Vitrification of *In Vitro* Produced Porcine Blastocysts: Influence of Cryoprotectants Toxicity and Embryo Age

Lain Uriel Ohlweiler, Joana Claudia Mezzalira & Alceu Mezzalira

ABSTRACT

Background: Porcine embryos are sensible to all assisted reproduction manipulations, especially the ones that involve cryopreservation. Despite the high cryoprotectant concentrations routinely applied, vitrification is the most effective technique to date. These substances toxicity can also play a negative role in embryo viability. During *in vitro* porcine embryo production, the speed of development is often unevenly distributed. It is possible that their development speed, affects embryo tolerance to cryoprotectants. This study aimed to evaluate the toxicity of porcine embryos of days 5 or 6 of culture to cryoprotectant agents; as well as to assess embryo survival to vitrification.

Materials, Methods & Results: Parthenogenetic porcine blastocysts and expanded blastocysts of days 5 and 6 of culture were exposed to toxicity tests (experiments 1 and 2) and vitrification (experiment 3) using different protocols. In the first experiment, three different cryoprotectants were used (Dimethyl sulfoxide - DMSO, Ethylene glycol - EG, and Sucrose - SUC), combined in three different associations (G1: 15% EG + 15% DMSO with 0.5 M SUC; G2: 16% EG + 16% DMSO with 0.4 M SUC; G3: 18% EG + 18% DMSO with 0.5 M SUC). In the fresh Control, embryos of day 6 are more sensible than the ones of day 5, whom showed a lower hatching rate (39.7 vs. 60.8%). After the toxicity (Experiment 1) test, the G1 showed better expansion rates in day 6 (50.0 vs 31.0 and 3.6% for G2 and G3) and higher hatching of day 6 compared to G2 and G3 (23.2, vs. 8.6 and 0.0% for G2 and G3). The fresh non hatched embryos at day 8, derived at day 6, had a lower percentage of cells with cleaved caspase-3 (20.2%) compared with the G1 (30.5%), G2 (31.4%) and G3 (30.5%). The hatched embryos of day 5 from G2 had lower total cell number (TCN) compared with the day 6 hatched embryos, whereas in G1 the TCN was not affected. The second experiment compared EG combined to one of these three extracellular cryoprotectants: Polyvinylpyrrolidone/sucrose/trehalose (respectively groups: PVP, SUC, TRE). The group SUC has raised the best results for day 5 embryos, whereas for day 6 embryos SUC and TRE were both best. The third experiment tested four vitrification protocols, being P1: EG+DMSO+TRE/warming with SUC; P2: EG+DMSO+TRE/warming TRE; P3: EG+TRE/ warming SUC; P4: EG+TRE/warming TRE. The expansion of vitrified day 5 embryos was higher in the P1 (20.0%) in comparison with the other three groups (4.3, 4.3 and 4.4% for P2, P3 and P4, respectively), with no difference for their hatching rates, been it lower comparing to the Control. Day 6 embryos showed no difference in expansion and hatching for the vitrified groups, been them lower than the Control.

Discussion: Embryos obtained on day 6 are more sensible than the ones of day 5, fact observed when the embryos were exposed to cryoprotectant solution, as well by the behavior of the no treated Control embryos. The toxicity increases as it does the concentration of intracellular cryoprotectant, where over 16% of the intracellular cryoprotectors already affected the day 6 embryos development. For the day 5 embryos however, 15 or 16% of the intracellular cryoptrotectors, had similar behavior to the embryos. For the extracellular solutions, however, it is variable according the embryos development speed. Indeed, it is necessary to adjust the cryoprotectors to be used to cryopreserve porcine *in vitro* produced embryos obtained at days 5 and 6 of culture.

Keywords: porcine embryos, cryopreservation, parthenogenesis, cryoprotector, vitrification.

INTRODUCTION

Porcine embryos, especially the *in vitro* produced, are very sensible to the reproductive assisted manipulations. This impairs their viability, as it hinders the application of some reproductive tools, especially embryo cryopreservation, one of the only ways, up to date, for genetic storing.

