PORCINE RESEARCH
International Journal of the Bioflux Society
Research article

Approaches in swine germplasm preservation: the effect of different cryoprotectant concentrations on immature oocyte vitrification

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Abstract. The aim of this study was to determine the effectiveness of using two different concentrations of cryoprotectants for the vitrification of immature swine oocytes. A total of 1394 oocytes were vitrified in super fine open pull straws, using two experimental designs. In experiment A the eggs were preserved stepwise in 15% (pre-vitrification solution) and 40% (vitrification solution) ethylene glycol, a permeating agent, combined with trehalose, a non-permeating agent (0.25 M and 0.5 M respectively). In the second experiment the concentration of the ethylene glycol was 20 and 50% and the trehalose concentrations were 0.5 and 0.75 M. After fluorescent staining, with fluorescein diacetate and propidium iodide, the cells were examined to test their viability by exposing them to the microscope's UV light. The results show that the increased concentration of cryoprotectants did not increase the rate of viable oocytes. The differences between two experiments are extremely significant ($p<0.001$).
Key Words: oocyte, swine, vitrification, cryoprotectants, ethylene glycol.

Introduction. Preservation of rare genotypes is important and costly (Critser & Russel 2000). Germplasm preservation can provide a cost-effective alternative to the maintenance of breeding colonies for the preservation of rare genotypes and can also provide the means to prevent the loss of rare genotypes. The value and feasibility of genome resource banking for the preservation of rare genotypes has been recognized for many years (Whittingham et al 1977). Having frozen oocyte banks could also improve research endeavors by providing freer access to oocytes and reduce the effects of seasonal variation in oocyte quality. In fact, it has been argued that "any improvements in the swine IVP (*in vitro* production) system would revolutionize not only the reproductive management of swine, but also increase the use of pigs for biotechnological and biomedical applications" (Wheeler et al 2004). A way to cryopreserve oocytes, spermatozoa or embryos is vitrification. The physical definition of vitrification is the solidification of a solution glass formation at low temperatures without ice crystal formation. This phenomenon can be explained as an extreme increase of viscosity and requires either rapid cooling rates or the use of cryoprotectant solutions, depressing ice crystal formation and increasing viscosity at low temperatures (Vajta 2000).

Porcine gametes and embryos cryopreservation seams to be much more challenging compared to other species (Rojas et al 2004). Swine oocytes are very sensitive to cooling below physiologic temperatures. The detrimental effects of cooling mammalian oocytes include: damage to lipid phase, changes in the oolemma (Gethler et al 2005) and developmental potential (Azambuja et al 1998). Because of the chilling sensitivity, rapid cooling methods have been the focus of attempts to preserve porcine oocytes (Fujihira et al 2005; Nagashima et al 1999).

In order to prevent intracellular ice formation at high cooling rates, a condition that is usually lethal (Mazur 1977, 1963), high concentrations of cryoprotectants are required

(Fahy et al 1984). Exposing cells to solutions containing high concentrations of solutes can damage due to the direct chemical toxicity of these compounds and as a result of the osmotic effects caused by the exposure. Although, osmotic effects can be controlled by modifying the manner by which cells are exposed to the compounds. More specifically, high concentrations of permeable solutes can be loaded into cells in a stepwise manner, like in our experiments, resulting in a reduced osmotic effect during each step (Paynter 2005). The field literature presents numerous cases describing the benefits of a stepwise approach to addition and/or removal of high concentrations of solutes compared to a single-step exposure. The optimal procedure for loading and unloading cryoprotectants will balance the chemical and osmotic effects by adding the cryoprotectants as quickly as possible (minimizing the chemical effects) but using a stepwise procedure to reduce the osmotic effects. Even though most reports regarding oocyte vitrification describe stepwise cryoprotectant agent (CPA) addition and removal procedures, the procedures employed are rarely designed to specifically take into account the osmotic tolerance and cell permeability characteristics of the cells. When these properties are taken into account, they can be used in a proactive manner to design theoretically optimal procedures for addition and removal of CPAs (Fuller & Paynter 2004).

