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Competence of vitrified porcine spermatozoa to produce zygotes and early embryo development*

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Porcine sperm cryopreservation allows genetic conservation but due to the difficulties for conventional freezing presented by this species, vitrification has been evaluated as an alternative conservation method, obtaining sperm with intact, condensed chromatin. The objective of this study was to evaluate if vitrified-warmed porcine sperm could maintain the capacity to generate zygotes and early embryo development *in vitro***. To this end, raw semen was vitrified using the spheres method, at a concentration of 5×10^6 sperm/mL in TALP medium supplemented with 1% bovine serum albumin (BSA), without cryoprotectants (treatment group) and using conventional frozen-thawed semen as a control. Using ICSI, both vitrified-warmed and frozen-thawed semen were injected into** *in vitro* **matured porcine oocytes, and subsequent** *in vitro* **embryo development was**

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monitored. No significant differences were observed between the treatment and control groups, both for the percentage of pronuclei (PN) obtained (p = 0.0682) and for embryo development attained (p=0.1669). Spheres vitrification in the absence of cryoprotectants is a simple, low-cost technique that allows porcine sperm preservation, conserving the capacity to produce zygotes and transferable embryos using ICSI.

KEY WORDS: embryo / ICSI / porcine / semen / vitrification

Interest for the porcine species has grown, both in the productive field by applying technology to improve genetics [Day 2000, Grossfeld *et al*. 2008, Roca *et al*. 2016], and in the biomedical and basic research fields, due to the biological similitude between this species and humans. Given this wide range of possibilities, it is necessary to have a method of sperm cryopreservation that allows genetic conservation of the species [Ikeda *et al*. 2002], easy transportation of the samples and insuring sanitary conditions as well. On the other hand, it is necessary to have tools that improve their production, increasing the possibility of obtaining viable embryos through the application of reproductive biotechnologies such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and embryo transfer.

It has been possible to preserve sperm chromatin condensation and integrity by vitrifying raw porcine sperm using the spheres method at a concentration of 5×10^6 sperm/mL in TALP medium supplemented with 1% bovine serum albumin (BSA) without the presence of cryoprotectants [Arraztoa *et al*. 2017a]. By conserving sperm genetic resources, vitrification is presented as an alternative method of cryopreservation for porcine spermatozoa, a species that is particularly sensitive to the changes suffered during conventional freeze-thawing.

Regarding applied reproductive biotechnology, ICSI can be used for different purposes, such as the production of transgenics or to resolve fertilization problems that occur in swine IVF systems [García Roselló *et al.,* 2008]. Production of porcine embryos using IVF is limited due to the high incidence of polyspermy [Coy *et al*. 2005], which is why ICSI becomes a very useful alternative technique to produce monospermic zygotes *in vitro* in the swine species [García Roselló *et al*. 2008]. Moreover, as long as the sperm nucleus retains its genetic integrity undamaged, ICSI allows viable offspring to be obtained, regardless of sperm concentration, morphology or motility [Yanagimachi 2005] as only one genetically undamaged sperm would be needed to fertilize an oocyte [García Roselló *et al*. 2008]. This is indicated by studies from Kim *et al.* [1998] and Nakai *et al.* [2003] in porcine, where isolated sperm heads were injected, obtaining initial embryo development and up to the blastocyst stage, respectively. Studies in our lab obtained early embryo development up to pronuclei (PN), after injecting *in vitro* matured slaughter-house oocytes with vitrified porcine sperm [Arraztoa *et al*. 2017b].

Many of the studies carried out in porcine ICSI use frozen-thawed semen [Katayama *et al.* 2002, Lee *et al*. 2003, Yong et al. 2003 and 2005, García Roselló *et al.* 2006, Tian *et al.* 2006, Suzuki *et al.* 2010], so it would be interesting to use this type of cryopreserved sperm as a control for studies using vitrified samples, to see if similar results can be obtained.

Therefore, the objective of this study was to evaluate whether porcine sperm cryopreserved using vitrification maintain their ability to generate zygotes and early embryo development *in vitro* and if it is similar to that obtained with frozen-thawed semen. Testing these hypothesis would validate another method of semen cryopreservation, in addition to conventional freeze-thawing, to be used in ICSI, with the advantages that vitrification offers: greater simplicity and less time and cost involved than conventional cryopreservation, while at the same time being an efficient method for preserving male genetic material.