Cryopreservation aids to preserve genetic material, as well as to disseminate it among different countries. Porcine embryos has already been successfully cryopreserved by vitrification [3,5,6,9,10,13]. The high cryoprotectant concentrations are responsible for providing cell protection, but its concentration and exposure time are critical points for survival. For instance, most protocols proposed for porcine embryo vitrification, when standardised using *in vivo* produced embryos, do not yield the same efficiency for the ones *in vitro*.

As it happens *in vivo*, during the *in vitro* development of porcine embryos, within the same batch, their growth can happen under different speeds. The embryos with best ability usually show faster development; therefore, embryo cryotolerance can be affected by their growing speed. We have hypothesized that fastest developing embryos would have better competence and higher cryotolerance.

This study aimed to assess the effect of different cryoprotectants combined in different vitrification protocols, in the toxicity and survival, for blastocysts of days 5 or 6 of culture.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich[1], unless otherwise noted.

Experimental design

Three experiments were conducted in order to access the cryoprotectants toxicity and embryos cryotolerance of days 5 and 6 parthenotes porcine embryos. In the first experiment, three different cryoprotectants were used (Dimethyl sulfoxide - DMSO, Ethylene glycol - EG, and Sucrose - SUC), combined in three different associations: G1: equilibration solution of 7.5% EG + 7.5% EG for 5-15 min, and vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC, warming was with 1 M SUC for 1 min followed by 0.5 M SUC for 3 min; G2: equilibration solution of 7.5% EG + 7.5% EG for 3 min, and vitrification solution with 16% EG + 16% DMSO with 0.4 M SUC for 1 min, warming was with 0.13 M SUC for 5 min; G3: equilibration solution of 10% EG + 10% EG for 3 min and vitrification solution of 18% EG + 18% DMSO with 0.5 M SUC for 1 min, warming was with 1 M SUC for 1 min followed by 0.5 M SUC for 3 min.

The second experiment compared EG combined to one of these three extracellular cryoprotectants: Polyvinylpyrrolidone/sucrose/trehalose (respectively groups: PVP, SUC, TRE). The equilibration solution was with 2 M EG for 5 min, and vitrification solution with 8 M EG associated with: 7% PVP-10 (group PVP), 0.4 M SUC (group SUC), or 0.6 M TRE (group TRE) in incubation for 1 min. Warming of all the groups were done with 0.13 M SUC for 5 min.

The third experiment evaluated four vitrification protocols for embryos of day 5: Prot 1- Equilibration solution with 7.5% EG + 7.5% DMSO incubated for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC incubated for 1 min, warmed with 0.13 M SUC for 5 min; Prot 2- Equilibration solution with 7.5% EG + 7.5% DMSO incubated for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC incubated for 1 min, warmed with 0.13 M TRE for 5 min; Prot 3- Equilibration solution with 2 M EG 5 min, vitrification solution with 8 M EG + 0.5 M SUC for 1 min, warmed with 0.13 M SUC for 5 min; Prot 4- Equilibration solution with 2 M EG 5 min, vitrification solution with 8 M EG + 0.5 M SUC for 1 min, warmed with 0.13 M TRE for 5 min; and four protocols for embryos of day 6: Prot 1- Equilibration solution with 7.5% EG + 7.5% DMSO for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.6 M TRE for 1 min, warmed with 0.13 M SAC for 5 min; Prot 2- Equilibration solution with 7.5% EG + 7.5% DMSO for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.6 M TRE for 1 min, warmed with 0.13 M TREA for 5 min; Prot 3- Equilibration solution with 2 M EG for 5 min, vitrification solution with 8 M EG + 0.6 M TRE for 1 min, warmed with 0.13 M SUC for 5 min; Prot 4- Equilibration solution with 2 M EG for 5 min, vitrification solution with 8 M EG + 0.6 M TRE for 1 min, warmed with 0.13 M TREA for 5 min.

For the three experiments, a group of fresh embryos was kept as Control.

