Accepted: 15 July 2016

DOI: 10.1111/and.12706

ORIGINAL ARTICLE

WILEY ANDROLOGIA

# Porcine sperm vitrification I: cryoloops method

C. C. Arraztoa[1,2] | M. H. Miragaya[1,2] | M. G. Chaves[1,2] | V. L. Trasorras[1,2] | M. C. Gambarotta[3] | C. H. Péndola[1] | D. M. Neild[1,2]

[1]Cátedra de Teriogenología, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina

[2]Instituto de Investigación y Tecnología en Reproducción Animal (INITRA), Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina

[3]Cátedra de Estadística, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina

**Correspondence**
Claudia Cecilia Arraztoa, Ciudad Autónoma de Buenos Aires (CABA), Buenos Aires, Argentina.
Email: carraztoa@fvet.uba.ar

**Summary**

The aims of this study were to evaluate porcine sperm vitrification in cryoloops, with and without two different cryoprotectants and assess two warming procedures. Extended ($n$ = 3; r = 4) and raw ($n$ = 5; r = 2) semen was diluted in media without and with cryoprotectants (4% dimethylformamide and 4% glycerol) to a final concentration of 20 × $10^6$ spermatozoa $ml^{-1}$ and vitrified using the cryoloops method. Two warming procedures were evaluated: rapid method (30 s at 37°C) and an ultra-rapid method (7 s at 75°C, followed by 30 s at 37°C). Total motility (phase contrast), sperm viability (6-carboxifluorescein diacetate and propidium iodide stain), membrane function (hypo-osmotic swelling test), acrosome integrity (phase contrast), chromatin condensation (toluidine blue stain) and chromatin susceptibility to acid denaturation (acridine orange stain) were evaluated before and after vitrification and analysed using Friedman's test. In all media, the only seminal parameters that were maintained after vitrification were chromatin condensation and integrity. Vitrification of porcine spermatozoon using cryoloops, both in the presence or absence of cryoprotectants and independent of the warming procedure used, permits conservation of sperm chromatin condensation and integrity. It would be interesting to further verify this by producing porcine embryos using vitrified spermatozoon with intracytoplasmic sperm injection.

**KEYWORDS**

cryoloop, cryopreservation, porcine, sperm, vitrification

## 1 | INTRODUCTION

In the last decade, interest in the porcine species has increased, both in the productive field applying technologies to improve genetics (Day, 2000; Grossfeld et al., 2008; Roca, Parrilla, Bolarin, Martinez, & Rodriguez-Martinez, 2016) and in the field of basic and biomedical research, due to the biological similarities between this species and humans. In this respect, porcine have been considered as a transgenic animal for the production of specific proteins or as a potential xenotransplant donor (García Rosello, García Mengual, Coy, Alfonso, & Silvestre, 2008; García Vázquez, García- Roselló, Gutiérrez-Adán, & Gadea, 2009). Faced with this wide array of possibilities, it becomes necessary to rely on a sperm cryopreservation method that permits genetic conservation of the species (Ikeda et al., 2002), while at the same time easing transport and the sanitary control of the semen samples. Nevertheless, porcine sperm cryopreservation has its difficulties because pig semen differs in many aspects from that of the rest of domestic species: it is produced in large volumes and is extremely vulnerable to cold shock or to freezing immediately after collection (Johnson, Weitze, Fiser, & Maxwell, 2000; Yeste, 2015). It is possible to cryopreserve and store porcine semen but freeze–thawing procedures induce sperm membrane changes that lead to its destabilisation, thereby affecting acrosome integrity and producing membrane lipid disorder (Yeste, 2015). The use of cryoprotectants for freezing is fundamental because they stabilise sperm plasma membranes, decrease both intra- and extracellular ice formation and restrict the "solution

  

effect" (Grossfeld et al., 2008; Medeiros, Gomes, Carmo, Papa, & Alvarenga, 2002). Many cryoprotectants have been tested in the porcine species, but as yet, the results obtained with glycerol have not been surpassed (Bianchi et al., 2008; Malo et al., 2012; Watson, 1995). Glycerol is highly soluble in water, a quality that allows it to penetrate the plasma membrane, but at a slow rate (Rodriguez & Wallgren, 2011). On the other hand, amides (formamide, methyl or dimethylformamide, acetamide, methyl or dimethylacetamide), because of their low molecular weight and viscosity, show greater membrane permeability, thus decreasing the damage caused by osmotic stress (Ball & Vo, 2001; Bianchi et al., 2008). Due to this characteristic, amides could replace glycerol in the composition of extenders used to cryopreserve porcine semen.

