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Porcine sperm vitrification II: Spheres method

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Summary

Owing to current problems in boar sperm cryopreservation, this study proposes to evaluate vitrification in spheres as an alternative cryopreservation procedure, comparing the use or not of permeable cryoprotectants and two warming methods. Extended (n = 3; r = 4) and raw (n = 5; r = 2) porcine spermatozoa were diluted in media, in the absence or presence of either 4% dimethylformamide or 4% glycerol, to a final concentration of 5×10^6 spermatozoa/ml and vitrified using the spheres method. Two warming procedures were evaluated: a rapid method (30 s at 37°C) and an ultrarapid method (7 s at 75°C, followed by 30 s at 37°C). Percentages of total motility (phase contrast), membrane function (hypo-osmotic swelling test), acrosome integrity (phase contrast), sperm viability (6-carboxyfluorescein diacetate and propidium iodide stain), chromatin condensation (toluidine blue stain) and chromatin susceptibility to acid denaturation (acridine orange stain) were evaluated in the samples before and after vitrification. Results, analysed using Friedman's test, suggest that rapid warming of raw porcine spermatozoa vitrified without permeable cryoprotectants may preserve DNA condensation and integrity better than the other processing methods studied in this work. Hence, porcine sperm vitrification using spheres could be used to produce embryos with ICSI to further validate this method.

KEYWORDS
cryopreservation, porcine, sperm, spheres, vitrification

1 | INTRODUCTION

The biological similarities between porcine and humans have increased the focus on pigs as donors for human-directed proteins and xenotransplants, leading to a notable growth in biomedical research (García Rosello, García Mengual, Coy, Alfonso, & Silvestre, 2008; García Vázquez, García- Roselló, Gutiérrez-Adán, & Gadea, 2009). In addition, the need to preserve porcine spermatozoa to be used as a bank of genetic resources, to maintain productive lines or to conserve endangered wild species, has become of increasing importance (Malo et al., 2012; Yoshida, 2000). Nevertheless, despite the fact that porcine sperm cryopreservation has been the object of continuous research (Grossfeld et al., 2008; Larsson & Einarsson, 1976; Polge, Salamos, & Wilmut, 1970; Roca, Parrilla, Bolarin, Martinez, & Rodriguez-Martinez, 2016; Yeste, 2015) and that reproductive performance outcomes following AI with frozen-thawed boar spermatozoa are currently acceptable (Yeste, 2015), there are still many weaknesses in the process. In addition, most of the progress obtained to date has simply counteracted boar sperm cryodamage (Yeste, 2015).

The purpose of slow cooling rates during conventional freezing is to maintain a very delicate balance between ice crystal formation and the resultant increased concentration of solutes. However, cell damage can occur due to crystallisation of intracellular water or osmotic and chilling injury (Sánchez et al., 2011). As a consequence, sperm membranes are affected, an increase in lipid peroxidation occurs, sperm motility and mitochondrial activity are decreased, and processes associated with cell death are induced (O'Conell, McClure, &

Lewis, 2002). Cryoprotective agents that permeate the cell membrane have been used to increase membrane fluidity and partially dehydrate the cell, lowering the freezing point and thus reducing the number and size of intracellular ice crystals (Jiménez-Rabadán et al., 2015). In porcine, glycerol has been widely used as a permeable cryoprotectant, while other studies have obtained similar results using dimethylamide and dimethylformamide (Bianchi et al., 2008), indicating that amides may be used as cryoprotectants in this species. However, Malo et al. (2012) reported that amides do not improve cryopreservation results, showing controversy in this area of study. The cryoprotectants themselves can have a toxic effect on spermatozoa, related to their concentration and the length of cell exposure (Swain & Smith, 2010). To solve this situation, some investigations have evaluated the effect of cryopreserving spermatozoa in the absence of cryoprotectant, in conjunction with other preservation methods such as vitrification (Isachenko, Isachenko, Katkov, Dessole, & Nawroth, 2003; Isachenko, Isachenko, Petrunkina, & Sanchez, 2012; Isachenko et al., 2004; Rosato & Iaffaldano, 2013).

