources that has been available for commercial use since 1975 [22,55].

The standard cryopreservation method used for preserving boar sperm is slow freezing [43]. This is performed using cryopreservation media, that contains substances that act as cryoprotectant, such as glycerol or egg yolk [57]. The media also contains non-penetrating substances that promote cell dehydration and form hydrogen bridges with the water molecules, reducing ice crystal formation [4]. Other molecules that act extracellularly as cryoprotectants are sugars, including sucrose which is a commonly used cryoprotectant in the cryopreservation of sperm cells across different species [11]. Despite this, the intracellular crystal formation during the cooling process is still very noticeable. Freezing produces significant damage to sperm cells and their membranes due to the low cryotolerance of sperm [32]. Moreover, the asymmetrical proportion of cholesterol between the layers of the boar sperm membranes leads to a greater susceptibility to thermal shock [40], and a lower sperm cryotolerance compared to other species [23].

The harmful effects of freezing produce a reduction in the different functional sperm parameters, such as motility and mitochondrial activity, while increasing the proportion of morphologic abnormalities in the samples and/or affecting DNA integrity [23,54]. All these can lead to reducing the fertilizing capacity of the frozen-thawed sperm by around 20–30% compared to semen in extender stored at 17 °C [14,26].

In recent years, vitrification has been extensively used to cryopreserve oocytes and embryos of different species [7,10,24,41]. Vitrification has also been increasingly applied as a technique to preserve sperm in smaller volumes. Plunging sperm directly into liquid nitrogen ($LN_2$) provides an ultra-fast cooling process that prevents intracellular ice crystal formation [20,21]. The first advances in this field were done on

* Corresponding author.
*E-mail address:* ms2104@kent.ac.uk (M. Serrano Albal).

https://doi.org/10.1016/j.cryobiol.2023.104583
Received 13 March 2023; Received in revised form 9 August 2023; Accepted 30 August 2023
Available online 31 August 2023

human samples [19] then were rapidly extended to non-human species [12,46].

The first sperm vitrification protocols simply involved a polystyrene box loaded with $LN_2$ where a metal strainer was immersed [19]. The sperm aliquots were pipetted directly into the $LN_2$ forming spheres that floated on the surface for a few seconds until they solidified, due to their appearance those spheres are referred to as pearls. Only one pearl at a time could be made, since having numerous pearls in the same strainer increased the risk of the pearls sticking together and forming drops of greater volume. Furthermore, in order to store the pearls in cryovials, the sperm spheres were collected by a spoon, resulting in a tedious collection process. New publications, showed the use of a device made by Kitazato BioPharma [36,38], that facilitates pearl formation. This device is a metal funnel that contains a metal grate facilitating the formation of multiple pearls simultaneously. Additionally, it has a cryovial screwed to the bottom of the device, simplifying the collection of the spheres and reducing the handling time [36]. However, a major limitation with the Kitazato device is that it is an open system, therefore the samples are in direct contact with $LN_2$, increasing the risk of cross-contamination between samples [36].

To perform vitrification in a more aseptic way, other studies have developed different packaging methods of sperm for cryopreservation [14]. The use of mini-straws to vitrify small volumes of sperm (30–50 μL) involves the use of straws with two different diameters, the smaller straw holds the sperm while the outer bigger straw has a protective function. Since the outer straws are heat-sealed on both sides, the samples remain isolated and they are not in direct contact with $LN_2$, reducing the risk of cross-contamination between the samples.

In terms of efficiency, other freezing methods (such as the freeze-dry method) have previously suggested to replace slow freezing [33,37]. However, when freeze-drying, spermatozoa lose their motility viability and showed increased DNA damage [29], while with vitrification the damage seems to be lower [1,19,36]. When conventional freezing methods had been compared to sperm vitrification on several occasions. In human samples, some studies carried out did not found significant differences in between both methods [8,34,49]. However, other studies have shown that some vitrification methods result in higher progressive motility, plasma membrane and acrosome integrity than conventional freezing [1,35,38].

Despite all these advances, when we focus on boar cryopreservation, there are not many studies comparing slow freezing with other methodologies. Only Arraztoa et al. have published a study comparing slow freezing and vitrification methods [3]; however, vitrification it has yet to be performed successfully on boar sperm [3].

The main objective of this study was to compare three different cryopreservation techniques (slow freezing, vitrification using pearls and vitrification using mini-straws) on boar sperm parameters. Furthermore, we present for the first time the use and effectiveness of the Kitazato device on boar sperm.

## 2. Material and methods

All chemicals and reagents used were purchased from Sigma-Aldrich (Gillingham, United Kingdom) except where specified otherwise.

### 2.1. Extenders

#### 2.1.1. Lactose and egg yolk based extender

Lactose and egg yolk-based extender was used in slow freezing. The preparation of this media is a two-step process: the first part of the extender was an egg yolk-based extender, that will be referred as LEY. LEY was composed of 80 mL 11% lactose and 20 mL egg yolk, to provide a 20% (vol/vol) egg yolk, 80% (vol/vol) lactose proportion, combined with 500 μg/mL streptomycin. A second medium, referred as LEY + GE, consisted of LEY, 9% glycerol, and 1.5% Equex-paste (Minitube, Tiefenbach, Germany).

After 2 h of cooling the samples during the slow freezing process, two parts of the sperm sample previously resuspended in LEY were mixed with one part of LEY + GE to provide a final concentration of 3% glycerol per sample.

#### 2.1.2. Porcine Gamete Medium

This extender was a non-commercial modified Porcine Gamete Medium (PGM [59]) that contained 108 mM NaCl, 10 mM KCl, 0.35 mM $KH_2PO_4$, 0.4 mM $MgSO_4$, 5 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 0.2 mM sodium pyruvate, 2 mM calcium lactate, 2.5 mM theophylline, 1 μM adenosine, 0.25 μM L-cysteine, 10 μg/ml gentamycin and 4 mg/ml BSA. The pH and the osmotic pressure of PGM was adjusted to be around 7.3–7.4 and 280 mOsm, respectively.

This media was used for sperm evaluation pre- and post-cryopreservation. Furthermore, PGM was selected to perform the vitrification protocols. In these cases, 0.3 M sucrose was added as a cryoprotectant prior to vitrification. PGM + sucrose had 500 mOsm of osmotic pressure.