When looking at substitute methods of cryopreservation, vitrification emerges as a possibility. Vitrification is a process by which liquids modify their state without the formation of ice crystals (Jiménez-Rabadán et al., 2015; Liebermann et al., 2002; Sánchez et al., 2011) acquiring a glassy or vitreous state (Gao & Critser, 2000; Katkov et al., 2006; Luyet & Hodapp, 1938; Rail & Fahy, 1985), thus presenting an alternative to conventional cryopreservation of cells or tissues. In addition, it is a simple procedure that requires less time and presents lower costs than conventional cryopreservation (Sánchez et al., 2011). Nevertheless, a high proportion of cryoprotectants, between 30% and 50% (combining permeable and nonpermeable cryoprotectants) is necessary to vitrify tissues and large cells, while slow freezing requires lower percentages, between 5% and 7% (Isachenko, Isachenko, Katkov, Dessole, & Nawroth, 2003). Unfortunately, it has not been possible to directly extrapolate vitrification to the male gamete, mostly due to the deleterious osmotic effect on spermatozoa produced by the high concentrations of the cryoprotectants required (Sánchez et al., 2011). In vitrification, there is an inverse relationship between the speed of cooling/warming and the concentration of cryoprotectant present in the media (Isachenko et al., 2003; Katkov et al., 2006). Therefore, higher velocities of cooling and warming require lower cryoprotectant concentrations to reach the vitreous state. As a result, vitrifying using very fast cooling and warming velocities in very small samples to avoid using high concentrations of cryoprotectants arises as an alternative (Isachenko et al., 2003). Consequently, a new vitrification technique that dispenses with cryoprotectants has been developed for human spermatozoa, using cryoloops and plunging the samples directly in liquid nitrogen (Isachenko, Isachenko, Katkov, Rahimi, et al., 2004; Nawroth et al., 2002) and as a result, using very high cooling rates (Isachenko et al., 2003).

It is also known that an incorrect warming can decrease sperm survival of the cryopreservation process (Gao & Critser, 2000; Johnson et al., 2000); therefore, this step should also be carried out very rapidly. For this reason, it would be interesting to evaluate the effects of rapid warming of vitrified samples (30 s at 37°C) (Isachenko et al., 2003; Isachenko, Isachenko, Katkov, Rahimi, et al., 2004; Nawroth et al., 2002) and compare them to those obtained with an ultra-rapid warming method used in equine semen cryopreservation (7 s at 75°C followed by 30 s at 37°C) (Cristanelli, Squires, Amann, & Pickett, 1984).

Hence, the objectives of this study were: (i) to evaluate vitrification in cryoloops, with and without two different cryoprotectants, as an alternative method for cryopreserving porcine spermatozoa and (ii) assess two warming procedures to ensure the maintenance of sperm survival of the cryopreservation process.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Eight terminal cross-breed males of proven fertility, 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, both places situated 34°36′S and 58°26′W, at sea level. In both cases, the animals were kept in individual pens, fed a balanced diet and had free access to water.

This study was approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol N° 2011/18.). All reagents were purchased from Sigma (St. Louis, MO, USA) except where stated otherwise.

### 2.2 | Semen collection and processing

Twenty-two ejaculates were collected using the gloved-hand method (King & Macpherson, 1973), and the sperm-rich fraction was obtained. The frequency of semen collection was every 15 days. Samples from the five boars housed in the locality of Cañuelas ($n$ = 5, r = 2) were diluted in Androstar Plus® (Minitube, Tiefenbach, Germany), were conserved and transported to the laboratory at a temperature of 17°C and upon arrival were warmed to 37°C for 20 min prior to their evaluation (extended semen). Another 12 ejaculates ($n$ = 3; r = 4) were collected from three boars housed at the Faculty of Veterinary Sciences of the University of Buenos Aires and were maintained at 37°C until evaluation 20 min later (raw semen).

### 2.3 | Vitrification of spermatozoa

Semen samples were centrifuged at 400 $g$ during 5 min and were then divided into aliquots to be diluted in media without cryoprotectants (TALP: NaCl 114 mmol $L^{-1}$; KCl 3.192 mmol $L^{-1}$; $NaH_2PO_4$ 0.3416 mmol $L^{-1}$; $NaHCO_3$ 2 mmol $L^{-1}$; $CaCl_2$-$2H_2O$ 2 mmol $L^{-1}$; $MgCl_2$-6H2O 0.5017 mmol $L^{-1}$; Lactato Na 10 mmol $L^{-1}$; Piruvato Na 0.2 mmol $L^{-1}$; Gentamicina 25 µg $ml^{-1}$; Glucosa 11.99 mmol $L^{-1}$, HEPES 10 mmol $L^{-1}$) and media with cryoprotectants (TALP + 4% DMF and TALP + 4% glycerol) to a concentration of 20 x $10^6$ spermatozoa $ml^{-1}$. Finally, samples were vitrified using the cryoloops method according to Isachenko, Isachenko, Katkov, Montag, et al. (2004). Briefly, manually made copper cryoloops with a diameter of 5 mm were submerged in each of the semen samples being assayed, obtaining a thin film of approximately 20 µl of sample contained in the loop (Fig. 1). This was directly placed into liquid nitrogen and conserved