Oocytes collection and in vitro *maturation (IVM)*

Gilt ovaries were transported from the abattoir to the laboratory where follicles measuring 3-6 Mm

in diameter were aspirated with the aid of a vacuum pump connected to an 18G needle.

After morphological classification (under stereomicroscope in centrifuged follicular fluid), the cumulus oophorous complexes (COCs) considered of good or excellent quality grade were selected (completely surrounded by a compact layer of cumulus cells, with none or very discrete cytoplasmic granulation), according to previously described [2].

Selected COCs were submitted to *in vitro* maturation (IVM) into 4-well dishes[2] containing 400 μL of maturation medium, in groups of 40-50 COCs per well. During the first 22 h of IVM, the medium consisted in: TCM-199 + 25% (v/v) of porcine follicular fluid (PFF) + 0.1 mg/mL cysteine + 10 ng/mL epidermal growth factor[3] + 0.91 mM of sodium pyruvate + 3.05 mM D-glucose + 0.5 mg/mL of luteinizing hormone (LH - Lutropin)[4] + 0.01 UI/mL follicle stimulating hormone (FSH - Folltropin)[4] + 1 mM of dibutiryl-cyclic-AMP (dbcAMP) + 100 IU/mL of penicilin G + 0.1 mg/mL of streptomycin sulphate. An additional 20-24 h IVM period had the medium depleted of LH, FSH and the dbcAMP. All incubations were performed at 38.5°C, in a 5% CO_2 atmosphere in air, and saturated humidity.

Parthenogenetic activation and in vitro *culture (IVC)*

The IVM oocytes were pipetted in manipulation medium (TCM-Hepes + 10% fetal calf serum - FCS) in order to remove their cumulus cells. Activation was through exposure to 15 μM of ionomycin (in TCM-Hepes) for 5 min, followed by a 4 h incubation in 2 mM of 6-dimethylaminopurine in PZM-3 medium [15]. Activated oocytes were transferred to the *in vitro* culture (IVC) steps, and the onset of IVC was considered as day 0.

The presumptive zygotes were cultured in medium PZM-3 under the same conditions as described for the IVM. On day 4, the medium was supplemented by adding 10% of FCS. Development to blastocyst was assessed on days 5, 6, 7 and 8 of IVC, according to the experiment.

Embryo toxicity to cryoprotectants

We tested different combinations of either intracellular (EG and DMSO), and extracellular cryoprotectants (SUC, TRE and PVP) dissolved in manipulation medium. Only blastocysts and expanded blastocysts obtained at days 5 and 6 of culture were used, being all of quality grade excellent (homogeneous cytoplasm without vacuoles or fragmentation) or good (homogeneous cytoplasm, with few vacuoles or fragmentation). The cryoprotectant concentrations and exposure times has been described previously in literature. This step of toxicity test was performed by exposing the embryos to equilibrium and vitrification solutions, followed by exposure to warming solutions, without plunging the embryos into the liquid nitrogen. Evaluations were performed up to day 8.

Vitrification of blastocysts

Only good or excellent blastocysts and expanded blastocysts of days 5 and 6 were vitrified, in groups of 3-5. Embryos were exposed to an equilibrium solution (with intra-cellular cryoprotectant), and then placed into the vitrification solution (with intra- and extracellular cryoprotectants). Embryos were loaded into an open glass pulled micropipette, by capillarity, and plunged into LN2, according to each treatment, as described in the experimental design.

For the warming steps, the micropipette tips with the embryos were quickly immersed into warming solution, being rinsed in manipulation medium for additional 10 min, and maintained in the IVC for further assessments.

Viability of blastocysts after toxicity tests and vitrification

Viability criteria were expansion (after 24 h) and hatching rates (assessed every 24 h until day 8). At day 8, all hatched and live non-hatched blastocysts were fixed in PBS + 4% formalin + 3% BSA, for assessment of the total cell number (TCN) and expression of apoptosis (by the expression of cleaved caspase-3 (CC3).