### 2.2. Sperm preparation and cryopreservation

There were 10 different extended boar semen ejaculates kept at 17.5 °C (for commercial AI) supplied by JSR Genetics Ltd. (Southburn, United Kingdom). Each sample was split into 4 equal fractions and were prepared separately at room temperature (RT) using a 35%/70% BoviPure discontinuous density gradient selection system (DGS; Nidacon, Göthenborg, Sweden) following manufacturer's instructions. Each fraction formed the following testing groups: 1) Pre- Test (PT); the sperm parameters of samples resuspended in PGM were evaluated without conducting any cryopreservation. 2) Slow freezing (SF). 3) Vitrification in pearls (VP) 0.4) Vitrification in mini-straws (VMS). In the results, the fifth group, labelled as Vitrification Pearls + DGS (VPD), arise from a change in the post - warming protocol (see section 2.4).

#### 2.2.1. Slow freezing method

The samples to be cryopreserved by slow freezing (SF) were resuspended in LEY at RT. For equilibration, samples were placed in a refrigerator for 2 h at 5 °C (Fig. 1A). After that, the second part of the media (LEY + GE), also keep at 5 °C for 2 h, was added to the samples.

After that, the sperm solutions were manually injected into 0.25 mL sperm straws using a 100–1000 μL manual single-channel pipette, and then heat-sealed. The sealed straws were cooled for 15min by exposure to liquid nitrogen ($LN_2$) vapours before being plunged into $LN_2$. Once the freezing process was complete, the samples were stored a $LN_2$ tank.

#### 2.2.2. Sperm vitrification

After DGS, samples were resuspended in modified PGM [59], and then equilibrated in the refrigerator for 1 h at 5 °C. After that, a 0.3 M sucrose solution, also equilibrated at 5 °C for 1 h, was added to the sample and then rapidly vitrified in the form of pearls or using the mini-straws.

*2.2.2.1. Pearls Vitrification method.* Pearls were formed by direct contact with $LN_2$. For this, 15 μL of sperm in vitrification medium were dropped, using 1–20 μL manual single-channel pipette, into a device designed by Kitazato Co [36,38], which was fully submerged in $LN_2$. Once the drops formed, they were funnelled through the canals of the device into a cryovial attached at the bottom (Fig. 1B.2).

*2.2.2.2. Mini-straws vitrification method (VMS).* 50 μL sperm in vitrification medium were manually injected, using a 1–100 μL micropipette, into a cut 0.25 mL sperm straw. Each of these straws were inserted into a bigger diameter straw (0.5 mL cut straw; Fig. 1B.1). After that, the straws were heat-sealed and directly plunged into $LN_2$. All samples were stored in the same $LN_2$ tank.

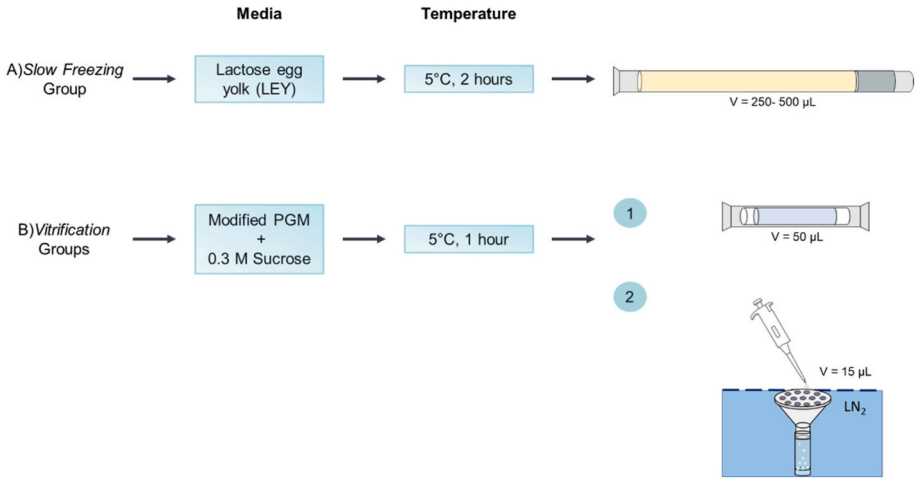



**Fig. 1.** Diagram of freezing protocols. A) Slow Freezing protocol, prepared sperm sample was diluted in LEY media and cooled to 5 °C for 2 h. Cooled samples were loaded in 0.5–0.25 ml straws and sealed. B) Samples were diluted in PGM and cooled at 5 °C for 1 h before supplementing with sucrose. B.1) Mini-straws vitrification method (VMS): 50 µL were injected into a 0.25 mL straw and sealed. B.2) Pearls Vitrification method: droplets were formed using a Kitazato device by direct contact with $LN_2$ and funnelled through the canals of the device into a cryovial submerged in $LN_2$.

### 2.3. Thawing/warming of the cryopreserved samples

Each cryopreservation method had its own thawing or warming process, as noted below.

#### 2.3.1. Thawing and post- thaw preparation of slow freeze samples

SL samples were thawed by placing the straws in warm water (37 °C for 30s) and resuspended in 500 µL of PGM (section 2.1). After, the samples were prepared using DGS, as explained above in section 2.2. Once the supernatant was removed the pellet was resuspended in 500 µL of fresh PGM.

#### 2.3.2. Warming and post-warming preparation of vitrified samples in mini-straws

VMS samples were warmed by immersing the straw in water at 37° for 30s. Then, the content of each mini-straw was diluted in 500 µL of warm PGM. After that, the samples were centrifuged for 7 min at 600G. Once the supernatant was removed the pellet was resuspended in 500 µL of fresh PGM.

#### 2.3.3. Warming and post-warming preparation of vitrified samples in pearls

Pearl samples were warmed by submerging 5 pearls of a sample into 500 µL of warm PGM (37 °C for 30s). Prior to sperm analysis, this samples were prepared in two different ways: centrifugation and DGS.

*2.3.3.1. Centrifugation.* Here, the warmed solution was centrifuged for 7 min at 600G. After that, the supernatant was removed and the pellet resuspended in 500 µL of fresh PGM. This method was used in the samples that formed the group labelled as VP.