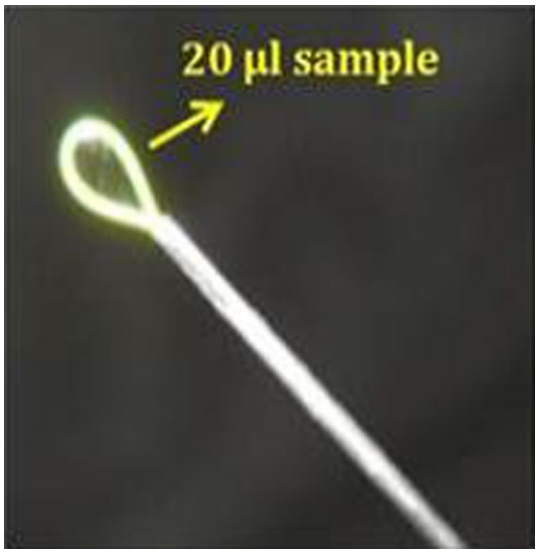


**FIGURE 1** 20 µl semen sample loaded on a cryoloop

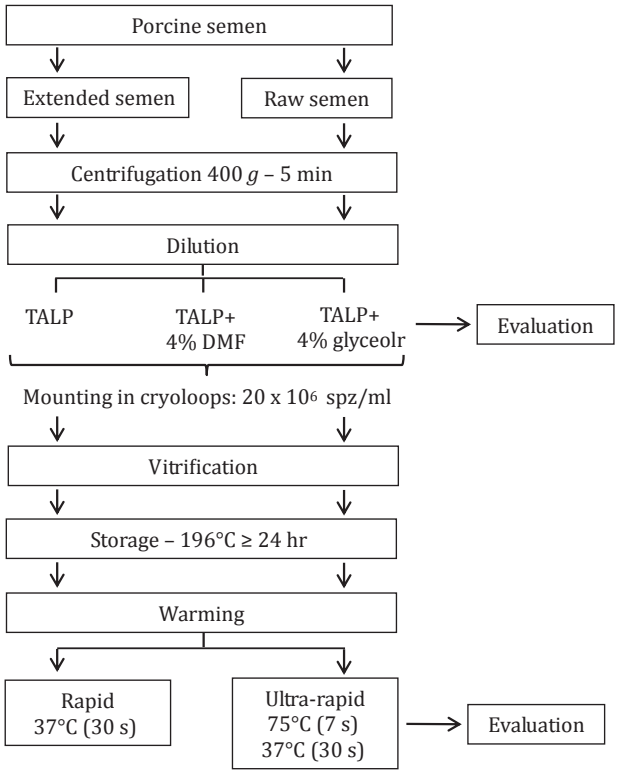


**FIGURE 2** Experimental design

at −196°C in 1.8 ml cryovials for a minimum of 24 hr before being evaluated.

For evaluation, vitrified samples were warmed using two procedures: a rapid method, directly plunging the cryoloop in TALP medium at 37°C for 30 s and an ultra-rapid method, submerging the cryoloop 7 s in TALP medium at 75°C, followed by 30 s at 37°C, applying soft stirring in both cases during the process. The experimental design can be observed in Fig. 2.

## 2.4 | Semen evaluation

The following semen parameters were evaluated in the samples diluted with and without cryoprotectant prior to vitrification and in vitrified/warmed samples: total motility, sperm viability, membrane function, acrosome integrity, chromatin condensation and chromatin susceptibility to acid denaturation.

### 2.4.1 | Total motility

The percentage of total sperm motility (progressive and circular) was evaluated using a warm stage and phase contrast microscopy (×100).

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

The viability of the samples was evaluated according to the technique described by Harrison and Vickers (1990) modified. Briefly, 100 µl of semen was incubated at 37°C during 15 min in 1 ml of saline solution (ClNa 140 mmol $L^{-1}$; Glucosa 10 mmol $L^{-1}$; ClK 2,5 mmol $L^{-1}$; PVP 0,5 mmol $L^{-1}$; HEPES 20 mmol $L^{-1}$. pH 7,55. 300 mOsm $kg^{-1}$) containing 20 µl of a stock solution of CFDA (0.5 mg $ml^{-1}$ in dimethylsulphoxide). Then, 20 µl of a stock solution of PI (0.5 mg $ml^{-1}$ 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

Sperm membrane function evaluation was carried out according to the technique described by 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 hypo-osmotic solution: fructose (9 mg $ml^{-1}$)—sodium citrate (4,9 mg $ml^{-1}$), adjusted to 100 mOsm. The percentage of spermatozoa with swelling in the tail (functional plasma 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 acrosome integrity was carried out according to the technique described by Pursel and Johnson (1974) modified. Semen samples were fixed in buffered formol 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 after evaluating 200 cells per sample.

### 2.4.5 | Sperm chromatin condensation: toluidine blue stain

The toluidine blue (TB) stain was carried out according to González et al. (2008) modified. Briefly, a smear was made with the semen sample, fixed with ethanol 96° for 1–2 min and then stained during 30 min with a 1:3 solution of TB in buffer pH 4 (1% TB stock solution). 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 while decondensed chromatin stains an intense blue-violet. Spermatozoa with a dark blue staining were classified as having intermediate chromatin decondensation and were considered damaged or altered.

A positive control of the TB stain was carried out at the same time. 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-minute 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 semen samples, obtaining spermatozoa with highly decondensed chromatin (intense dark blue-violet staining).

### 2.4.6 | Sperm chromatin susceptibility to acid denaturalisation: acridine orange stain

The acridine orange (AO) stain was carried out according to Tejada, Mitchell, Norman, Marik, and Friedman (1984) modified. Briefly, a semen smear was fixed in ethanol 96°, air-dried and stained in the dark with a solution of AO (0.19 mg $ml^{-1}$; 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 450–490 nm and LP 515 nm filters. A total of 200 spermatozoa were evaluated per sample, observing three staining patterns: spermatozoa with green fluorescence (non-susceptible to denaturation), spermatozoa with light orange colouring in the post-acrosome region (slightly susceptible to denaturation) and spermatozoa with orange fluorescence (highly susceptible to denaturation).