Vitrification is a process by which liquids acquire a glassy state without the formation of ice crystals (Gao & Critser, 2000; Katkov et al., 2006; Kumar Gupta, Jun Uhm, & Taek Lee, 2007; Luyet & Hodapp, 1938). It is a simple, less time-consuming and less costly alternative to conventional cryopreservation. However, because of the deleterious osmotic effect that high concentrations of cryoprotectants have on spermatozoa (30%–50% combining permeable and nonpermeable cryoprotectants), vitrification cannot be extrapolated directly to the male gamete (Sánchez et al., 2011). Essentially, the success of vitrification is based on working with very high cooling/warming rates, decreasing the volume or increasing the viscosity of the samples to be vitrified (Arav et al., 2002). Thus, a new vitrification technique has been developed for human, canine, salmon and rabbit spermatozoa using very small samples (spheres) to be able to dispense with the addition of toxic cryoprotectants (Isachenko et al., 2008; Merino et al., 2011; Rosato & Iaffaldano, 2013; Sánchez et al., 2011). Furthermore, a cell that has survived cooling to low subzero temperatures is still challenged during warming and thawing, which can exert effects on survival comparable with those of cooling (Gao & Critser, 2000; Johnson, Weitze, Fiser, & Maxwell, 2000; Mazur, 1984). For this reason, we propose to assay two warming methods: a rapid method, warming the samples 30 s at 37°C proposed by the reports on vitrification in other species (Isachenko et al., 2008; Merino et al., 2011; Sánchez et al., 2011), and an ultrarapid one, warming the samples 7 s at 75°C followed by 30 s at 37°C. This last method is based on work by Cristanelli, Squires, Amann, and Pickett (1984) in equines, where the temperature descent for freezing spermatozoa is very swift, compared to that used in other species. They proposed to thaw using this ultrarapid procedure to counterbalance the fast temperature descent, as they observed an improvement in progressive motility with this method.

Thus, it was of interest to evaluate porcine sperm vitrification as an alternative cryopreservation procedure, using the spheres method in the absence of cryoprotectants, and to compare two warming methods, rapid versus ultrarapid.

2 | MATERIALS AND METHODS

2.1 | Animals

Eight terminal cross-breed males of proven fertility (88%–92% pregnancy rate), between 3 and 4 years old, were used for this study. Five of the males were lodged in the locality of Cañuelas, in the Province of Buenos Aires, and the other three in the Faculty of Veterinary Sciences of the University of Buenos Aires, in the capital city, both situated 34°36′S and 58°26′W, at sea level. In all cases, the animals were kept in individual pens, fed a balanced diet and had free access to water.

The Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires approved this study (protocol N° 2011/18).

All reagents were purchased from Sigma (St. Louis, MO, USA) except where stated otherwise.

2.2 | Semen collection and processing

All twenty-two ejaculates were collected between March and October, using the gloved-hand method (King & Macpherson, 1973), and the sperm-rich fraction was obtained. The frequency of semen collection from each boar was every 15 days, and samples were not pooled. Samples from the five boars housed in the locality of Cañuelas (n = 5, r = 2) were diluted in a commercial extender: Androstar Plus® (Minitube, Germany). The diluted samples were maintained and transported to the laboratory at a temperature of 17°C and upon arrival (40 min later) were warmed to 37°C for 20 min prior to their evaluation (extended semen). Another twelve ejaculates (n = 3; r = 4) were collected from three boars housed at the Faculty of Veterinary Sciences of the University of Buenos Aires. In this case, the samples were processed in the laboratory and were maintained at 37°C for 20 min, until evaluation and processing (raw semen).

2.3 | Vitrification of spermatozoa

Sperm samples were centrifuged at 400 ***g*** during 5 min and were then divided into aliquots to be diluted to a concentration of 5×10^6 spermatozoa/ml, in one step in TALP medium (NaCl 114 mmol/L; KCl 3,192 mmol/L; NaH_2PO_4 0.3416 mmol/L; $NaHCO_3$ 2 mmol/L; $CaCl_2$-$2H_2O$ 2 mmol/L; $MgCl_2$-$6H_2O$ 0.5017 mmol/L; lactate Na 10 mmol/L; pyruvate Na 0.2 mmol/L; gentamicin 25 µg/ml; glucose 11.99 mmol/L, HEPES 10 mmol/L) without cryoprotectants or with cryoprotectants (4% dimethylformamide (DMF) or 4% glycerol, final concentrations). The samples were kept at 37°C for 15 min until vitrification using the spheres method according to Isachenko et al. (2008). Briefly, small aliquots of 20–30 µl of diluted sperm samples were dropped directly into liquid nitrogen, keeping a minimum distance of 10 cm between the pipette and the surface of the liquid. The microdroplets formed spheres upon contact with the liquid nitrogen and spontaneously sank after 4 s (Figure 1a,b). The solid spheres remained submerged in liquid nitrogen during individual transfer to the