*2.3.3.2. DGS.* This method was use in the samples that form the VPD group. Here, the warmed solution was prepared by DGS centrifugation (as explained above in the section 2.2). After that, the supernatant was removed, and the pellet was resuspended in 500 µL of fresh PGM.

### 2.4. Sperm evaluation

The following tests for semen assessment were performed before and after vitrification: sperm total and progressive motilities, morphology, concentration, viability, acrosome integrity, mitochondrial membrane potential, DNA damage and protein alteration in sperm nuclei.

#### 2.4.1. Total motility

Total motility (progressive and non-progressive) was evaluated using a warm stage and phase-contrast microscopy phase-contrast at x200 total magnification. Spermatozoa that showed stationary flagellation, curved motion and twitching were considered as motile but non-progressive, while those spermatozoa that swam in a straight line were classified as motile and progressive.

#### 2.4.2. Sperm concentration

The sperm count of each sample was performed on a Makler chamber and the data was presented as million sperm cells/mL.

#### 2.4.3. Sperm morphology

Sperm samples fixed in 4% PFA (for 30 min at 4 °C) were visualised using phase-contrast microscopy on an Olympus BX61 microscope equipped with a cooled CCD camera at x200 total magnification, and using SmartCapture3 imaging software (Digital Scientific UK, Cambridge, United Kingdom). The sperm was classified as: 1) normal, those sperm with normal size and shape of the head and acrosome, and a long and straight tail; 2) tail abnormalities, when sperm had coiled or bend tails; 3) head abnormalities, when they had misshapen heads, absent or malformed acrosomes; or 4) cytoplasmic drops, sperm showing a cytoplasmic droplet in the distal or proximal region of the tail.

#### 2.4.4. Analysis of seminal parameters by flow cytometry

Flow cytometric analyses were performed on a BD Accuri C6 Plus (BD Biosciences, Wokingham, Berkshire, United Kingdom) that have 14.7 mW a laser power 640 nm wavelengths for the Diode Red Laser and 20 mW laser power and 488 nm wavelengths for the Solid State Blue Laser. Data from 10,000 events per sample were collected in each test. Flow cytometric data were analysed using the BD C6 Plus Software (BD Biosciences, Wokingham, Berkshire, United Kingdom) using a gate in forward and side scatter to exclude remaining debris or aggregates.

*2.4.4.1. Sperm viability and acrosome integrity.* Sperm viability was assessed using propidium Iodide (PI) and acrosome status was determined using fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Invitrogen™, Inchinnan, United Kingdom) following the protocol explained in Robles and Martinez-Pastor (2013) [44].

*2.4.4.2. Sperm chromatin structure assay.* The detection of sperm DNA damage and protein alteration in sperm nuclei was assessed via flow cytometry of acridine orange stained sperm following a previously published protocol [16].

*2.4.4.3. Mitochondrial membrane potential assay.* The assessment of the mitochondria membrane potential (MMP) was done using Mitotracker Red CMXRos (Invitrogen™, Inchinnan, United Kingdom) following a protocol previously published [42].

### 2.5. Statistical analysis

Data were analysed on SPSS (Version 25, IBM). The statistical analysis of the samples, before and after three different cryopreservation techniques (slow freezing, vitrification using pearls and vitrification using mini-straws), and two different methods of post-thaw (VP and VPD; section 2.3) were assessed using one-way ANOVA in those parameters that had a normal distribution. The data parameters that did not have a normal distribution were tested using the Krustal-Wallis Test. The data in tables are presented as mean ± SEM. Results were considered to be statistically significant when $p < 0.05$.

## 3. Results

Comparing with the average motility of the samples before cryopreservation (PT = 80.20 ± 3.8%), all the studied cryopreservation methods (two vitrification methods, plus slow freezing) significantly decreased the progressive and total motility after thawing (Fig. 2). However, SF group had the highest total and progressive motility (42.2 ± 4.3% and 25.3 ± 4.4%, respectively). When comparing only vitrification groups, the total motility of the vitrified samples using the pearls method was significantly higher than when using mini-straws (26.15 ± 2.8% and 9.39 ± 0.9% for VP and VMS, respectively; Fig. 2.A). Moreover, this parameter decreased significantly when the samples were prepared using DGC after warming (VP = 26.15 ± 2.8% and VPD = 5.93 ± 1.5%). However, when comparing vitrified groups with slow freezing, slow freezing had a significantly higher percentage of total motility (SF = 42.20 ± 4.3%). Progressive motility did not differ between the vitrification methods, but it was significantly higher in the slow freezing group (VP = 8.31 ± 1.4%, VPD = 2.78 ± 1.1% VMS = 1.44 ± 0.6% and SF = 25.28 ± 4.4%; Fig. 2.B).

Mitochondrial activity is also severely affected by the cryopreservation (PT = 29.46 ± 7.13%). The results illustrated in Fig. 3 showed no differences in mitochondrial activity between the vitrification methods, but mitochondrial membrane potential was significantly higher in the slow freezing group (VP = 5.70 ± 1.1%, VPD = 5.01 ± 1.2%, VMS = 2.10 ± 0.5% and SF = 15.73 ± 3.4%).

The evaluation of the sperm morphology, performed before and after freezing (Table 1), showed significantly higher proportions of anomalies in all the cryopreservation groups relative to PT (17.8 ± 2.6%). The highest number of abnormalities were found in VPD and SF (39.6 ± 3.8% and 40.4 ± 1.9%, respectively), where the samples were prepared using DGC after warming. The %abnormalities were not significantly different to the VMS group (35.2 ± 1.8%) but %abnormalities in the VP group were significantly lower than all cryopreservation groups (28.6 ± 2.1%). Looking into the different types of abnormalities, our results showed no significant differences in the percentage of presence of cytoplasmic drop (CIT DROP) between all the studied groups. When looking at tail abnormalities, the percentage found in the control group (PT = 5.7 ± 1.5%) was significantly lower than the cryopreservation groups. There was no significant difference in tail abnormalities amongst the different cryopreservation groups (VP = 18.3 ± 2.0%, VPD = 23.2 ± 3.1% VMS = 18.2 ± 2.9% and SF = 26.7 ± 2.5%). The percentage of head abnormalities showed more variability between the groups. VP (8.8 ± 0.9%) was the second group, after the control (5.9 ± 1.1%), with the lowest levels of head anomalies and the differences among these 2 groups were not statistically significant. The proportion of head abnormalities in the group SF (10.0 ± 1.2%) is significantly higher than in PT but it did not significantly differ from VP. VPD (12.6