A positive control for the AO stain was carried out at the same time. Spermatozoa were subjected to alkaline denaturation to obtain single chain DNA (orange fluorescence). To this end, a semen 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 so the formol interacts with the exposed nitrogenous bases preventing their reunion. Finally, the smear was washed at room temperature with a solution of PBS + BFS, dried protected from the light and stained with AO. 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

For each of the different types of sample (raw and extended), sperm evaluation results, before and after vitrification, were analysed within each cryopreservation media 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).

### 3.2 | Sperm chromatin condensation. TB stain

In the extended semen samples, no significant differences ($p > .05$) were observed for chromatin condensation between the samples prior to vitrification and the corresponding vitrified/warmed samples when using either DMF or glycerol, independently of the warming method used. Nevertheless, the samples vitrified using only TALP medium (without cryoprotectant) showed a significant decrease ($p < .05$) in chromatin condensation when compared to the corresponding samples prior to vitrification for both warming methods (Fig. 3a).

When comparing the warming methods, a significant decrease ($p < .05$) in chromatin condensation was observed when using the TALP medium and the ultra-rapid warming method (Fig. 3 a,b).

In raw semen samples, no significant differences ($p > .05$) were observed between samples before vitrification and the corresponding samples vitrified with cryoprotectants (both DMF and glycerol) and without cryoprotectant, independently of the warming method used (Fig. 3b).

### 3.3 | Sperm chromatin susceptibility to acid denaturation: AO stain

No significant differences ($p > .05$) were observed between the degree of sperm chromatin susceptibility to acid denaturation in vitrified/warmed samples and the corresponding samples prior to vitrification, for any of the media (with or without cryoprotectants) or warming methods used (Fig. 4), both in vitrified extended and raw semen samples.

## 4 | DISCUSSION

This work represents the first study on vitrification in porcine spermatozoa. It was possible to cryopreserve pig spermatozoa using cryoloops and maintain sperm chromatin condensation and integrity. These results were similar to those reported by Isachenko, Isachenko, Katkov, Rahimi, et al. (2004) and Isachenko, Isachenko, Katkov,

**TABLE 1** Results obtained for the evaluation of sperm total motility, viability, plasma membrane integrity and function and acrosome integrity in extended (A) and raw (B) porcine semen samples, before and after vitrification (mean ± standard error)

| | Total motility (%) | | Viability (%) | | Membrane function (%) | | Acrosome integrity (%) | |
|---|---|---|---|---|---|---|---|---|
| Samples | A | B | A | B | A | B | A | B |
| Original sample | 57.5 ± 7.9 | 60.8 ± 3.8 | 53.8 ± 4.1 | 67 ± 3.7 | 37.9 ± 6.1 | 44.5 ± 3.8 | 91.3 ± 2.4 | 92.8 ± 2 |
| TALP Pre | 57.5 ± 5.9 | 60 ± 3.9 | 45.8 ± 2.4 | 46.3 ± 3.8 | 68.4 ± 4.3 | 48.3 ± 4.8 | 91.6 ± 3.3 | 90 ± 3.1 |
| TALP + DMF Pre | 54 ± 5.5 | 45.8 ± 2.9 | 45.5 ± 2.8 | 44.2 ± 3.7 | 69.4 ± 5.3 | 56.5 ± 4.2 | 89.5 ± 3.1 | 86.3 ± 4.5 |
| TALP + Gly. Pre | 58 ± 6.5 | 44.2 ± 4.3 | 46.1 ± 3.5 | 46 ± 3.4 | 72.7 ± 4.5 | 47.6 ± 5.1 | 91.3 ± 2.7 | 86.3 ± 3.9 |
| TALP R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 ± 0.1 |
| TALP UR | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| TALP + DMF R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 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 |
| TALP + Gly. UR | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 ± 0.1 |

Original sample: extended semen (A) and raw semen (B). TALP Pre: semen diluted in TALP medium, before vitrification. TALP+DMF Pre: semen diluted in TALP medium + 4% DMF, before vitrification. TALP+Gly. Pre: 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 ultra-rapidly. 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 ultra-rapidly. 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 ultra-rapidly.