Material and methods

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 No. 2011/18).

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

Semen processing

An ejaculate from each of three different males (n=3), between 3 and 4 years old, with proven fertility, was used. Animals belonged to a private establishment located in the province of Buenos Aires, Argentina. Raw semen was collected using the gloved-hand method [King and Macpherson 1973] and the sperm-rich fraction was split in two: one aliquot was vitrified and the rest was frozen.

Vitrification raw semen samples were centrifuged at 400 *g* for 5 min to obtain a pellet which was then diluted in TALP medium supplemented with 1% BSA (final concentration: 5 x 10^6 sperm/ml). Vitrification was carried out using the spheres method according to Arraztoa *et al*. [2017a]. Briefly, small aliquots of 20-30 µl of diluted semen were dropped directly into a container with liquid nitrogen. The microdroplets formed spheres upon contact with the liquid nitrogen and sank spontaneously after 4 sec. The solid spheres were kept in cryovials at -196°C for a minimum of 24 h before use. For warming, the spheres were submerged one by one (not more than 5 spheres at a time) during 30 sec in a 15 ml tube containing 5 ml of TALP with 1% BSA at 37°C, accompanied by gentle stirring for 5-10 sec after each addition of spheres. After warming, samples were centrifuged at 300 g for 5 min and the pellet was resuspended in TALP medium with 1% BSA for use in ICSI (treatment group).

Freeze-thawing was carried out according to Caldevilla *et al*. [2016]. Briefly, raw semen was diluted in the commercial medium Androstar® plus (Minitub, Germany) and was stabilized at 17ºC for 2 h, then centrifuged at 800 g for 15 min and the supernatant was removed. The pellet was re-diluted to 300 x 10^6 sperm/ml with the following diluent: 3% glycerol, 11% lactose, 20% egg yolk and 0.5% Equex®. The diluted semen was then equilibrated 2 h, in a refrigerator at 5 ºC, packaged in 0.5 cc straws and then placed in an isolated Styrofoam box, 5 cm above the liquid nitrogen

for 20 min, to be finally submerged in liquid nitrogen until use. Straws were thawed at 37ºC for 1 min and diluted in 1 ml of Androstar® plus (Minitub, Germany).

For ICSI, because the samples had egg yolk, they were filtered through glass wool according to Sterzik *et al*. [1998] modified. Briefly, 20 mg of glass wool were placed in a 1 ml syringe, delicately compressing the plunger to the 0.2 cc mark. It was then washed with 5 ml of PBS warmed to 37ºC, to remove any loose glass wool fibres, and finally 1 ml of the sample was filtered. The first two droplets were discarded and the following ones were collected in an Eppendorf tube containing 250 µl of TALP with 1% BSA (control group).

Semen evaluation

Before and after sperm conservation, the following semen parameters were evaluated:

- 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.
- 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; KCl 2.5 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 6-carboxifluorescein diacetate (0.5 mg/ml CFDA in dimethylsulphoxide). Then, 20 µl of a stock solution of propidium iodide (0.5 mg/ml PI 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) (Fig. 1, A1-A2). A total of 200 spermatozoa were evaluated in each sample.
- Membrane function evaluation, using the hypoosmotic swelling test (HOS test), was carried out according to Vázquez *et al*. [1997] modified. Briefly, 25 µl of semen sample was incubated at 37°C during 10 min in 1 ml of 100 mOsm hypoosmotic 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×) (Fig. 1B). A total of 200 spermatozoa were evaluated per sample.
- 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 (Fig. 1 – C1-C2-C3-C4).
- Chromatin condensation evaluation, using the Toluidine blue stain (TB), was