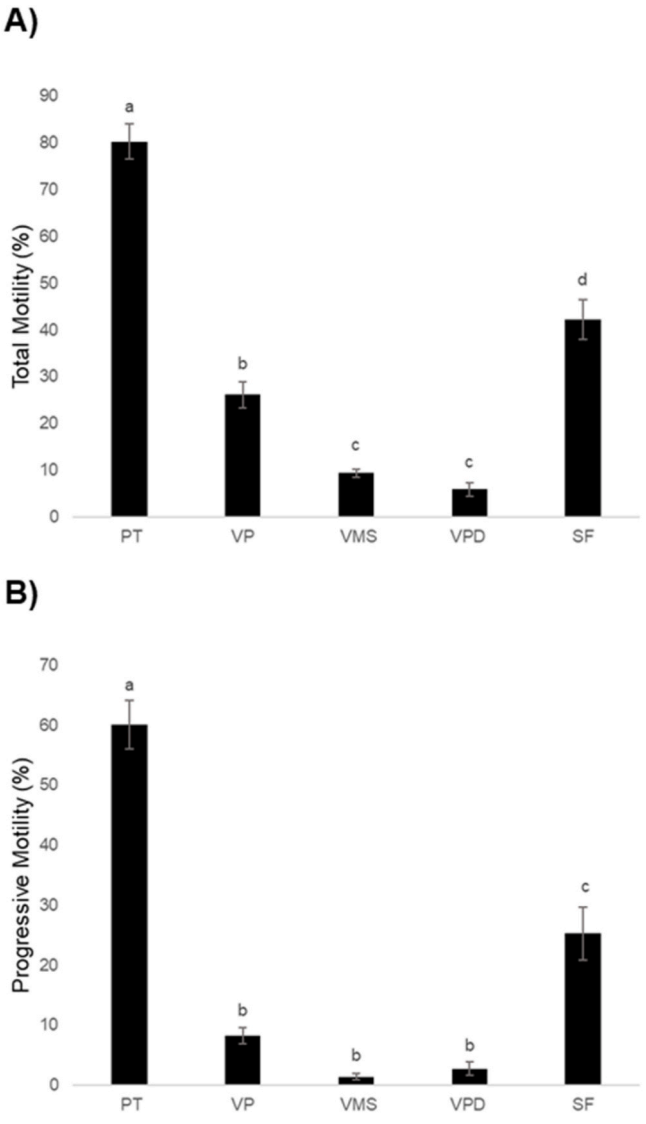



**Fig. 2.** Proportion of total (A) and progressive motility (B) of each group prior and post cryopreservation. PT=Pre-Test; VP= Vitrification Pearls; VMS = Mini-Straws; VPD= Vitrification Pearls + DGS; SF=Slow Freezing. Data are shown as mean% ± SEM. N = 13 replicates for each group. Krustal-Wallis Test (gl = 4, $p < 0.001$; $H_{\text{Total Motility}} = 42.119$; $H_{\text{Progressive Motility}} = 37.165$). $^{a,b,c,d}$ Different superscript letters indicate significant differences between groups: samples that share the same letter are statistically indistinguishable from each other.

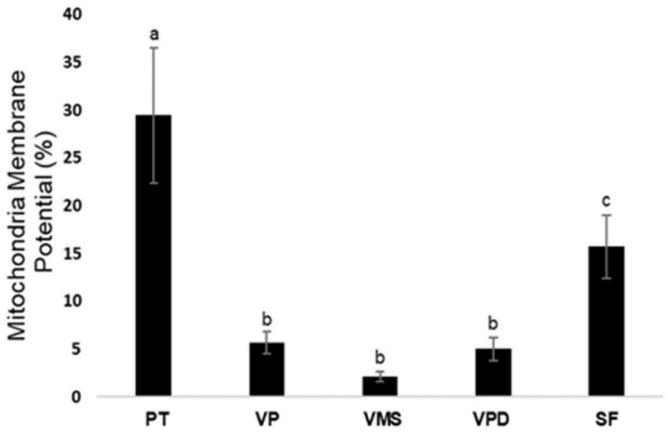



**Fig. 3.** Mitochondria Membrane Potential (MMP) of each group prior and post cryopreservation. PT=Pre-Test; VP= Vitrification Pearls; VMS = Mini-Straws; VPD= Vitrification Pearls + DGS; SF=Slow Freezing. Data are shown as mean% ± SEM. N = 13 replicates for each group. Krustal-Wallis Test (gl = 4, p = 0.002; H = 16.803). $^{a,b,c,}$Different superscript letters indicate significant differences amongst the groups; samples that share the same letter are statistically indistinguishable from each other.

**Table 1**
Results obtained from the evaluation of the sperm morphology and sperm concentration before and after cryopreservation.

| Group | Abnormal Mean % ± SEM | Head Abn. Mean % ± SEM | Tail Abn. Mean % ± SEM | CIT DROP Mean % ± SEM | [] sperm Million/mL Mean ± SEM |
|---|---|---|---|---|---|
| PT | 17.8 ± 2.6[a] | 5.9 ± 1.1[a] | 5.7 ± 1.5[a] | 6.0 ± 1.9 | 12.9 ± 2.6[a] |
| VP | 28.6 ± 2.1[b] | 8.8 ± 0.9[ab] | 18.3 ± 2.0[b] | 1.5 ± 0.6 | 0.8 ± 0.1[b] |
| VMS | 35.2 ± 1.8[c] | 13.0 ± 1.6[d] | 18.2 ± 2.9[b] | 4.0 ± 1.3 | 1.1 ± 0.1[b] |
| VPD | 39.6 ± 3.8[c] | 12.6 ± 1.5[cd] | 23.2 ± 3.1[b] | 3.9 ± 1.8 | 0.5 ± 0.1[b] |
| SF | 40.4 ± 1.9[c] | 10.0 ± 1.2[bc] | 26.7 ± 2.5[b] | 3.7 ± 1.0 | 2.6 ± 0.6[b] |
| p value[a] | <0.001 | 0.002 | <0.001 | 0.261 | <0.001 |
| F[a] | 15.176 | 5.162 | 11.969 | 1.370 | 17.320 |

Data are shown as mean ± SEM (N = 13 replicates for each group).
Abbreviations: PT=Pre-Test; VP= Vitrification Pearls; VMS = Mini-Straws; VPD= Vitrification Pearls + DGS; SF=Slow Freezing.
[a,b,c,d] Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are statistically indistinguishable from each other. F = calculated as variation between sample means/variation within the samples.
[a] ANOVA Test (gl = 4).