Expression of cleaved caspase-3 (CC3) and blastocysts total cell number (TCN)

The expression of CC3 indicates the programmed cell death. To assess that, embryos were fixed in 4% formalin + 3% BSA in PBS, for at least 20 min, and incubated in PBS + 3% BSA and 0.5% Triton X-100 (permeabilization solution) at 37°C, for 1h. After a 2x rinse in PBS + 0.2% tween-20 + 3% BSA (blocking solution), embryos were overnight exposed to the primary antibody anti-cleaved caspase-3 (1:400) in rabbit, and diluted in blocking solution. Embryos were then washed 3x (20 min each) in blocking solution, incubated (for 2 h) at 37°C, in

secondary antibody goat anti-rabbit Alexa Fluor 488 (1:400). Then, after one rinse (for 20 min) in blocking solution, and another (20 min) rinse in DAPI (1:1000) in blocking solution, embryos were mounted between slide and coverslip in a droplet of mowiol, to be observed under an inverted epi-fluorescent microscope (Olympus IX51)[5].

The incidence of apoptosis in each embryo was estimated by the difference between the cells expressing CC3 (stained by Alexa Fluor 488) and the total cell count (stained by DAPI).

Statistical analysis

Data were analyzed using the JMP (software version 5, 2002)[6]. The proportion of positive cells for CC3 and the percentages of blastocysts expansion and hatching were analysed by Chi-square. The TCN values were submitted to ANOVA, being normalized, when necessary, by the arc sin of the square root of the data to be compared by the Tukey test. To all the experiments, the significance level was 5%.

RESULTS

Experiment 1: *Toxicity test for EG, DMSO and SUC in blastocysts of days 5 or 6 of culture*

The Control of day 5 showed higher ($P \leq 0.05$) expansion (100.0%) and hatching (60.8%) rates, compared to the treated groups. The groups G1 and G2 showed similar expansion rates (respectively 50.0 and 52.8%) between them, both higher than G3 (35.6%). The hatching rates for treated day 5 embryos were similar amongst all treated embryos, respectively 15.3, 9.7 and 5.5% for G1, G2 and G3 (Table 1).

For day 6 embryos, the expansion rate for the Control (84.5%) was higher than all treated groups, as follows: G1 (50.0%) higher than G2 (31.0%) and G3 (3.6%), and G2 higher than G3. The hatching rate for the Control (39.7%) was similar to G1 (23.2%), being them both higher than G2 (8.6%). The group G3 has the lowest hatching rate ($P \leq 0.05$) [0.0%].

Expansion rates of day 6 Control embryos was (84.5%) lower ($P < 0.05$) than for day 5 embryos (100.0%) [Table 1]. When G2 and G3 were compared between days 5 and 6, the expansion rates were higher for day 5.

Table 1. Viability of parthenote porcine embryos exposed or not to toxicity tests using EG, DMSO and SUC for blastocysts of days 5 and 6 of culture.

Group	Embryos of day 5 of culture			Embryos of day 6 of culture		
	Blastocysts	Expanded	Hatched	Blastocysts	Expanded	Hatched
	n	n (%)	n (%)	n	n (%)	n (%)
Control	74	74 (100.0)[aA]	45 (60.8)[aX]	58	49 (84.5)[aB]	23 (39.7)[aY]
G1	72	36 (50.0)[bc]	11 (15.3)[b]	56	28 (50.0)[b]	13 (23.2)[a]
G2	72	38 (52.8)[bA]	7 (9.7)[b]	58	18 (31.0)[cB]	5 (8.6)[b]
G3	73	26 (35.6)[cA]	4 (5.5)[b]	56	2 (3.6)[dB]	0 (0.0)[c]

G1: Vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC, warmed with 1 M SUC for 1 min followed by 3 min in 0.5 M SUC. G2: Vitrification solution with 16% EG + 16% DMSO + 0.4 M SUC, warmed with 0.13 M SUC for 5 min. G3: Vitrification solution with 18% EG +18% DMSO + 0.5 M SUC, warmed with 1 M SUC for 1 min followed by 3 min in 0.5 M SUC. [a,b]Distinct letters in the same column shown difference ($P < 0.05$). [A,B]Distinct letters in the same row shown difference in expanded blastocysts rates ($P < 0.05$). [X,Y]Distinct letters in the same row shown difference in hatched blastocysts rates ($P < 0.05$).