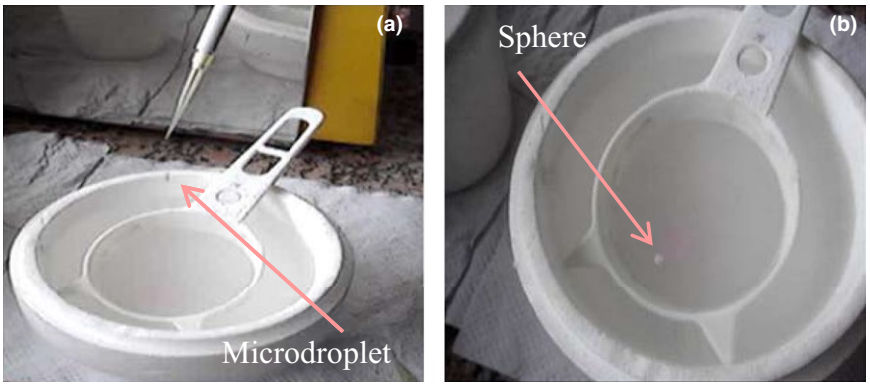

FIGURE 1 (a) 20–30 µl of diluted semen samples, dropped directly into liquid nitrogen. (b) Sphere exchanging heat with liquid nitrogen

cryovials using a dissecting forceps. The cryovials and the dissecting forceps used to manipulate the spheres were all previously cooled with liquid nitrogen and kept submerged throughout the process (thus avoiding exposure of the spheres to the air). The solid spheres were maintained in cryovials at −196°C for a minimum of 24 hr before being evaluated.

For post-thaw evaluation, vitrified samples were warmed using two procedures: (i) a rapid method, submerging the spheres in an cryovial containing 500 µl of TALP medium at 37°C for 30 s, and (ii) an ultrarapid method, submerging the spheres in an cryovial containing 500 µl of TALP medium at 75°C for 7 s, followed by transfer of the cryovial to a thermostatic water bath at 30 s at 37°C. In both cases, the spheres were submerged one by one (not more than five spheres per warming time) and accompanied by gentle vortexing for 5–10 s after each addition. The experimental design can be observed in Figure 2.

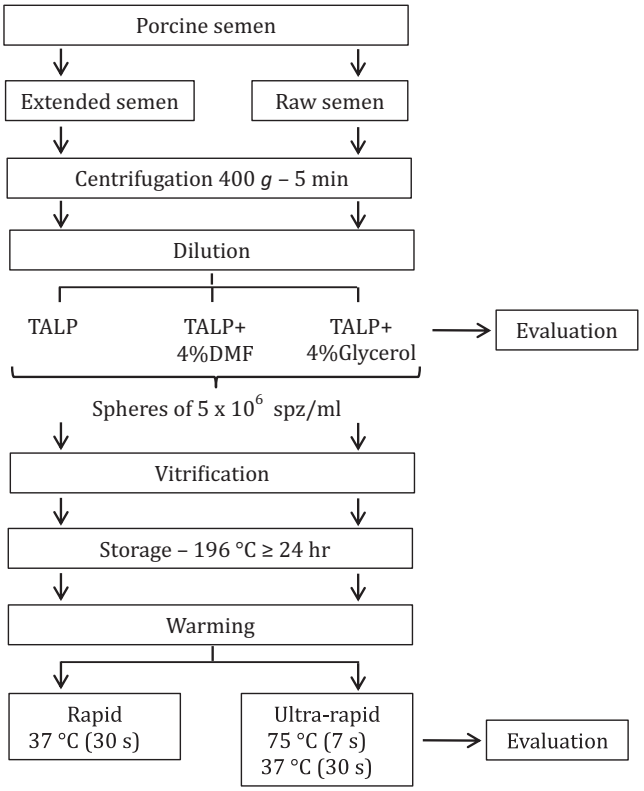

FIGURE 2 Experimental design

2.4 | Sperm evaluation

The following parameters were evaluated in sperm samples before and after vitrification: percentage of total motility, sperm viability, membrane function, acrosome integrity, chromatin condensation and chromatin susceptibility to acid denaturation.