± 1.5%) did not significantly differ from SF but it did from the groups PT and VP. Lastly, VMS (13.0 ± 1.6%) showed a significant higher proportion of head anomalies with all the groups except VPD.

In terms of sperm DNA damage (Fig. 4), the data obtained showed that VP, VPD and SF groups had similar levels of % DNA fragmentation index (DFI) to the control group (1.2 ± 0.2%, 1.2 ± 0.4%, 1.5 ± 0.3% and 1.6 ± 0.2%, respectively). However, VMS had a significantly higher percentage (5.1 ± 0.1.7%). The VPD group had the lowest percentage of immature spermatozoa, identified as spermatozoa having high DNA stainability (HDS) (0.8 ± 0.1%) and it is significantly different to the VMS and VP groups. The highest HDS levels were found in VMS (2.2 ± 0.2%), and was significantly higher than the levels found in VPD and SF groups.

The total percentage of alive sperm cells (Table 2) in SF is not significantly different to the obtained in the control group (PT = 81.6 ± 5.4%). The groups VP and VPD showed similar percentages (32.0 ± 3.0% and 33.7 ± 8.3%, respectively), and they were significantly lower than the ones in PT and SF. The lowest percentage of alive sperm was in the VMS group, this group was significantly different to the other studied groups. The proportion of alive sperm with reacted acrosome (Alive-Acr) is not significantly different between the groups, with the highest percentages being found in the PT and VP groups (15.8 ± 8.2% and 15.4 ± 4.3%, respectively).

## 4. Discussion

It is commonly accepted that cryopreservation causes damage to spermatozoa, leading to changes in membrane lipid composition, acrosome status, sperm motility, and viability [17,39]. In this study we agree with the fact that vitrification, as any other type of cryopreservation, decreases the viability of the vitrified samples after thawing [56]. Our results showed a better outcome when performing boar sperm vitrification compared to the current literature [3], not only showing live rates after thawing of between 10 and 33%, but having progressive motility up to 8% in the best vitrification group with a 21% of total motility.

In humans, DFI over 30%, as measured by the sperm chromatin structure assay, has been shown to decrease fertility in vivo and in vitro [9,28,51]. Though, when considering boar sperm samples, DFI levels higher than 5% are considered poor quality [31]. In this study, vitrification using mini-straws was the sole method that resulted in DFI levels higher than 5%. Additionally, vitrification using mini-straws showed the lowest number of viable sperm following thawing. Thus, according to results produced in our study, we can confirm vitrification with mini-straws is not ideal for use in boar sperm. This is different to what was published by Díaz-Jiménez [12] in vitrification of donkey sperm, where straws showed a better efficiency than pearls. Furthermore, in humans the literature showed sperm vitrified in bigger volumes

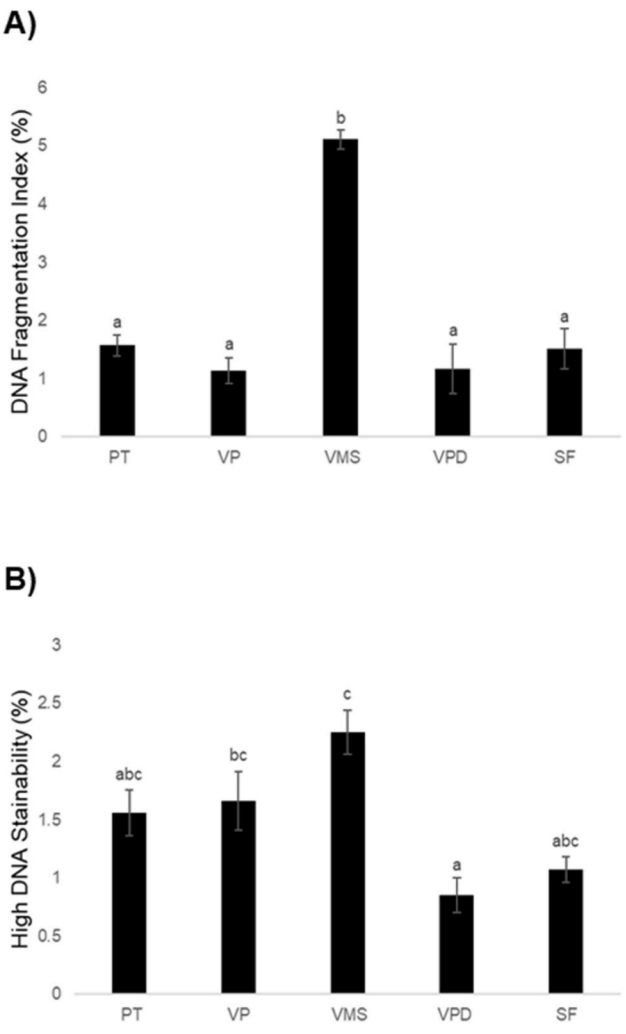



**Fig. 4.** Results obtained from the evaluation of DNA Fragmentation Index (DFI; A) and high DNA stainability (HDS; B) by flow cytometry before and after cryopreservation. Data are shown as mean ± SEM (N = 13 replicates for each group). Abbreviations: PT=Pre-Test; VP= Vitrification Pearls; VMS = Mini-Straws; VPD= Vitrification Pearls + DGS; SF=Slow Freezing. Krustal-Wallis Test (gl = 4; $p_{DFI} = 0.025$, $p_{HDS} < 0.001$; $H_{DFI} = 11.131$, $H_{HDS} = 21.004$.). [a,b,c,d] Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are statistically indistinguishable from each other. H was calculated as variation between sample means/variation within the samples.