The amount of total cells in day 5 embryos was higher for G1 (94.0 ± 17.0) than for G3 (45.8 ± 12.6), without significant differences amongst other groups (Table 2). Conversely, for day 6 embryos, the Control and G1 TCN were similar between them and higher than G2 (respectively 75.0 ± 3.6; 65.0 ± 4.2; and 33.0 ± 6.2). The live non-hatched embryos of days 5 and 6 had similar TCN at day 8, despite the treatment.

For all treatments of both days 5 and 6, there was a higher proportion of CC3 in the non-hatched, in comparison to the hatched blastocysts (Table 2). Within the non-hatched, the higher incidence of CC3 was at day 6; whereas within the hatched, no differences were observed in the incidence of CC3, despite the treatment (including controls) for either day 5 and 6 (Table 2).

Table 2. Total cell number (TCN) and percentage of cells expressing cleaved caspase-3 (CC3) at day 8 in blastocysts and expanded blastocysts after been exposed tor not to toxicity test at days 5 or 6 of culture.

Group	Toxicity test	Not hatched embryos		Hatched embryos	
		TCN ± SE	CC3 %	TCN ± SE	CC3 %
Control		43.3 ± 8.1	21.5^X	75.3 ± 7.1^{ab}	7.4^Y
G1	D5	34.0 ± 9.8	22.5^X	94.0 ± 17.0^{a}	7.7^Y
G2		49.5 ± 12.2	21.0^{X*}	$67.7 \pm 12.2^{ab*}$	10.3^Y
G3		36.0 ± 25.3	24.0^{X}	45.8 ± 12.6^{b}	9.7^Y
Control		39.6 ± 8.4	20.2^{Xy}	75.0 ± 3.6^{x}	8.1^Y
G1	D6	35.0 ± 10.8	30.5^{Xx}	65.0 ± 4.2^{x}	10.1^Y
G2		39.8 ± 9.4	31.4^{Xx*}	$33.0 \pm 6.2^{y*}$	6.1^Y
G3		34.8 ± 9.8	30.5^{x}	0.0	0.0

G1: Vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC, warmed with 1 M SUC for 1 min followed by 3 min in 0.5 M SUC. G2: Vitrification solution with 16% EG + 16% DMSO + 0.4 M SUC, warmed with 0.13 M SUC for 5 min. G3: Vitrification solution with 18% EG +18% DMSO + 0.5 M SUC, warmed with 1 M SUC for 1 min followed by 3 min in 0.5 M SUC. [X,Y]Distinct letters in the same row shown difference for CC3 ($P < 0.05$). [a,b]Distinct letters in the same column shown difference for the TCN for embryos of day 5 ($P < 0.05$). [x,y]Distinct letters in the same column shown difference for the TCN for embryos of day 6 ($P < 0.05$). *In the same column show difference between embryos of day 5 and 6 for the same group ($P < 0.05$).

Experiment 2: *The effect of extracellular cryoprotectants: PVP, SUC and TRE*

Under our conditions, PVP and SUC (68.7 vs 65.9%) for day 5 embryos provided the best expansion rates in comparison to TRE (43.8%), and all lower than the Control (89.9%). The hatching rates were similar between SUC (28.0%) and PVP (15.7%), but higher than TRE (12.5%), whilst PVP and TRE were similar between them (Table 3).

For day 6, TRE (58.5%) kept an expansion rate similar to the Control (73.2%). By the other hand, the group SAC (42.6%) was similar to TRE and to PVP (39.3%), been PVP lower than TRE and Control. For day 6 embryos, hatching was similar among Control (12.5%), SUC (5.6%) and TRE (5.5%), being PVP (1.8%) similar to SUC and TRE but lower than the Control (Table 3).