2.4.1 | Total motility

Total motility (progressive and circular) was evaluated using a warm stage and phase-contrast microscopy (100×). Spermatozoa that showed stationary flagellation and twitching were not considered motile for this study.

2.4.2 | Sperm viability: 6-carboxyfluorescein diacetate and propidium iodide (CFDA/PI) stain

The viability stain of the samples was processed according to Harrison and Vickers (1990) modified. Briefly, 100 µl of semen was incubated at 37°C during 15 min in 1 ml of saline solution (NaCl 140 mmol/L; glucose 10 mmol/L; KCl2,5 mmol/L; PVP 0.5 mmol/L; HEPES 20 mmol/L. pH 7.55. 300 mOsm/kg) containing 20 µl of a stock solution of CFDA (0.5 mg/ml in dimethylsulphoxide). Then, 20 µl of a stock solution of PI (0.5 mg/ml in isotonic saline) was added and the sample was incubated a further 15 min. The percentage of viable spermatozoa (PI negative) was evaluated using an epifluorescence microscope (Leica® DMLS model; 400×), with the corresponding filters (CFDA: BP 450–490 nm and LP 515 nm; PI: BP 515–560 nm and LP 590 nm). A total of 200 spermatozoa were evaluated in each sample.

2.4.3 | Membrane function: hypo-osmotic swelling (HOS) test

The membrane function evaluation was carried out according to Vázquez, Martinez, Martinez, García-Artiga, and Roca (1997) modified. Briefly, 25 µl of semen sample was incubated at 37°C during 10 min

in 1 ml of 100 mOsm hypo-osmotic solution: fructose (9 mg/ml)–sodium citrate (4.9 mg/ml) diluted in ultrapure water. The percentage of spermatozoa with swelling in the tail (functional membrane) was evaluated using a phase-contrast microscope (400×). A total of 200 spermatozoa were evaluated per sample.

2.4.4 | Acrosome integrity

Evaluation of sperm acrosome integrity was carried out according to Pursel and Johnson (1974) modified. Sperm samples were fixed in buffered formalin saline (BFS) and were evaluated using a phase-contrast microscope (1,000×). Acrosome integrity was expressed as the percentage of spermatozoa with an intact acrosome (dark, smooth, crescentic apical ridge) after evaluating 200 cells per sample.

2.4.5 | Sperm chromatin condensation: toluidine blue (TB) stain

The TB stain was carried out according to González et al. (2008) modified. Briefly, a smear was made with the sperm sample, air-dried and fixed with ethanol 96% for 1–2 min and then stained during 30 min with a 1:3 working solution of TB (one part of 1% TB stock solution with three parts of a citric acid, sodium hydroxide and hydrogen chloride buffer, pH 4; Merck, Darmstadt, Germany). Samples were then washed with distilled water, air-dried and observed using light microscopy (1,000×), evaluating a total of 200 spermatozoa per sample. Condensed chromatin stains light blue (unaltered DNA), while decondensed chromatin stains an intense blue-violet (altered DNA). Spermatozoa with a dark blue staining were classified as having intermediate chromatin decondensation and were considered altered.

A positive control of the TB stain was carried out for each batch of slides. To this end, equal quantities of semen were incubated with 1% dithiothreitol (DTT) for 2 min at room temperature. This was followed by a 2-min incubation at room temperature with an equal proportion of 1% *N*-lauryl sarcosine sodium salt (SDS—detergent) to facilitate the entry of DTT into the spermatozoa. A smear was made and dried to stop the reaction and was then fixed in ethanol 96%. Staining of the positive control was carried out at the same time as the sperm samples, obtaining spermatozoa with highly decondensed chromatin (intense dark blue-violet staining).

2.4.6 | Sperm chromatin susceptibility to acid denaturation: acridine orange (AO) stain

The AO stain was carried out according to Tejada, Mitchell, Norman, Marik, and Friedman (1984) modified. Briefly, a sperm smear was fixed in ethanol 96%, air-dried and stained in the dark with a solution of AO (0.19 mg/ml; pH 2.5). It was then rinsed with distilled water, dried protected from the light and mounted to be evaluated under epifluorescence using a Leica® model DMLS microscope (1,000×) with BP 450- to 490-nm and LP 515-nm filters. A total of 200 spermatozoa were evaluated per sample, observing three staining patterns: spermatozoa with green fluorescence (nonsusceptible to denaturation), spermatozoa with light orange colouring in the post-acrosome region (slightly susceptible to denaturation), spermatozoa with orange fluorescence (highly susceptible to denaturation). The last two patterns were considered to have altered DNA.