**Table 2**
Results obtained from the evaluation of vitality and the acrosome status of the sample by flow cytometry before and after cryopreservation.

| Group | Alive Mean % ± SEM | Alive-Acr Mean % ± SEM | Total Alive Mean % ± SEM |
|---|---|---|---|
| PT | 65.8 ± 9.0[a] | 15.8 ± 8.2 | 81.6 ± 5.4[a] |
| VP | 16.6 ± 3.3[b] | 15.4 ± 4.3 | 32.0 ± 3.0[b] |
| VMS | 6.7 ± 1.5[b] | 3.8 ± 1.2 | 10.6 ± 2.5[c] |
| VPD | 25.4 ± 6.8[b] | 8.3 ± 2.7 | 33.7 ± 8.3[b] |
| SF | 71.4 ± 2.3[a] | 6.1 ± 1.4 | 77.5 ± 2.8[a] |
| p value[a] | <0.001 | 0.138 | <0.001 |
| H[a] | 31.878 | 6.965 | 37.333 |

Data are shown as mean ± SEM (N = 13 replicates for each group).
Abbreviations: PT=Pre-Test; VP=Pearls; VMS = Mini-Straws; VPD= Pearls + DGS; SF=Slow Freezing.
[a,b,c] Within each column, different superscript letters indicate significant differences amongst the groups; samples that share the same letter are statistically indistinguishable from each other. H = calculated as variation between sample means/variation within the samples.
[a] Krustal-Wallis Test (gl = 4).

(100–300 μL) resulted in similar motility, and similar or higher viability and mitochondrial membrane potential, with lower DNA damage, when the samples were warmed at a higher temperature (42 °C [34,52]).

The low concentration obtained on the vitrification groups (Table 1) could be a limitation for the use of this method for IUI. However, this will not be a problem for other in vitro techniques as IVF or ICSI [18]. Also, it can be evaluated the possibility of increasing the concentration of the samples, as was done in the study Saravia et al. in 2005 and 2011 [47,48].

When sperm morphology was evaluated, the results revealed a higher proportion of structural damage in the slow freeze boar sperm samples than when the most appropriate vitrification technique (VP) was used. The slow freezing process promotes the formation of intra- and extracellular ice crystals, which can cause morphological damage [57]. The ultra-fast drop in temperature induced by direct contact of samples with $LN_2$ during the vitrification process reduces the time samples are between −5 °C and −60 °C to prevent ice crystal formation [25]. These results agreed with the results published by Le et al., in 2019 [30], where it was shown that conventional freezing resulted in higher sperm's vitality and motility, while vitrification showed better morphology, reducing the incidence of sperm head, midpiece, and tail abnormalities. When comparing VP to VMS, the increased proportion of abnormalities in VMS may be due to the larger sperm volumes used and the insulation given by the straws, which slows down the speed of the vitrification process. The differences between VP and VPD can only be attributed to the warming protocol since the vitrification method is the same; the significant decrease in viability of the vitrified sperm could increase susceptibility to damage and decrease tolerance to the damage caused by centrifugation or DGS solutions.

Despite the reduction in morphological abnormalities and the preservation of DNA integrity of the VP samples, slow freezing is still more efficient than vitrification as mitochondrial activity deteriorates with cold shock in vitrification groups, decreasing the membrane potential radically when compared to slow freezing. The protection of sperm mitochondrial activity is very important to preserve the quality of the samples since it is directly related with a reduction in motility [5]. One way to mitigate this damage could be to modify the thawing method used. The standard thawing in boar sperm is 37 °C for 20–30 s [3,13,57]. However, vitrification protocols of oocytes or blastocyst, showed a shorter and drastic change of temperature, reducing ice formation and osmotic stress post-thaw [55,56]. Moreover, other publications have showed that shorter periods of time at higher temperatures shown an improved in the sperm parameters post thaw [2,6,15,53].

Furthermore, the media used for slow freezing has been extensively studied and implemented for decades [50]. In this study, the media used for vitrification was a standard IVF media, so the addition of antioxidants or chelating agents might be useful to prevent cooling damage and increase the efficiency of vitrification [2,50]. We can confirm that the use of sucrose as a cryoprotectant during boar sperm vitrification preserves sperm function, as happened in other species [11,19,36].

This study demonstrates that vitrification it is also a method available for boar sperm cryopreservation and opens a new line of investigation to improve its efficacy. Slow freezing is still more sustainable and efficient to cryopreserve boar sperm so ongoing research is needed to bring vitrification at the same level.

## Funding

This work was supported by a studentship provided by Topigs Norsvin and the Biotechnology and Biological Sciences Research Council (BBSRC) LINK awards scheme (BB/R00708X/1).

## Author contributions

Conceptualization: MSA; Formal analysis: MSA; Funding acquisition: DKG; Investigation: MSA, MCA; Methodology: MSA; Resources: LJZ; Supervision: DKG, LJZ, PJE; Writing-original draft: MSA; Writing -review & editing: MSA, MCA, DKG, PJE, LJZ. All the authors have read and agreed to publish this version of the manuscript.

## Institutional review board statement

This project only involved the use of commercial samples of sperm and so did not fall within the Animals (Scientific Procedures) Act 1986. It thus did not require review by an Animal Welfare and Ethical Review Body.

## Informed consent statement

Not applicable.

## Data availability

*The data underlying this article are available in the article.*

## Declaration of competing interest

The authors declare no conflict of interest.

## Acknowledgments

The authors wish to thank JSR Genetics Ltd. for their assistance in sourcing the samples required for this study. Also, they wish to thank Dr. Helen O'Neill at University College London for providing the vitrification device.

## References

[1] J. Aizpurua, L. Medrano, M. Enciso, J. Sarasa, A. Romero, M.A. Fernández, M. J. Gómez-Torres, New permeable cryoprotectant-free vitrification method for native human sperm, Hum. Reprod. 32 (2017) 2007–2015.
[2] F. Amidi, A. Pazhohan, M. Shabani Nashtaei, M. Khodarahmian, S. Nekoonam, The role of antioxidants in sperm freezing: a review, Cell Tissue Bank. 17 (2016) 745–756.
[3] C.C. Arraztoa, M.H. Miragaya, M.G. Chaves, V.L. Trasorras, M.C. Gambarotta, D. M. Neild, Porcine sperm vitrification II: spheres method, Andrologia 49 (2017), e12738.
[4] L.M. Ávila-Portillo, J.I. Madero, C. López, M.F. León, L. Acosta, C. Gómez, L. G. Delgado, C. Gómez, Fundamentos de criopreservación, Rev. Colomb. Obstet. Ginecolog. 57 (2006) 291–300.
[5] G. Carvajal, C. Cuello, M. Ruiz, J.M. Vázquez, E.A. Martínez, J. Roca, Effects of centrifugation before freezing on boar sperm cryosurvival, J. Androl. 25 (2004) 389–396.