Experiment 3: *Post-warming survival of porcine embryos, vitrified using different combinations of intra- and extra-cellular cryoprotectant agents*

For day 5 embryos, the Fresh Controls showed the highest expansion and hatching rates. Amongst only the vitrified protocols, the expansion rate (20.0%) of protoco1 (P1) was higher than all the other vitrified counterparts: P2, P3 and P4 (respectively 4.3%, 4.3% e 4.4%). For the vitrified ones, the hatching rates were similar amongst them all (Table 4).

For day 6 embryos, the expansion and hatching rates after vitrification had no significant difference, although all they were significantly lower than the Fresh Control (Table 5).

Table 3. Porcine parthenotes embryos of days 5 and 6 exposed or not to toxicity test to the extracellular cryoprotectors PVP, SUC and TRE.

Group	D5			D6		
	Blastocysts	Expanded	Hatched	Blastocysts	Expanded	Hatched
	n	n (%)	n (%)	n (%)	n (%)	n (%)
Control	79	$71 (89.9)^{aA}$	$41 (51.9)^{aX}$	56	$41 (73.2)^{aB}$	$7 (12.5)^{aZ}$
PVP	83	$57 (68.7)^{bA}$	$13 (15.7)^{bcX}$	56	$22 (39.3)^{cB}$	$1 (1.8)^{bZ}$
SAC	82	$54 (65.9)^{bA}$	$23 (28.0)^{bX}$	54	$23 (42.6)^{bcB}$	$3 (5.6)^{abZ}$
TRE	80	$35 (43.8)^{c}$	$10 (12.5)^{c}$	58	$34 (58.6)^{ab}$	$3 (5.2)^{ab}$

[a,b,c]Distinct letters in the same column shown difference ($P \leq 0.05$). [A,B]Distinct letters in the same row shown difference for the expansion rates ($P \leq 0.05$). [X,Z]Distinct letters in the same row shown difference for the hatching rates ($P \leq 0.05$).

Table 4. Day five porcine parthenotes embryos viability after vitrification in four different protocols.

Group	D5 Blastocysts	Expanded	Hatched
	n	n (%)	n (%)
Control	46	44 (95.7)[a]	19 (41.3)[a]
Prot 1	45	9 (20.0)[b]	1 (2.2)[b]
Prot 2	47	2 (4.3)[c]	0 (0.0)[b]
Prot 3	46	2 (4.3)[c]	0 (0.0)[b]
Prot 4	45	2 (4.4)[c]	0 (0.0)[b]

Prot 1: Equilibration solution with 7.5% EG + 7.5% DMSO incubated for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC incubated for 1 min, warmed with 0.13 M SUC for 5 min. Prot 2: Equilibration solution with 7.5% EG + 7.5% DMSO incubated for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.5 M SUC incubated for 1 min, warmed with 0.13 M TRE for 5 min. Prot 3: Equilibration solution with 2 M EG 5 min, vitrification solution with 8 M EG + 0.5 M SUC for 1 min, warmed with 0.13 M SUC for 5 min. Prot 4: Equilibration solution with 2 M EG 5 min, vitrification solution with 8 M EG + 0.5 M SUC for 1 min, warmed with 0.13 M TRE for 5 min. [a,b,c]Distinct letters in the same column shown difference ($P \leq 0.05$).

Table 5. Day six porcine parthenotes embryos viability after vitrification in four different protocols

Group	D6 Blastocysts	Expanded	Hatched
	n	n (%)	n (%)
Control	60	52 (86.7)[a]	23 (38.3)[a]
Prot 1	62	7 (11.3)[b]	0 (0.0)[b]
Prot 2	58	2 (3.4)[b]	0 (0.0)[b]
Prot 3	60	8 (13.3)[b]	0 (0.0)[b]
Prot 4	60	5 (8.3)[b]	0 (0.0)[b]

Prot 1: Equilibration solution with 7.5% EG + 7.5% DMSO for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.6 M TRE for 1 min, warmed with 0.13 M SAC for 5 min. Prot 2: Equilibration solution with 7.5% EG + 7.5% DMSO for 5-15 min, vitrification solution with 15% EG + 15% DMSO + 0.6 M TRE for 1 min, warmed with 0.13 M TREA for 5 min. Prot 3: Equilibration solution with 2 M EG for 5 min, vitrification solution with 8 M EG + 0.6 M TRE for 1 min, warmed with 0.13 M SUC for 5 min. Prot 4: Equilibration solution with 2 M EG for 5 min, vitrification solution with 8 M EG + 0.6 M TRE for 1 min, warmed with 0.13 M TREA for 5 min. [a,b]Distinct letters in the same column shown difference ($P \leq 0.05$).