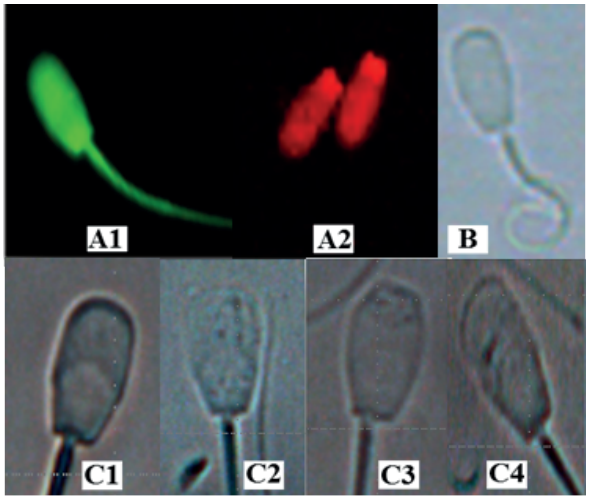

Fig. 1. Semen evaluation techniques. A - CFDA/PI viability stain, A1 viable spermatozoa (PI negative), A2 non-viable spermatozoa (PI positive). B - functional membranes with HOS test, HOS-positive spermatozoa (tails with swelling). C - acrosome integrity, C1 intact acrosome (dark, smooth, crescentic apical ridge), C2 acrosome absent and C3-C4 altered acrosome (irregular apical ridge).

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, Fig. 2 – A1), while decondensed chromatin stains an intense blue-violet (altered DNA, Fig. 2 – A3). Spermatozoa with a dark blue staining were classified as having intermediate chromatin decondensation and were considered altered (Fig. 2 – A2). 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).

– The chromatin susceptibility to acid denaturalization using the acridine orange stain (acridine orange test, AOT), was carried out according to Tejada *et al.*

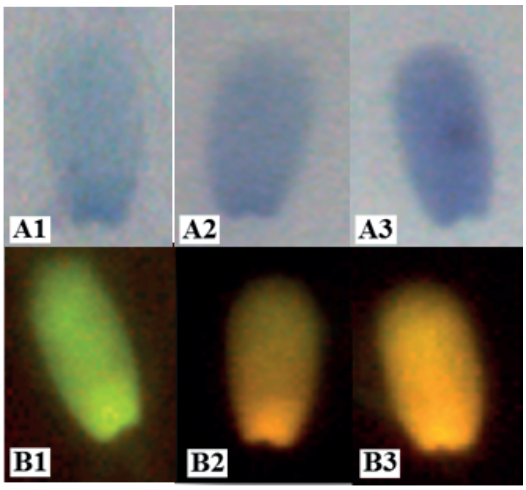

Fig. 2. DNA evaluation techniques. A - chromatin condensation with Toluidine Blue stain, A1 condensed chromatin (light blue), A2 intermediate chromatin decondensation (dark blue) and A3 decondensed chromatin (intense blue-violet). B - chromatin susceptibility to acid denaturalization with Acridine Orange stain, B1 non-susceptible to denaturation (green fluorescence), B2 slightly susceptible to denaturation (light orange in the post-acrosome region), B3 highly susceptible to denaturation (orange fluorescence).

[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 (non-susceptible to denaturation, Fig. 2 – B1), spermatozoa with light orange colouring in the post-acrosome region (slightly susceptible to denaturation, Fig. 2 – B2), spermatozoa with orange fluorescence (highly susceptible to denaturation, Fig. 2 – B3). 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 and 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.

In vitro **oocyte maturation**

Cumulus-oocyte complexes (COCs) were obtained by aspiration of antral follicles (3-8 mm) from slaughterhouse ovaries. Maturation was carried out in TCM-199 medium supplemented with 57 mM cysteine, 50 mg/L gentamicin sulfate, 0.5 mg/l porcine follicular stimulating hormone (pFSH; Folltropin-V®; Bioniche, Belleville, ON, Canada), 0.5 mg/L porcine luteinizing hormone (pLH; Lutropin-V®; Bioniche) and 10% follicular fluid [Abeydeera 2001], for 48 h under mineral oil, at 39°C, with 5% CO_2 and 100% humidity. Oocytes were denuded with 0.1% hyaluronidase, evaluated under a stereomicroscope and those having a visible polar body and a homogenous cytoplasm, were selected for ICSI.

ICSI and embryo development

Micromanipulation was carried out using an inverted Leica® DMIL microscope equipped with Narishige® micromanipulators. Briefly, one spermatozoon was immobilized by crushing the midpiece with the tip of the injection pipette and then was aspirated tail first into the pipette. The mature oocyte was held with the holding pipette, placing the polar body either in the 12 or 6 o'clock position, to avoid damaging the metaphasic plate with the injection pipette. This pipette was pushed through the zona pellucida and subsequently through the oolemma into the cytoplasm at the 3 o'clock position. A small amount of ooplasm was aspirated into the injection pipette to verify oocyte membrane penetration and subsequently, the immobilized spermatozoon was released into the oocyte. Randomly, one group of mature oocytes were injected with vitrified-warmed sperm (treatment group) and the other group was injected with frozen-thawed sperm (control group). The microinjection was carried out in HEPES-TALP droplets supplemented with 0.3% BSA, while both semen suspensions were placed in drops of 10% polyvinylpyrrolidone (PVP). Temperature was maintained at 38°C throughout the process. As a control for the ICSI technique and to show if any parthenogenetic activation was present, a group of mature oocytes were injected without depositing any sperm in the cytoplasm (Sham group). All injected oocytes were cultured at 38°C in a defined SOF medium supplemented with essential and non-essential aminoacids and BSA [Lagutina *et al*. 2006], using tri-gas atmosphere (5% CO_2, 5% O_2 and 90% N_2) and 100% humidity.