A positive control for the AO stain was carried out for each batch of slides. Spermatozoa were subjected to alkaline denaturation to obtain single-chain DNA (orange fluorescence). To this end, a sperm smear was fixed in ethanol 96% and, once dry, was incubated in a 0.3 M solution of NaOH for 20 min at room temperature (Morris & Shertzer, 1985). BFS was added to the solution before the end of the incubation to allow the formaldehyde to interact with the exposed nitrogenous bases, thus preventing their reunion. Finally, the smear was washed at room temperature with a solution of PBS + BFS, dried and stained with AO, at all times protected from the light. The positive control was processed and evaluated at the same time as the semen samples, observing spermatozoa with a reddish orange fluorescence due to the metachromatic binding of the stain with the single-strand DNA that was produced.

2.5 | Statistical analysis

Sperm evaluation results, before and after vitrification, were analysed for each of the different types of sample (raw and extended) using Friedman's test and the R 2.2.1 program, considering significant p values < .05.

3 | RESULTS

3.1 | Sperm motility, plasma membrane integrity and function and acrosome integrity

Sperm motility, viability and acrosome integrity were not maintained in any of the vitrified/warmed samples evaluated (see Table 1). With regard to sperm viability, some live porcine spermatozoa were observed (range 0%–2%) both in the raw and extended samples vitrified in the absence of cryoprotectant or in the presence of DMF. Only when vitrifying raw porcine semen were acrosome-intact spermatozoa obtained after warming (range 0%–8%).

3.2 | Sperm chromatin condensation. TB stain

In the extended semen samples, there was a decrease (p < .05) in the percentage of sperm exhibiting normally condensed chromatin in all vitrified/warmed samples, independent of the use of cryoprotectants or the warming method implemented, when compared to the samples prior to vitrification (Figure 3).

In the raw semen samples, a decrease (p = .0498) in spermatozoa exhibiting normally condensed chromatin was only observed in the samples vitrified with DMF and warmed with both methods under evaluation, when compared with the sample prior to

TABLE 1 Results obtained from the evaluation of sperm total motility, viability, plasma membrane integrity and function and acrosome integrity in porcine semen samples, before and after vitrification (mean ± *SE*)

	Total motility (%)		Viability (%)		Membrane function (%)		Acrosome integrity (%)	
Samples	Extended	Raw	Extended	Raw	Extended	Raw	Extended	Raw
Original sample	57.5 ± 7.9	60.8 ± 3.8	53.8 ± 4.1	67.0 ± 3.7	37.9 ± 6.1	44.5 ± 3.8	91.3 ± 2.4	92.8 ± 2.0
Pre-TALP	67.5 ± 6.8	62.5 ± 4.6	41.6 ± 2.8	40.9 ± 3.3	68.6 ± 4.6	49.6 ± 5.4	90.6 ± 3.0	82.8 ± 5.7
Pre-TALP + DMF	56.5 ± 6.3	50.0 ± 4.3	46.7 ± 2.8	38.5 ± 3.0	72.5 ± 3.9	59.0 ± 4.5	89.8 ± 3.4	83.5 ± 5.3
Pre-TALP + Gly	58.5 ± 7.2	51.7 ± 4.9	41.7 ± 4.9	39.0 ± 3.6	72.8 ± 4.2	50.4 ± 4.9	90.5 ± 2.7	86.6 ± 3.6
TALP R	0	0	0.3 ± 0.1	0.5 ± 0.2	0	0	0	0.1 ± 0.1
TALP UR	0	0	0.1 ± 0.1	0.3 ± 0.1	0	0	0	0.8 ± 0.7
TALP + DMF R	0	0	0	0.1 ± 0.1	0	0	0	0.2 ± 0.1
TALP + DMF UR	0	0	0	0	0	0	0	0.2 ± 0.1
TALP + Gly R	0	0	0	0	0	0	0	0.7 ± 0.7
TALP + Gly UR	0	0	0	0	0	0	0	0

Pre-TALP, semen diluted in TALP medium, before vitrification; Pre-TALP + DMF, semen diluted in TALP medium + 4% DMF, before vitrification; Pre-TALP + Gly, semen diluted in TALP medium + 4% glycerol, before vitrification; TALP R, semen diluted in TALP medium, vitrified and warmed rapidly; TALP UR, semen diluted in TALP medium, vitrified and warmed ultrarapidly; TALP + DMF R, semen diluted in TALP medium + 4% DMF, vitrified and warmed rapidly; TALP + DMF UR, semen diluted in TALP medium + 4% DMF, vitrified and warmed ultrarapidly; TALP + Gly R, semen diluted in TALP medium + 4% glycerol, vitrified and warmed rapidly; TALP + Gly UR, semen diluted in TALP medium + 4% glycerol, vitrified and warmed ultrarapidly.