DISCUSSION

In order to provide an adequate cell cryoprotection, the concentration of cryprotectant agents shall be the most effective but safe, to avoid any toxic effect to the cells. In the porcine embryos it was found that 15% of intracellular cryoprotectant might not be enough to protect the cells [4]. Nevertheless, under our conditions, this concentration provided (for the toxicity tests) the highest expansion and hatching rates for the most sensible (day 6) embryos, and 16% already damaged the embryos. One possible explanation for the best results might be the higher concentration of SUC (0.5 M) in comparison to what they (0.4 M) used; even though it is a slight difference, the potential to dehydrate the cells can be importantly affected. These results confront what the same authors found when

the association of 17% of intracellular cryoprotectants impaired the cell proliferation capacity of *in vivo* produced embryos; this treatment is equivalent to our experimental group G3, which consisted of 18% of each intracellular cryoprotectant.

The expression of CC3 is a reliable indicator of programmed cell death. According to results of the toxicity tests combining EG, DMSO and SUC, the embryos showing ability to hatch also presented similar cell viability, since they had similar patterns of CC3 expression. In spite of the highest sensibility of day 6 blastocysts and expanded blastocysts, it was possible to observe a higher incidence of CC3 expression in the embryos exposed to cryoprotectants, in comparison to their Controls, when all non-hatched (but morphologically alive) counterparts

were analysed. This difference was not observed when day 5 embryos were assessed for CC3, what can be attributed to their better resistance, compared to day 6.

The use of EG as the only intracellular cryoprotectant for porcine embryo has been reported [1,4], often combined with some extracellular cryoprotectant, in steps of vitrification or warming. Between the extracellular cryoprotectants PVP [1] and SUC [4], the warming steps are more commonly held by SUC, during warming steps.

The use of TRE has provided very encouraging results, in spite of its known properties, such as the capacity to stabilize cell membranes under stressful conditions [14], or even its specific interactions with membrane phospholipids [7]. However, when combined as a cryoprotectant in our system, the expansion rate obtained was not above the satisfactory, not playing any positive role in the embryo hatching rates.

Our results also disagree from what Morató *et al*. [12] found, where day 6 blastocysts had not only better quality, but also higher survival (expansion) rates when compared to day 5 counterparts. By the other hand, embryos of day 6 are more sensible to specific culture medium [11]. As evidenced by the clear difference in sensitivity showed by embryos that reach the blastocyst stage under different developing speeds (at days 5 or 6), there is the need for directing specific vitrification protocols for either developing kinetics; the production system, howsoever, must be considered when one intends to select the best cryopreservation protocol.

CONCLUSIONS

In conclusion, day 5 embryos are more viable and resistant to cryoprotectors than day 6 embryos. Notwithstanding, the intracellular cryoprotectors EG and DMSO when used together are less toxic to embryos of day 5 of culture when used in a concentration up to 16%. However, for embryos of day 6, this limit goes up to 15% of each cryoprotector, whereas 16% already increases caspase in no hatched embryos, and reduces TCN of the hatched ones.

Besides the extracellular cryoprotectors showed a slight difference on its cell toxicity, the cryotolerance of the less toxic extracellular agents was not effective to promote embryo hatching.

MANUFACTURERS

[1]Sigma Chemical Co. St. Louis, MO, USA.

[2]SPL Lifescience Inc. Eumhyeon, South Korrea.

[3]Invitrogen Brasil Ltda. São Paulo, SP, Brazil.

[4]Bioniche Animal Health. Belleville, ON, Canada.

[5]Olympus Corporation. Tokyo, Japan.