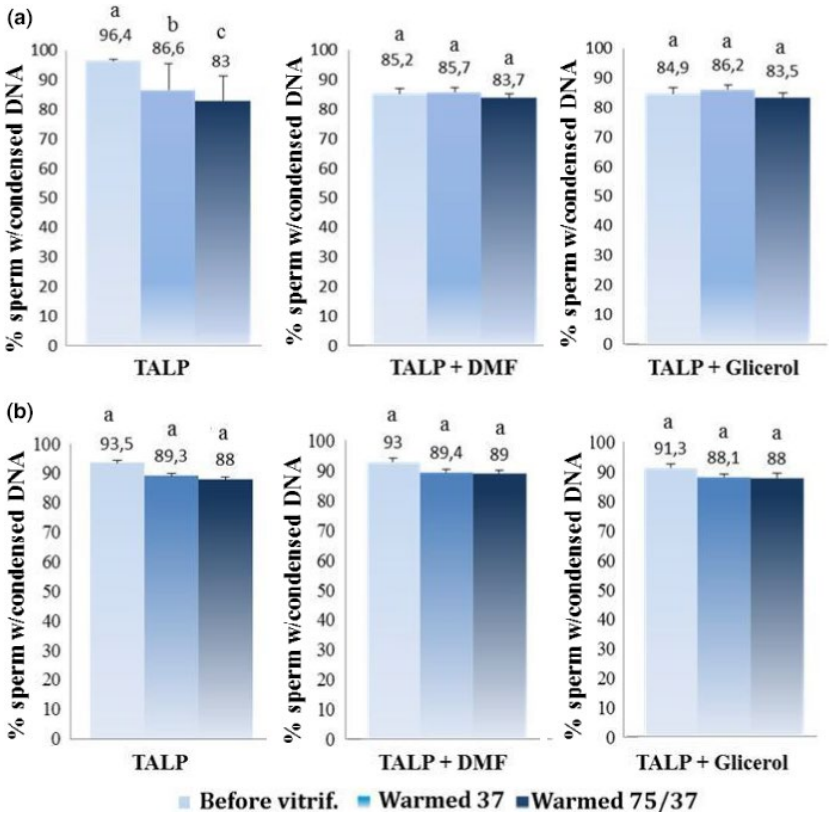


**FIGURE 3** Percentage of spermatozoa with condensed DNA in extended (a) and raw (b) vitrified/warmed porcine semen samples. [a,b,c] Different superscripts, between different warming methods and samples before vitrification, indicate significant differences ($p < .05$) within each cryopreservation media

Montag, et al. (2004), when evaluating human sperm chromatin using the Comet technique after vitrification with cryoloops. In these studies, no significant differences ($p > .05$) were observed between the semen samples prior to vitrification and the samples vitrified in the presence or absence of cryoprotectants.

The absence of motility and viability in vitrified/warmed porcine spermatozoa contrasts with the results reported by Nawroth et al. (2002), Isachenko, Isachenko, Katkov, Rahimi, et al. (2004), Isachenko, Isachenko, Katkov, Montag, et al. (2004)in human spermatozoa vitrified in cryoloops without cryoprotectants and warmed at 37°C. These

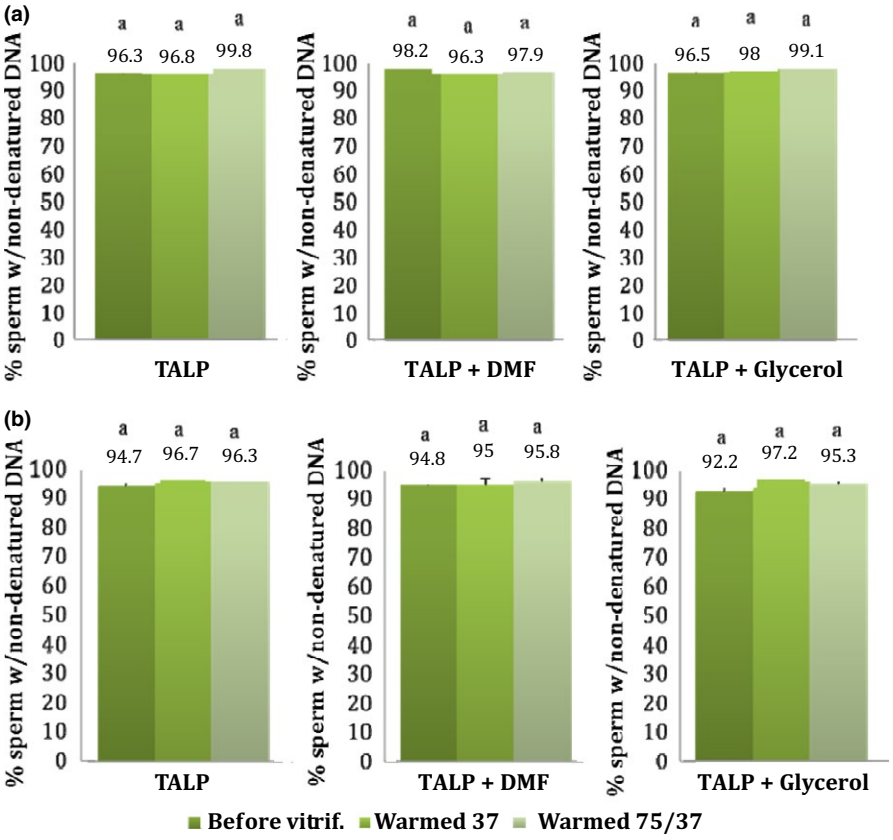


**FIGURE 4** Percentage of spermatozoa with nondenatured DNA in extended (a) and raw (b) vitrified/warmed samples ($p > .05$)

authors reported motility percentages of 57.5 ± 18.1 and 51.5 ± 4.5, respectively (mean ± standard deviation), and although this motility was significantly inferior to that of the samples prior to vitrification, it was not absent as in our study in porcine semen. Isachenko, Isachenko, Katkov, Montag, et al. (2004) corroborated viability in human vitrified spermatozoa using in vitro fertilisation (IVF) and obtaining embryo development to the early blastocyst stage.

The absence of sperm positive to the membrane function test (HOS + spermatozoa) was also confirmed in all vitrified/warmed samples, observing the presence of coiled or bent tails present in the morphological evaluation carried out after warming that were attributed to alterations suffered during the process of cryopreservation.