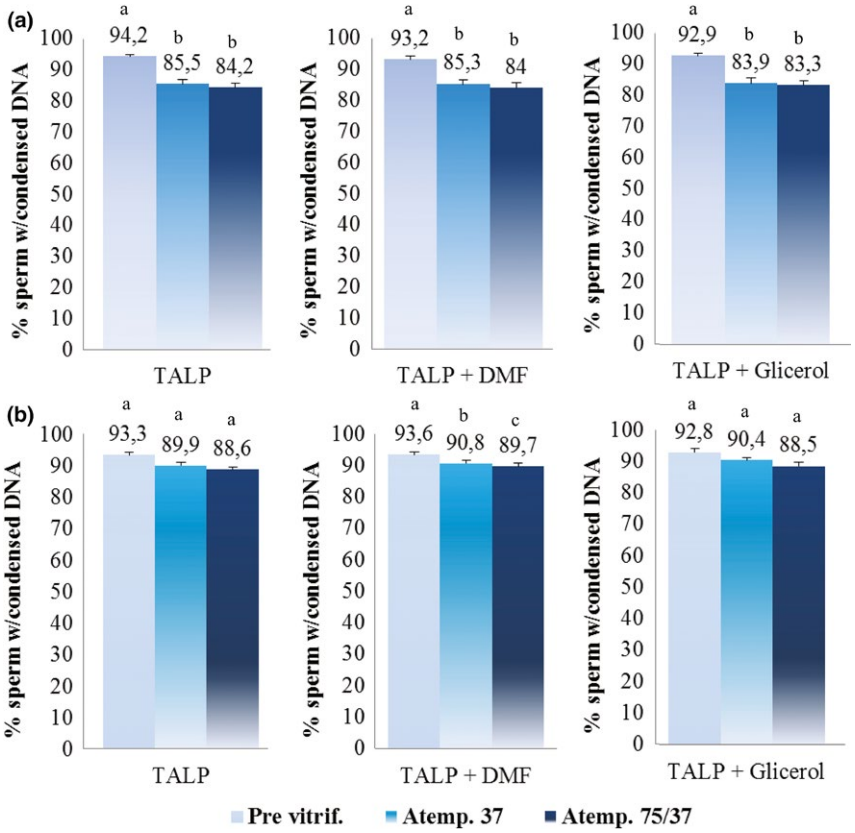

FIGURE 3 Percentage of spermatozoa with condensed DNA in extended (a) and raw (b) vitrified/warmed porcine semen samples. [a,b,c]Different superscripts, between samples before vitrification and between different warming methods, indicate significant differences ($p < .05$)

vitrification. Nevertheless, when comparing the warming methods, a decrease ($p < .05$) in spermatozoa exhibiting normally condensed chromatin was observed when using the ultrarapid warming method (Figure 3).

3.3 | Sperm chromatin susceptibility to acid denaturation: acridine orange (AO) stain

There were no differences ($p > .05$) in the percentage of spermatozoa exhibiting green nuclear fluorescence (intact DNA) among pre- and

TABLE 2 Percentage of sperm with non-denatured DNA in extended vitrified/warmed samples ($p > .05$) (mean ± standard error). Different superscripts between samples, within each medium, indicate differences ($p < .05$)

	TALP	TALP + DMF	TALP + Glycerol
Pre vitrif.	97.4 ± 2.8[a]	95.9 ± 3.8[a]	97.1 ± 1.9[a]
Warmed 37	97.6 ± 1.7[a]	97.6 ± 1.3[a]	98.9 ± 0.7[a]
Warmed 75/37	99.6 ± 0.3[a]	99.2 ± 0.6[a]	98.4 ± 0.8[a]

TALP + DMF: TALP medium + 4% DMF.
TALP + Glycerol: TALP medium + 4% Glycerol.