To evaluate PN formation, ICSI was carried out on a total of 118 *in vitro* matured porcine oocytes: 41 were injected with vitrified-warmed porcine semen (treatment), 35 were injected with frozen-thawed porcine semen (control) and 42 oocytes were subjected to the Sham test. Oocytes were then evaluated 18 h after ICSI to determine PN formation. Presumptive zygotes were fixed for 15 min (2% glutaraldehyde in PBS), stained with 1% Hoechst 33342 in PBS for 15 min, washed in PBS that contained 1 mg/ml polyvinylpyrrolidone and mounted on glass slides. Oocytes were examined under epifluorescence microscopy using 330 to 380 nm (excitation) and 420 nm (emission) filters [Coy *et al*. 2005].

A total of 150 *in vitro*-matured porcine oocytes were injected to assess early embryo *in vitro* development: 54 with vitrified-warmed porcine semen, 52 with frozen-thawed semen and 44 were subjected to the Sham test. Injected oocytes were monitored on days 2 and 7 of *in vitro* culture, using an inverted stereomicroscope, to evaluate the development achieved *in vitro*. On day 7 embryos were stained and fixed, as described above for PN evaluation, to determine the number of blastomeres present.

Statistical analysis

The percentage of zygotes and of *in vitro* embryo development was analyzed using a non-parametric Chi square test

Normal distribution and variance homogeneity of the seminal parameters were examined using the Shapiro-Wilk and the Levene tests, respectively. Sperm motility data did not present a normal distribution so were analyzed with the Kruskal Wallis test. The rest of the seminal variables were compared using one-way analysis of variance (ANOVA) considering one factor and three levels: pre cryopreservation, post vitrification and post thawing.

In both cases, statistical analysis was carried out using the Infostat program. For detecting differences in the percentages of zygotes and of embryo development, significance level was set at 0.05% ($p<0.05$) and a statistical trend was set at 0.1% ($p<0.1$). The level of significance was set at 0.01% ($p<0.01$) for the analysis of the seminal data.

Results and discussion

Seminal parameters of motility, viability, membrane function and acrosome integrity decreased significantly in both vitrified-warmed and frozen-thawed semen samples compared to raw semen prior to cryopreservation. Regarding DNA quality, both chromatin condensation and integrity remained constant in samples before and after vitrification and freezing (Tab. 1). The seminal parameters evaluated in the vitrified samples showed similar results to those reported in previous studies [Arraztoa *et al*. 2017a], where chromatin condensation and integrity were the only seminal characteristics to be maintained with no significant differences compared to the same samples before vitrification. Regarding frozen-thawed semen, literature reports considerable differences in the survival of sperm after thawing. Although these differences could be attributed to the different freezing methods employed, the main determinant seems to reside in the ejaculate itself. In fact, each ejaculate exhibits a unique cryotolerance, with clear differences between individuals and even between ejaculates from the same individual [Yeste *et al.* 2017]. However, post-thaw seminal parameters were similar to those reported by Caldevilla *et al*. [2020] and Flores *et al.* [2009] for total motility; to Flores *et al*. [2009] for viability and acrosome integrity and to Caldevilla *et al*. [2020] and Malo *et al*. [2012] for membrane function. With regard

Table 1. Seminal parameters evaluated in the samples used for ICSI (means with their standard deviations)