These very different results between human and porcine vitrified spermatozoa could be due to various factors. It is known that the particular phospholipid and fatty acid composition of sperm membranes in each species is responsible for the differential behaviour during freeze–thawing (Lessig et al., 2004). According to Paulos and White (1973), the lipid composition of porcine and human sperm membranes is similar, although porcine spermatozoa present less phospholipid. When comparing susceptibility to cold shock, human spermatozoa are quite resistant to rapid cooling to 0°C, while porcine spermatozoa are very sensitive to these changes. This situation suggests that the distribution of phospholipids in the membranes would be different between these species, especially in the areas most affected by temperature descent such as the acrosome and the plasma membrane. It would be interesting to confirm the possibility that, despite their similar composition, these species have differences in membrane lipid distribution. Another factor that influences sperm sensitivity to freeze–thawing is the percentage of cholesterol present, because its presence confers greater rigidity to membranes. Human spermatozoa, that are proven to be more resistant to cold shock, possess a cholesterol:phospholipid ratio of 0.9–1, whereas in porcine spermatozoa, the ratio is 0.2 (Parks, 1997). This difference could also explain the greater sensitivity that porcine spermatozoa show towards deep freezing and vitrification. Furthermore, Parks and Lynch (1992) evidenced an inverse relationship between sensitivity to cold shock and the quantity of proteins present in sperm membranes. These authors found that porcine sperm membranes have greater protein content than bovines and equines, while roosters (a species that is very resistant to temperature changes) show the lowest protein content of all. In addition, it has been reported that membrane proteins also suffer alterations during temperature changes, modifying their function and thus also in the cell (Watson, 2000).

According to Nawroth et al. (2002) and Katkov (2012), the shape and size of the sperm head could be factors that define cell cryo-sensitivity. Comparative studies carried out by Nauk (1991) in sperm cryo-properties in different mammalian species (among which were human and porcine) showed a negative correlation between the size of the head and cryo-sensitivity. According to Gao, Mazur, and Critser (1997), human spermatozoa have the smallest measurements and therefore the maximum cryo-stability, reinforcing this hypothesis. Although many characteristics define sperm head

morphometry, if we use the area to compare species, we find the following values: 31.35 ± 2.53 $\mu m^2$ for rabbits, 34.9 ± 2.65 $\mu m^2$ for rams, 15.4 ± 1.34 $\mu m^2$ for dogs (Yániz, Soler, & Santolaria, 2015) and 7.25 ± 0.59 $\mu m^2$ for trout (Tuset et al., 2008). If we look at the sperm head areas of the species that have been vitrified to date, it is not surprising to find that the best results obtained with vitrification of spermatozoa have been in dogs (Sánchez et al., 2011) and trout (Merino et al., 2011) in contrast to that obtained in rams (Jiménez-Rabadán et al., 2015), rabbits (Rosato & Iaffaldano, 2013) and porcine in our study. Both these species (dog and trout) possess smaller sperm head areas and therefore more similar to that of humans (18.35 ± 1.37 $\mu m^2$) than to that of porcine (34.85 ± 1.76 $\mu m^2$). This reaffirms the hypotheses proposed by Nauk (1991), Gao et al. (1997), Nawroth et al. (2002) and Katkov (2012) that the shape and size of the sperm head could be factors that define cell cryosensitivity showing a negative correlation between the size of the head and cryosensitivity.

When evaluating sperm chromatin, we observed that the susceptibility of porcine spermatozoa to acid denaturation (acridine orange stain) was unaltered in samples vitrified in cryoloops in all media and methods of warming used, while on the other hand, chromatin condensation (toluidine blue stain) could be affected during vitrification in this species. However, the only samples that presented chromatin decondensation were those from extended semen, vitrified in the absence of cryoprotectants (TALP media). This result could be indicating that it is advisable to vitrify porcine spermatozoa using raw semen samples rather than after cooling to 17°C, because DNA condensation is better preserved when using the fresh ejaculate. Previous exposure of pig semen to 16–18°C could be increasing sperm chromatin susceptibility to the changes suffered during the cryopreservation process, and this could then be evidenced in the altered DNA condensation after vitrification. Boe-Hansen, Ersboll, Greve, and Christensen (2005) evaluated sperm chromatin integrity in diluted porcine semen samples preserved at 18°C during 0, 24, 48 and 72 hr, observing a significant increase in the fragmentation index in the samples preserved for 72 hr (total average: 7.9%; range: 0.3–83.9). These authors suggest that this damage to the DNA could be owing to two factors: 1) an alteration of the degree of sperm chromatin compaction or condensation, inherent to each individual, which makes it vulnerable to external aggressions and 2) the presence of old spermatozoa, or those damaged during the process of cryopreservation, could generate reactive oxygen species that further damage sperm membranes and oxidise thiol groups, thus altering chromatin condensation, although this latter hypothesis is not supported by the findings of Guthrie and Welch (2006). With regard to the warming of vitrified samples, Gao and Critser (2000) and Johnson et al. (2000) suggested that sperm survival could be decreased if it is carried out incorrectly. Our study showed similar results with both warming methods, with the rapid method (30 s at 37°C) being better for preserving DNA quality than the ultra-rapid method (7 s at 75°C followed by 30 s at 37°C) only in extended samples vitrified without cryoprotectants. Nevertheless, as the rapid method is practical, simple and easily repeatable, this makes it the reasonable choice for routine use with this biotechnology. Isachenko, Isachenko, Katkov, Montag, et al. (2004) proposed that, due to the slimness of the sample contained in the cryoloops, an instantaneous warming is obtained by direct immersion in the same media at 37°C. Our results would seem to confirm this.