[6]SAS Institute Inc. Cary, NC, USA.

REFERENCES

1 **Beebe L.F.S., Cameron R.D.A., Blackshaw A.W. & Keates H.L. 2005.** Changes to porcine blastocyst vitrification methods and improved litter size after transfer. *Theriogenology*. 64: 879-890.

2 **Brogni C.F., Ohlweiler L.U., Klein N., Mezzalira J.C., Cristani J. & Mezzalira A. 2016.** Pré-incubação dos espermatozoides suínos diminui polispermia e aumenta a produção embrionária em oócitos de baixa qualidade. *Ciencia Rural*. 46(6): 1113-1118.

3 **Cameron R., Beebe L., Blackshaw A., Higgins A. & Nottle M. 2000.** Piglets born from vitrified early blastocysts using a simple technique. *Australian Veterinary Journal*. 78: 195-206.

4 **Cuello C., Sanchez-Osorio J., Almiñana C., Gil M.A., Perals M.L., Lucas X., Roca J., Vazquez J.M. & Martinez E.A. 2008.** Effect of the cryoprotectant concentration on the in vitro embryo development and cell proliferation of OPS vitrified porcine blastocysts. *Cryobiology*. 56: 189-194.

5 **Dobrinsky J. 1997.** Cryopreservation of pig embryos. *Journal of Reproduction and Fertility*. (Suppl). 52: 301-312.

6 **Du Y., Zhang T., Li J., Kragh P.M., Kuwayama M., Ieda S., Zhang X., Schmidt M., Bøgh I.B., Purup S., Pedersen A.M., Villemoes K., Yang H., Bolund L. & Vajta G. 2007.** Simplified cryopreservation of porcine cloned blastocysts. *Cryobiology*. 54: 181-187.

7 **Hu J.H., Li Q.W., Li G., Jiang Z.L., Bu S.H., Yang H. & Wang L.Q. 2009.** The cryoprotective effect of trehalose supplementation on boar spermatozoa quality. *Animal Reproduction Science*. 112: 107-118.

8 Li R., Liu Y., Pedersen H.S., Kragh P.M. & Callesen H. 2013. Development and quality of porcine parthenogenetically activated embryos after removal of zona pellucida. *Theriogenology.* 80: 58-64.

9 Martinez E.A., Martinez C.A., Nohalez A., Sanchez-Osorio J., Vazquez J.M., Roca J., Parrilla I., Gil M.A. & Cuello C. 2015. Nonsurgical deep uterine transfer of vitrified, *in vivo*-derived, porcine embryos is as effective as the default surgical approach. *Scientific Reports.* 5: 10587.

10 Men H., Zhao C., Si W., Murphy C.N., Spate L., Liu Y., Walters E.M., Samuel M.S., Prather R.S. & Critser J.K. 2011. Birth of piglets from in vitro-produced, zona-intact porcine embryos vitrified in a closed system. *Theriogenology.* 76: 280-289.

11 Mito T., Yoshioka K., Noguchi M., Yamashita S., Misumi K., Hoshi T. & Hoshi H. 2015. Birth of piglets from in vitro-produced porcine blastocysts vitrified and warmed in a chemically defined medium. *Theriogenology.* 84(8): 1314-1320.

12 Morató R., Castillo-Martin M., Yeste M. & Bonet S. 2014. Cryotolerance of porcine in vitro-produced blastocysts relies on blastocyst stage and length of *in vitro* culture prior to vitrification. *Reproduction Fertility and Development.* 28(7): 886-892.

13 Nagashima H., Kashiwazaki N., Ashman R.J., Grupen C.G. & Nottle M.B. 1995. Cryopreservation of porcine embryos. *Nature.* 374: 416.

14 Sampedro J.G. & Uribe S. 2004. Trehalose-enzyme interactions result in structure stabilization and activity inhibition. The role of viscosity. *Molecular and Cellular Biochemistry.* 256: 319-327.

15 Yoshioka K., Susuki C., Tanaka A., Anas I. & Iwamura S. 2002. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biology of Reproduction.* 66: 112-119.