Item	Pre cryopreservation		Post vitrification		Post freezing		p value
	mean	SD	mean	SD	mean	SD	
Total motility (%)	88.3[a]	2.9	0[b]		20.0[c]	0	0.0036
Viability (%)	66.7[a]	1.5	0[b]		40.5[c]	2.5	<0.0001
Membrane function (%)	65.0[a]	9.1	2.3[b]	1.2	25.0[c]	3	0.0005
Acrosome integrity (%)	99.0[a]	0.6	0[b]		24.7[c]	7.0	<0.0001
DNA condensation (%)	96.7[a]	0.6	93.7[a]	2.0	91.0[a]	1.0	0.0165
DNA integrity (%)	98.3[a]	0.6	97.3[a]	2.5	97.7[a]	1.2	0.8150
DNA susceptibility to denaturalization (%)	1.7[a]	0.6	2.7[a]	2.5	2.3[a]	1.2	0.8150

Pre cryopreservation – seminal parameters in semen samples diluted in the cryopreservation media used, prior to being vitrified or frozen.
Post vitrification – seminal parameters in vitrified-warmed semen samples.
Post freezing – seminal parameters in frozen-thawed samples.
[abc]Values with different superscripts within a row indicate differences ($p<0.01$) among experimental treatment groups (pre vs post vitrification and pre vs post freezing) (n=3).

to DNA analysis, so far there are no reports of the use of Toluidine blue for evaluating frozen-thawed porcine spermatozoa. Regarding the Acridine Orange test, this study showed similar results to those reported by Boe-Hansen *et al*. [2005], Chanapiwat *et al.* [2010] and Buranaamnuay *et al*. [2011]. What is interesting to note is that for both methods of cryopreservation, the only seminal parameter that was preserved when compared to the sample prior to cryopreservation, was DNA quality.

Regarding the experiment to evaluate the *in vitro* production of PN, of the 41 oocytes injected with vitrified-warmed semen, 16 PN (39%) were obtained and of the 35 oocytes injected with frozen-thawed semen, 21 PN (60%) were obtained. Although the percentage of PN obtained with vitrified-warmed sperm was lower than that reported by our group in previous studies in this species (39 vs. 42.9% - Arraztoa *et al.* [2017b]), this difference was not significant ($p=0.6794$). The percentage of PN for frozen-thawed semen (60%) was equal to that expected according to García Roselló *et al.* [2008]. However, no significant differences ($p=0.0682$) were observed in the percentage of pronuclei obtained when injecting *in vitro* matured porcine oocytes with either vitrified-warmed or frozen-thawed semen. Concerning the 42 oocytes subjected to the Sham test, 5 PN (12%) were obtained and this percentage of PN was significantly lower to both the treatment ($p=0.0045$) and control ($p<0.0001$) groups.

This study represents the first report demonstrating the ability of porcine vitrified-warmed sperm to generate zygotes and to develop embryos *in vitro* using ICSI and *in vitro*-matured porcine oocytes. Of the 54 *in vitro* matured porcine oocytes injected with vitrified-warmed porcine semen, forty 2-cell embryos were obtained (74%) and of the 52 oocytes injected with frozen-thawed semen, thirty-two 2-cell embryos were obtained (62%). Seventeen parthenogenetic embryos were obtained from the 44 oocytes subjected to the Sham test (39%) – Table 2 and Figure 3. No significant

differences were observed in the percentages of in vitro embryo development obtained with the oocytes injected with vitrified-warmed semen and those injected with frozen-thawed semen. Although embryo development was observed in the Sham group, this was significantly lower to both the treatment and control groups for the 2-cell and 4-cell stages (p=0.017 and p=0.0483 respectively). The different embryo development obtained in vitro is detailed in Table 2.

Table 2. Embryo development at each stage during 7 days of *in vitro* culture using ICSI with vitrified-warmed, frozen-thawed porcine semen and *in vitro*-matured porcine oocytes

Treatment	Total injected oocytes	Unfertilized oocytes (%)	Embryos developed to			
			2 cell (%)	4 cell (%)	6 cell (%)	8 cell (%)
Vitrified semen	54	14[a] (26)	40[a] (74)	22[a] (41)	7[a] (13)	2[a] (4)
Frozen semen	52	20[a] (38)	32[a] (62)	22[a] (42)	5[a] (10)	1[a] (2)
Sham	44	27[b] (61)	17[b] (39)	9[b] (20)	3[a] (7)	0[a] (0)
p value	-	0.0017	0.0017	0.0483	0.5974	0.8307

[ab]Values with different superscripts within a column differ significantly at p<0.05 (replicates=6).