Due to the fact that in this study, the only porcine seminal parameter that was maintained after vitrification was the chromatin, these samples would be suitable for use in reproductive biotechnologies such as intracytoplasmic sperm injection (ICSI), where it is indispensable to have intact DNA.

## 5 | CONCLUSIONS

Vitrification of porcine spermatozoa using cryoloops, both in the presence or absence of cryoprotectants and independent of the warming procedure used, is a technique that permits conservation of sperm chromatin condensation and integrity. Although this method is simple, rapid and inexpensive, it remains necessary to better adapt this protocol to the porcine species to obtain live motile spermatozoa.

## ACKNOWLEDGMENTS

This research was supported by a grant from the University of Buenos Aires UBACyT 20020110100160. The authors wish to thank people from the Biophysics Area of the Faculty of Veterinary Sciences, University of Buenos Aires, EDUVIPA S.A. and Los Talas farm, for providing the semen samples.

## CONFLICT OF INTEREST

None.

## REFERENCES

Ball, B., & Vo, A. (2001). Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability and mitochondrial membrane potential. *Journal of Andrology*, *22*, 1061–1069.

Bianchi, I., Calderam, K., Maschio, E., Madeira, E., Ulguim, R., Corcini, C., … Correa, M. (2008). Evaluation of amides and centrifugation temperature in boar semen cryopreservation. *Theriogenology*, *69*, 632–638.

Boe-Hansen, G., Ersboll, A., Greve, T., & Christensen, P. (2005). Increasing storage time of extended boar semen reduces sperm DNA integrity. *Theriogenology*, *63*, 2006–2019.

Cristanelli, M., Squires, E., Amann, R., & Pickett, B. (1984). Fertility of stallion semen processed, frozen and thawed by a new procedure. *Theriogenology*, *22*, 39–45.

Day, B. (2000). Reproductive biotechnologies: Current status in porcine reproduction. *Animal Reproduction Science*, *60–61*, 161–172.

Gao, D., & Critser, J. (2000). Mechanisms of cryoinjury in living cells. *ILAR Journal*, *41*, 187–196.

Gao, D., Mazur, P., & Critser, J. K. (1997). Fundamental cryobiology of mammalian spermatozoa. In A. M. Karow, & J. K. Critser (Eds.), *Reproductive tissue banking: Scientific principles* (pp. 263–328). London: Academic Press.

García Rosello, E., García Mengual, E., Coy, P., Alfonso, J., & Silvestre, M. (2008). Intracytoplasmic sperm injection in livestock species: An update. *Reproduction in Domestic Animals*, *44*, 143–151.

García Vázquez, F., García- Roselló, E., Gutiérrez-Adán, A., & Gadea, J. (2009). Effect of sperm treatment on efficiency of EGFP-expressing porcine embryos produced by ICSI-SMGT. *Theriogenology*, *72*, 506–518.

González, L., Campi, S., Cisale, H., Torres, P., García, C., & Ferrari, M. (2008). Descondensación *in vitro* de núcleos espermáticos porcinos. *InVet*, *10*, 174.

Grossfeld, R., Sieg, B., Struckmann, C., Frenzel, A., Maxwell, W. M. C., & Rath, D. (2008). New aspects of boar semen freezing strategies. *Theriogenology*, *70*, 1225–1233.

Guthrie, H. D., & Welch, G. R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *Journal of Animal Science*, *84*, 2089–2100.

Harrison, R. A., & Vickers, S. E. (1990). Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of Reproduction and Fertility*, *88*, 343–352.

Ikeda, H., Kikuchi, K., Noguchi, J., Takeda, H., Shimada, A., Mizokami, T., & Kaneko, H. (2002). Effect of preincubation of cryopreserved porcine epididymal sperm. *Theriogenology*, *57*, 1309–1318.

Isachenko, E., Isachenko, V., Katkov, I., Dessole, S., & Nawroth, F. (2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: From past practical difficulties to present success. *Reproductive Biomedicine Online*, *6*, 191–200.

Isachenko, V., Isachenko, E., Katkov, I., Montag, M., Dessole, S., Nawroth, F., & Van der Ven, H. (2004). Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: Effect on motility, DNA integrity, and fertilization ability. *Biology of Reproduction*, *71*, 1167–1173.

Isachenko, E., Isachenko, V., Katkov, I., Rahimi, G., Schöndorf, T., Mallmann, P., … Nawroth, F. (2004). DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Human Reproduction*, *19*, 932–939.

Jiménez-Rabadán, P., García-Álvarez, O., Vidal, A., Maroto-Morales, A., Iniesta-Cuerda, M., Ramón, M., … Soler, A. (2015). Effects of vitrification on ram spermatozoa using free-egg yolk extenders. *Cryobiology*, *71*, 85–90.

Johnson, L., Weitze, K., Fiser, P., & Maxwell, W. (2000). Storage of boar semen. *Animal Reproduction Science*, *62*, 143–172.