[6] I. Casas, S. Sancho, M. Briz, E. Pinart, E. Bussalleu, M. Yeste, S. Bonet, Freezability prediction of boar ejaculates assessed by functional sperm parameters and sperm proteins, Theriogenology 72 (2009) 930–948.
[7] R.P. Cervera, F. Garcia-Ximenez, Vitrification of zona-free rabbit expanded or hatching blastocysts: a possible model for human blastocysts, Hum. Reprod. 18 (2003) 2151–2156.
[8] H.J. Chang, J.R. Lee, S.J. Chae, B.C. Jee, C.S. Suh, S.H. Kim, Comparative study of two cryopreservation methods of human spermatozoa: vitrification versus slow freezing, Fertil. Steril. 90 (2008) S280.
[9] M. Cissen, M. van Wely, I. Scholten, S. Mansell, J.P. de Bruin, B.W. Mol, D. Braat, S. Repping, G. Hamer, Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: a systematic review and meta-analysis, PLoS One 11 (2016), e0165125.
[10] A.F. Davidson, J.D. Benson, A.Z. Higgins, Mathematically optimized cryoprotectant equilibration procedures for cryopreservation of human oocytes, Theor. Biol. Med. Model. 11 (2014) 1–19.
[11] M. Diaz-Jimenez, J. Dorado, C. Consuegra, I. Ortiz, B. Pereira, J.J. Carrasco, V. Gomez-Arrones, A. Domingo, M. Hidalgo, Optimization of donkey sperm vitrification: effect of sucrose, sperm concentration, volume and package (0.25 and 0.5 mL straws), Anim. Reprod. Sci. 204 (2019) 31–38.
[12] M. Diaz-Jimenez, J. Dorado, B. Pereira, I. Ortiz, C. Consuegra, M. Bottrel, E. Ortiz, M. Hidalgo, Vitrification in straws conserves motility features better than spheres in donkey sperm, Reprod. Domest. Anim. 53 (2018) 56–58.
[13] J. Ding, M. Pry, N. Rana, W.P. Dmowski, Improved outcome of frozen-thawed blastocyst transfer with Menezo's two-step thawing compared to the stepwise thawing protocol, J. Assist. Reprod. Genet. 21 (2004) 203–210.
[14] B.M. Eriksson, H. Petersson, H. Rodriguez-Martinez, Field fertility with exported boar semen frozen in the new flatpack container, Theriogenology 58 (2002) 1065–1079.
[15] B.M. Eriksson, H. Rodriguez-Martinez, Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws, Anim. Reprod. Sci. 63 (2000) 205–220.
[16] D.P. Evenson, Sperm chromatin structure assay (SCSA®), in: Spermatogenesis, Springer, 2013, pp. 147–164.
[17] C. Gangwar, A. Saxena, A. Patel, S.P. Singh, S. Yadav, R. Kumar, V. Singh, Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen, Anim. Reprod. Sci. 192 (2018) 171–178.
[18] M.A. Gil, C. Alminana, J. Roca, J.M. Vázquez, E.A. Martinez, Boar semen variability and its effects on IVF efficiency, Theriogenology 70 (2008) 1260–1268.
[19] E. Isachenko, V. Isachenko, J.M. Weiss, R. Kreienberg, I.I. Katkov, M. Schulz, A. Lulat, M.J. Risopatrón, R. Sánchez, Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose, Reproduction 136 (2008) 167–174.
[20] E. Isachenko, V. Isachenko, I.I. Katkov, S. Dessole, F. Nawroth, Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success, Reprod. Biomed. Online 6 (2003) 191–200.
[21] V. Isachenko, E. Isachenko, M. Montag, V. Zaeva, I. Krivokharchenko, F. Nawroth, S. Dessole, I.I. Katkov, H. van der Ven, Clean technique for cryoprotectant-free vitrification of human spermatozoa, Reprod. Biomed. Online 10 (2005) 350–354.
[22] L.A. Johnson, Fertility results using frozen boar spermatozoa 1970 to 1985, Deep Freez. Boar Semen. (1985) 199–222.
[23] L.A. Johnson, K.F. Weitze, P. Fiser, W.M.C. Maxwell, Storage of boar semen, Anim. Reprod. Sci. 62 (2000) 143–172.
[24] M. Kasai, T. Mukaida, Cryopreservation of animal and human embryos by vitrification, Reprod. Biomed. Online 9 (2004) 164–170.
[25] T. Kasai, K. Ogawa, K. Mizuno, S. Nagai, Y. Uchida, S. Ohta, M. Fujie, K. Suzuki, S. Hirata, K. Hoshi, Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential, Asian J. Androl. 4 (2002) 97–104.
[26] R.V. Knox, Artificial insemination in pigs today, Theriogenology 85 (2016) 83–93.
[27] P. Kumar, N. Sharma, S. Sharma, N. Mehta, A.K. Verma, S. Chemmalar, A.Q. Sazili, In-vitro meat: a promising solution for sustainability of meat sector, J. Anim. Sci. Technol. 63 (2021) 693.
[28] A. Kumaresan, A. Johannisson, E.M. Al-Essawe, J.M. Morrell, Sperm viability, reactive oxygen species, and DNA fragmentation index combined can discriminate between above-and below-average fertility bulls, J. Dairy Sci. 100 (2017) 5824–5836.
[29] I.-K. Kwon, K.-E. Park, K. Niwa, Activation, pronuclear formation, and development in vitro of pig oocytes following intracytoplasmic injection of freeze-dried spermatozoa, Biol. Reprod. 71 (2004) 1430–1436.
[30] M.T. Le, T.T.T. Nguyen, T.T. Nguyen, T.T.A. Nguyen, V.Q.H. Nguyen, N.T. Cao, Cryopreservation of human spermatozoa by vitrification versus conventional rapid freezing: effects on motility, viability, morphology and cellular defects, Eur. J. Obstet. Gynecol. Reprod. Biol. 234 (2019) 14–20.
[31] C. López-Fernández, B. Pérez-Llano, P. García-Casado, R. Sala, A. Gosálbez, F. Arroyo, J.L. Fernández, J. Gosálvez, Sperm DNA fragmentation in a random sample of the Spanish boar livestock, Anim. Reprod. Sci. 103 (2008) 87–98.