TABLE 3 Percentage of sperm with non-denatured DNA in raw vitrified/warmed samples ($p > .05$) (mean ± standard error). Different superscripts between samples, within each medium, indicate differences ($p < .05$)

	TALP	TALP + DMF	TALP + Glycerol
Pre vitrif.	91.2 ± 5.2[a]	97.2 ± 1.0[a]	92.5 ± 3.2[a]
Warmed 37	97.2 ± 1.9[a]	94.7 ± 2.4[a]	93.7 ± 3.4[a]
Warmed 75/37	93.1 ± 4.6[a]	91.0 ± 7.5[a]	96.7 ± 2.0[a]

TALP + DMF: TALP medium + 4% DMF.
TALP + Glycerol: TALP medium + 4% Glycerol.

post-vitrification samples, both in the extended (see Table 2) and the raw (see Table 3) semen samples, regardless of cryoprotectant treatment or warming method.

4 | DISCUSSION

This work represents the first study on boar sperm vitrification using the spheres method. It was possible to vitrify boar spermatozoa in spheres and maintain normally condensed chromatin and chromatin integrity. Similar results have been reported in rabbits by Rosato and Iaffaldano (2013) who evaluated sperm DNA quality using the acridine orange technique and obtained an average of 94.5 ± 1.4% (mean ± *SD*) spermatozoa with intact DNA after vitrifying in spheres in the absence of cryoprotectants. Sánchez et al. (2011) analysed DNA quality using the TUNEL technique in vitrified canine semen samples, and these authors did not observe significant differences either between the original samples and those vitrified in the presence or absence of cryoprotectants with the spheres method.

In our study, no motile spermatozoa were observed after vitrification with any of the protocols assayed. These results resemble those reported by Rosato and Iaffaldano (2013) in rabbits, where samples vitrified in the absence of cryoprotectants and warmed rapidly showed a motility range of 0–1%, and those reported by Sánchez et al. (2011), who obtained a low average motility in canine spermatozoa vitrified in the absence of cryoprotectants (1.66 ± 0.3% progressive motility; mean ± *SE*). Jiménez-Rabadán et al. (2015) reported 0 ± 0.73% motility (mean ± *SE*) in ram spermatozoa vitrified in straws with glycerol and sucrose as cryoprotectants. It is noteworthy that despite the different media and vitrification method used, the results in post-warming motility are similar. The only species where results were analogous to those reported in humans by Isachenko et al. (2008) (45%–57% motility) was in salmon, where Merino et al. (2011) obtained a maximum motility of 85.7% in semen samples vitrified using the spheres method without cryoprotectants and warmed at 37°C.

With regard to sperm viability, some live porcine spermatozoa were observed (range 0%–2%) both in the raw and extended samples vitrified in the absence of cryoprotectant or in the presence of DMF. These values were similar to those obtained in rabbits by Rosato and Iaffaldano (2013), who reported a range of 1%–5% membrane integrity in spermatozoa vitrified in the absence of cryoprotectants. Although these authors obtained better results after vitrifying in the presence of bovine serum albumin (BSA) and sucrose (a nonpermeable cryoprotectant), the values did not exceed 6%. Similar results were obtained by Jiménez-Rabadán et al. (2015) who reported for the different media they assayed, mean intact membranes (live spermatozoa) ranging between 0.24 ± 2.95 and 1.29 ± 2.95% (means ± *SE*) in vitrified ram semen.

When evaluating membrane function with HOS test, the absence of sperm swelling was also confirmed in the vitrified/warmed samples. Although some coiled or bent tails were observed in the post-warming morphological evaluation of the samples, as only 0%–2% of the cells were viable after warming, these morphological changes were attributed to alterations suffered during the process of cryopreservation and not to a positive reaction to the HOS test.

Only when vitrifying raw porcine semen were acrosome-intact spermatozoa obtained after warming. Binh, Van Thuan, and Miyake (2009) suggested that the sperm membranes suffer a certain degree of damage during the process of cooling to 17°C, especially in the region of the head. This partial damage could be responsible for the greater susceptibility of these samples to the considerable temperature changes suffered during vitrification.