Katkov, I. (2012). Kinetic vitrification of spermatozoa of vertebrates: what can we learn from nature? In I. Katkov (Ed.), *Current frontiers in cryobiology* (pp. 3–40). Rijeka, Croatia: InTech.

Katkov, I., Isachenko, V., Isachenko, E., Kim, M., Lulatd, A., Mackaye, A., & Levine, F. (2006). Low and high-temperature vitrification as a new approach to biostabilization of reproductive and progenitor cells. *International Journal of Refrigeration*, *29*, 346–357.

King, G., & Macpherson, J. (1973). A comparison of two methods for boar semen collection. *Journal of Animal Science*, *36*, 563–565.

Lessig, J., Gey, C., Suss, R., Schiller, J., Glander, H., & Arnhold, J. (2004). Analysis of the lipid composition of human and boar spermatozoa by MALDI-TOF mass spectrometry, thin layer chromatography and 31P NMR spectroscopy. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, *137*, 265–277.

Liebermann, J., Nawroth, F., Isachenko, V., Isachenko, E., Rahimi, G., & Tucker, M. J. (2002). Potential importance of vitrification in reproductive medicine. *Biology of Reproduction*, *67*, 1671–1680.

Luyet, B., & Hodapp, R. (1938). Revival of frog spermatozoa vitrified in liquid air. *Experimental Biology and Medicine*, *39*, 433–434.

Malo, C., Gil, L., Cano, R., Martínez, F., García, A., & Jerez, R. (2012). Dimethylformamide is not better than glycerol for cryopreservation of boar semen. *Andrologia*, *44*, 605–610.

Medeiros, A., Gomes, G., Carmo, M., Papa, F., & Alvarenga, M. (2002). Cryopreservation of stallion sperm using different amides. *Theriogenology*, *58*, 273–276.

Merino, O., Risopatrón, J., Sánchez, R., Isachenko, E., Figueroa, E., Valdebenito, I., & Isachenko, V. (2011). Fish (*Oncorhynchus mykiss*) spermatozoa cryoprotectant – free vitrification: Stability of mitochondrion as criterion of effectiveness. *Animal Reproduction Science*, *124*, 125–131.

Morris, S., & Shertzer, H. (1985). Rapid analysis of DNA strand breaks in soft tissues. *Environmental and Molecular Mutagenesis*, *13*, 151–163.

Nauk, V. (1991). *Structure and function of spermatozoa from farm animals by cryopreservation*. Shtiinca Kishineu 1991; 1–198.

Nawroth, F., Isachenko, V., Dessole, S., Rahimi, G., Farina, M., Vargiu, N., … Isachenko, E. (2002). Vitrification of human spermatozoa without cryoprotectants. *Cryo Letters*, *23*, 93–102.

Parks, J. (1997). Hypothermia and mammalian gametes. In A. Karow, & J. K. Critser (Eds.), *Reproductive tissue banking: Scientific principles* (pp. 229–261). San Diego, CA: Academic Press.

Parks, J., & Lynch, D. (1992). Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membranes. *Cryobiology*, *29*, 255–266.

Paulos, A., & White, I. (1973). The phospholipid composition of human spermatozoa and seminal plasma. *Journal of Reproduction and Fertility*, *35*, 265–272.

Pursel, V., & Johnson, L. (1974). Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation. *Theriogenology*, *1*, 63–68.

Rail, W. F., & Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature*, *313*, 573–575.

Roca, J., Parrilla, I., Bolarin, A., Martinez, E. A., & Rodriguez-Martinez, H. (2016). Will AI in pigs become more efficient? *Theriogenology*, *86*, 187–193.

Rodriguez, H., & Wallgren, M. (2011). Advances in boar semen cryopreservation. *Veterinary Medicine International*, 2011, 1–5.

Rosato, M., & Iaffaldano, N. (2013). Cryopreservation of rabbit semen: Comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor. *Theriogenology*, *79*, 508–516.

Sánchez, R., Risopatrón, J., Schulz, M., Villegas, J., Isachenko, V., Kreinberg, R., & Isachenko, E. (2011). Canine sperm vitrification with sucrose: Effect on sperm function. *Andrologia*, *43*, 233–241.

Tejada, R., Mitchell, J., Norman, A., Marik, J., & Friedman, S. (1984). A test for the practical evaluation of male fertility by acridine orange fluorescence. *Fertility and Sterility*, *42*, 87–91.

Tuset, V. M., Dietrich, G. J., Wojtczak, M., Słowinska, M., de Monserrat, J., & Ciereszko, A. (2008). Comparison of three staining techniques for the morphometric study of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. *Theriogenology*, *69*, 1033–1038.

Vázquez, J., Martinez, E., Martinez, P., García-Artiga, C., & Roca, J. (1997). Hypoosmotic swelling of boar spermatozoa compared to other methods for analyzing the sperm membrane. *Theriogenology*, *47*, 913–922.

Watson, P. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction, Fertility, and Development*, *7*, 871–891.

Watson, P. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, *60*, 481–492.

Yániz, J. L., Soler, C., & Santolaria, P. (2015). Computer assisted sperm morphometry in mammals: A review. *Animal Reproduction Science*, *156*, 1–12.

Yeste, M. (2015). Recent advances in boar sperm cryopreservation: State of the art and current perspectives. *Reproduction in Domestic Animals*, *50*, 71–79.