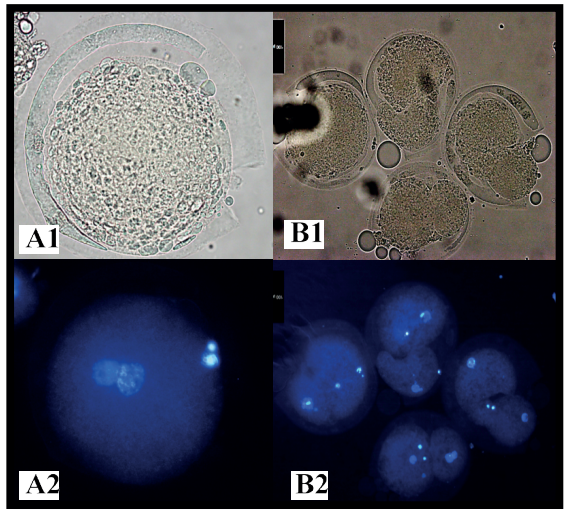

Fig. 3. A - pronuclei stage zygotes. B: embryos with two blastomeres. Row 1 - observation using bright field microscopy. Row 2 - observation using epifluorescence microscopy.

The results obtained in this study indicate that porcine vitrified-warmed semen presents the same competence as frozen-thawed semen to produce embryos *in vitro*, as no significant differences were observed either in the percentages of PN obtained or in the degree of embryo development attained. Although embryos of different stages were obtained, blastocysts were not achieved in any of the groups, unlike what was reported by García Roselló *et al*. [2008] with frozen-thawed semen (0 vs. 10 to 36.7%). A lower cleavage rate was also observed compared to that reported by these authors (10 vs. 26 to 72.8%). The differences observed between the results in this

study and those reported for *in vitro* embryo development to transferable stages could be due to several factors. Among them, the following could perhaps be involved: a possible failure in either oocyte nuclear or cytoplasmatic *in vitro* maturation, different *in vitro* culture media or atmosphere used in the respective studies [Nagai *et al*. 2006, Dang-Nguyen *et al*. 2011] or the fact that in this study we did not chemically [Swain and Pool 2008] or electrically [Liu *et al.* 2015] activate oocytes after ICSI.

Embryo development was arrested as from the 4-cell stage, both in the control and treatment groups, and this block coincides with the moment in which the embryo genome is activated (EGA) – Stroband and Van der Lende [1990], which occurs in the third cell cycle of porcine embryos, 30 h after cleavage [Dorthe *et al*. 2002]. Moreover, *in vitro* conditions for porcine embryo development significantly alter the RNA profiles present before EGA and these changes could originate from the oocyte stage, when they were aspirated or during *in vitro* maturation. For this reason, the embryos produced *in vitro* have an altered content of apoptotic factors, cell cycle regulation factors, spindle components and transcription factors, all of which could contribute to reducing their development [Østrup *et al*. 2013].

Considering that in the porcine species embryos enter the uterus 2-3 days after ovulation, at the stage of 4 cells [Stroband and Van der Lende 1990], the embryos obtained in our study after ICSI of IVM oocytes with vitrified spermatozoa could be transferred to the uterus of recipient sows and thus further evaluate their capacity to develop *in vivo*. Although the non-surgical embryo transfer (ET) technique is currently being improved in sows, there are records of pregnancies and live births using both the surgical and non-surgical techniques for transferring 4-cell embryos and blastocysts [Hazeleger and Kemp 2001].

The degree of DNA damage in porcine semen samples is presumed to be an indicator of fertility because it shows a positive correlation with the size of the litter and with the farrowing rate [Broekhuijse *et al*. 2012]. In addition, Yanagimachi [2005] reported that ICSI allows one to obtain viable offspring if the sperm nucleus is conserved intact, independently of sperm concentration, morphology or motility. Thus, taking into consideration these reports, the results of this study indicate that both vitrified and frozen samples maintained the same competence to produce embryos *in vitro* using ICSI. It would be interesting to evaluate if the early embryo stages obtained *in vitro* maintain the ability to develop a full-term pregnancy in recipient sows after ET.

Conclusion

Porcine semen vitrified in spheres at a concentration of 5×10^6 sperm/ml in TALP medium supplemented with 1% BSA and warmed at 37°C for 30 seconds retains the capacity to produce zygotes and embryos of transferrable stages *in vitro* comparable to that of frozen-thawed semen, using ICSI with *in vitro*-matured porcine oocytes.

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