[32] A. Medrano, P.F. Watson, W.V. Holt, Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope, Reproduction-Cambridge 123 (2002) 315–322.
[33] N.T. Men, K. Kikuchi, M. Nakai, A. Fukuda, F. Tanihara, J. Noguchi, H. Kaneko, N. V. Linh, B.X. Nguyen, T. Nagai, Effect of trehalose on DNA integrity of freeze-dried boar sperm, fertilization, and embryo development after intracytoplasmic sperm injection, Theriogenology 80 (2013) 1033–1044.
[34] M.S.A. Mohamed, Slow cryopreservation is not superior to vitrification in human spermatozoa; an experimental controlled study, Iran, J. Reprod. Med. 13 (2015) 633.
[35] S.I. Moskovtsev, A.G.M. Lulat, C.L. Librach, Cryopreservation of human spermatozoa by vitrification vs. slow freezing: Canadian experience, Curr. Front. Cryobiol. (2012) 77–100.
[36] H.C. O'Neill, M. Nikoloska, H. Ho, A. Doshi, W. Maalouf, Improved cryopreservation of spermatozoa using vitrification: comparison of cryoprotectants and a novel device for long-term storage, J. Assist. Reprod. Genet. 36 (2019) 1713–1720.
[37] M. Olaciregui, L. Gil, Freeze-dried spermatozoa: a future tool? Reprod. Domest. Anim. 52 (2017) 248–254.
[38] D. Pabón, M. Meseguer, G. Sevillano, A. Cobo, J.L. Romero, J. Remohi, M.J. de Los Santos, A new system of sperm cryopreservation: evaluation of survival, motility, DNA oxidation, and mitochondrial activity, Andrology 7 (2019) 293–301.
[39] D. Paoli, F. Lombardo, A. Lenzi, L. Gandini, Sperm cryopreservation: effects on chromatin structure, Genet. Damage Hum. Spermatozoa. (2014) 137–150.
[40] J.E. Parks, D.V. Lynch, Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes, Cryobiology 29 (1992) 255–266.
[41] L. Parmegiani, C. Tatone, G.E. Cognigni, S. Bernardi, E. Troilo, A. Arnone, A. M. Maccarini, G. Di Emidio, M. Vitti, M. Filicori, Rapid warming increases survival of slow-frozen sibling oocytes: a step towards a single warming procedure irrespective of the freezing protocol? Reprod. Biomed. Online 28 (2014) 614–623.
[42] F.J. Peña, B.A. Ball, E.L. Squires, A new method for evaluating stallion sperm viability and mitochondrial membrane potential in fixed semen samples, Cytometry B Clin. Cytometry 94 (2018) 302–311.
[43] F. Pezo, F. Romero, F. Zambrano, R.S. Sánchez, Preservation of boar semen: an update, Reprod. Domest. Anim. 54 (2019) 423–434.
[44] V. Robles, F. Martínez-Pastor, Flow cytometric methods for sperm assessment, in: Spermatogenesis, Springer, 2013, pp. 175–186.
[45] J.E. Rodríguez-Gil, E. Estrada, Artificial insemination in boar reproduction, in: Boar Reprod., Springer, 2013, pp. 589–607.
[46] R. Sánchez, J. Risopatrón, M. Schulz, J. Villegas, V. Isachenko, R. Kreinberg, E. Isachenko, Canine sperm vitrification with sucrose: effect on sperm function, Andrologia 43 (2011) 233–241.
[47] F. Saravia Ramos, Deep Freezing of Concentrated Boar Semen for Intra-uterine Insemination, 2011.
[48] F. Saravia, M. Wallgren, S. Nagy, A. Johannisson, H. Rodríguez-Martínez, Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability, Theriogenology 63 (2005) 1320–1333.
[49] K.R. Saritha, A. Bongso, Comparative evaluation of fresh and washed human sperm cryopreserved in vapor and liquid phases of liquid nitrogen, J. Androl. 22 (2001) 857–862.
[50] C.G. Silva, E.R. Cunha, G.R. Blume, J.V. Malaquias, S.N. Báo, C.F. Martins, Cryopreservation of boar sperm comparing different cryoprotectants associated in media based on powdered coconut water, lactose and trehalose, Cryobiology 70 (2015) 90–94.
[51] L. Simon, B.R. Emery, D.T. Carrell, Diagnosis and impact of sperm DNA alterations in assisted reproduction, Best Pract. Res. Clin. Obstet. Gynaecol. 44 (2017) 38–56.
[52] M. Slabbert, S.S. Du Plessis, C. Huyser, Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa, Andrologia 47 (2015) 594–599.
[53] C. Tomás, J. Gómez-Fernández, E. Gómez-Izquierdo, E. de Mercado, Effect of the holding time at 15° C prior to cryopreservation, the thawing rate and the post-thaw incubation temperature on the boar sperm quality after cryopreservation, Anim. Reprod. Sci. 144 (2014) 115–121.
[54] M.L. Vadnais, G.C. Althouse, Characterization of capacitation, cryoinjury, and the role of seminal plasma in porcine sperm, Theriogenology 76 (2011) 1508–1516.
[55] P. Westendorf, L. Richter, H. Treu, Deep freezing of boar sperma. Laboratory and insemination results using the Hülsenberger paillete method, DTW.Deutsche Tierarztl. Wochenschrift. 82 (1975) 261–267.
[56] I.G. White, Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review, Reprod. Fertil. Dev. 5 (1993) 639–658.
[57] M. Yeste, Sperm cryopreservation update: cryodamage, markers, and factors affecting the sperm freezability in pigs, Theriogenology 85 (2016) 47–64.
[58] M. Yeste, J.E. Rodríguez-Gil, S. Bonet, Artificial insemination with frozen-thawed boar sperm, Mol. Reprod. Dev. 84 (2017) 802–813.
[59] K. Yoshioka, C. Suzuki, A. Onishi, Defined system for in vitro production of porcine embryos using a single basic medium, J. Reprod. Dev. 54 (2008) 208–213.