According to Rosato and Iaffaldano (2013), sperm capacity for surviving vitrification in the absence of permeable cryoprotectants would seem to depend on species, on the vitrification procedure (speed of cooling/warming and the volume of the sample) and on the structural composition of the spermatozoa. In effect, the human spermatozoon is one of the smallest mammalian germinal cells and it has almost no residual histones, thus allowing for a very compact DNA. These characteristics of having a small and structurally compact sperm head would make it less vulnerable to the damage produced by ultrarapid cooling which is evident in other mammalian species such as rabbits and porcine (Gao, Mazur, & Critser, 1997; Katkov, 2012; Nauk, 1991; Nawroth et al., 2002).

In addition, human spermatozoa have a cholesterol:phospholipid ratio between 0.9 and 1, while porcine spermatozoa have 0.2 (Parks, 1997). This larger proportion of cholesterol present in human spermatozoa could grant the cells a greater rigidity and thus a better resistance to temperature changes. Moreover, during temperature changes, membrane proteins also suffer alterations that affect their function, thus also modifying cell behaviour (Watson, 2000). Parks and Lynch (1992) showed that porcine spermatozoa present greater

protein content in their membranes than bovine, equine and roosters, with this last species being the most resistant to temperature changes and the one with the least quantity of membrane proteins. Therefore, the greater intrinsic membrane protein content in porcine spermatozoa could increase their susceptibility to cryopreservation damage.

Regarding the warming method, it has been reported that if a very rapid curve of temperature descent is used, the thawing should be equally fast (Cochran, Amann, Froman, & Pickett, 1984; Gao & Critser, 2000; Johnson et al., 2000; Mazur, 1984). This did not seem to be true for warming after vitrification, at least under the conditions of our study. Our results indicate that raw semen vitrified in media with DMF and warmed rapidly (30 s at 37°C) preserved sperm DNA condensation better than ultrarapid warming (7 s at 75°C followed by 30 s at 37°C). The ultrarapid method of warming proved to be difficult to apply to samples vitrified in spheres, because in this method, the samples do not have a physical support as do the samples vitrified with cryoloops or cryotops. Changing temperatures after a very short time of exposition accompanied by gentle vortexing proved to be complex when manipulating spheres. Consequently, rapid warming (not ultrarapid warming) would be the method of choice, not only because it preserves the samples better, but also because it is a repeatable, simple and more practical method that minimises the possibility of damaging the samples during the warming process.

Our results, based on DNA quality, indicate that it would be preferable to vitrify raw porcine semen samples with the spheres method in the absence of cryoprotectants because sperm chromatin condensation and integrity is conserved and, in addition, some live sperm cells are obtained. Although the use of glycerol as cryoprotectant did not affect DNA condensation or integrity, no live cells were obtained when this cryoprotectant was present. Thus, this method, performed without cryoprotectants, avoids the negative effects of these substances (Isachenko et al., 2004, 2012) and saves time during warming as it becomes unnecessary to add sperm washing steps to remove the toxic cryoprotectants from the media. Considering that in our study boar spermatozoa vitrified in spheres preserved their chromatin condensation and integrity, those cells would only be apt for use in reproductive biotechnologies such as intracytoplasmic sperm injection (ICSI) where, according to Yanagimachi (2005), ICSI would allow one to obtain viable offspring as long as the sperm nucleus is conserved intact regardless of concentration, morphology or sperm motility. Furthermore, porcine in vitro embryo production using abattoir ovaries has been limited by the high incidence of polyspermy that is observed; hence, ICSI is presented as an alternative for in vitro production of monospermic zygotes (García Rosello et al., 2008).

When referring to the type of samples to vitrify, according to our results, vitrified raw porcine samples preserve DNA quality better than those that were extended and conserved at 17°C. Despite dilution in an extender, prior exposure to 16–18°C would seem to promote greater chromatin susceptibility, making the spermatozoa more vulnerable to the processes of vitrification and warming. This hypothesis would seem to be supported by the report from Boe-Hansen, Ersboll, Greve, and Christensen (2005) who observed a significant increase in the DNA fragmentation index (using the sperm chromatin structure assay) in porcine semen samples extended and conserved at 18°C for 72 hr (range: 0.3%–83.9%).

5 | CONCLUSIONS

Vitrifying raw porcine spermatozoa, in the absence of permeable cryoprotectants and using a rapid warming, preserves sperm DNA condensation and integrity, allowing the use of these cells in intracytoplasmic sperm injection. Current studies are being carried out to verify the possibility of producing porcine embryos using spermatozoa vitrified without cryoprotectants with the spheres method.

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