The permeating cryoprotectants, which form hydrogen bonds with water molecules, can prevent ice crystallization and protect cells from solution effects. They act to depolymerize microfilaments and microtubules and may be beneficial for protecting cytoskeletal components during osmotic stresses induced by exposure to, or removal of, cryoprotectants. Ethylene glycol (EG), with high permeability and low toxicity, is one of the most effective permeating CPA (Guang-Bin & Ning 2009) reason why we choose it four our study. Trehalose was used in our research as a non-permeating CPA. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage.

Materials and Methods

Medium preparation. *Base medium (BM)*, for a 10 mL working solution, consists of 7.2 mL pure water, 800 µL PBS 10X (Phosphate buffered saline solution) and 2 mL bovine fetal serum.

Vitrification solution: for experiment A, for pre-vitrification (first step): 850 µL BM, 150 µL EG representing 15%, trehalose 0.25 M, assuming 1000 µL working solution are prepared; vitrification solution (second step): BM – 600 µL, EG – 400 µL, and trehalose 0.5 M, in 1000 µL. For experiment B pre-vitrification (first step): BM – 800 µL, EG – 200 µL (20%), trehalose 0.5 M; vitrification solution (second step): BM – 500 µL, EG – 500 µL (50%), trehalose 0.75 M.

Warming solution: oocytes are warmed up in three different concentrated solutions containing PBS and trehalose in each experimental design.

Experiment A: step 1 (pre-vitrification): 1 mL PBS and trehalose 0.5 M; step 2: 1 mL PBS + trehalose 0.25 M; step 3: 1 mL PBS+ 0.125 M trehalose.

Experiment B: step 1: 1mL PBS and trehalose 0.75 M; step 2: 1 mL PBS +trehalose 0.5 M; step 3: 1 mL PBS and trehalose 0.25 M.

Oocyte collection. The ovaries were collected from prepuberal gilts from a slaughterhouse near Cluj-Napoca, Cluj county and were transported in saline solution (0.9% NaCl w/v) supplemented with antibiotic (gentamicine) at 37 °C within 2 hours. The oocytes-cumulus complexes were collected with a syringe by follicular punction and washed in plastic Petri dishes containing TCM 199 medium (Sigma-Aldrich, Germany). Only cumulus layer rich oocytes were taken to further processing.

Oocyte vitrification. Swine immature cumulus-oocyte complexes were cryopreserved in two steps using two experimental designs.

Experiment A: a number of 722 cumulus-oocyte complexes were cryopreserved by vitrification in two steps. The washed oocytes were transferred in 10 µL of solution containing 15% ethylene glycol (EG) and 0.25 M trehalose (Sigma-Aldrich, Germany) for 4 minutes and then placed into 2 µL vitrification solution containing 40% EG and 0.5 M trehalose for 40 seconds. After equilibration the oocytes where placed in superfine open

pull straws (SOPS – Minitüb, Germany). Loading into the tip of the SOPS was done by means of the capillarity effect by simply touching the 2 µL drop of vitrification solution containing the oocytes. Plunging the SOPS into the liquid nitrogen achieved rapid cooling.

Experiment B: The procedure is the same as in the first experiment but the concentration of cryoprotectants differs. A total of 672 swine oocytes were used. In the first step of equilibration the EG was increased to 20% compared to 15% and the exposure time to 2 minutes vs. 4 minutes. In the next step of vitrification the EG concentration increase to 50% vs. 40% (experiment A) and the equilibration time decreased from 40 seconds (experiment A) to 20 seconds.

Six repeats were performed per each experiment. Table 1 shows the number of oocytes per each repetition. The number of oocytes is not equal in every repetition because of the quantity and the quality of the ovaries/oocytes can not be managed.

Oocyte warming. Oocytes are warmed up in three different concentrated solutions containing PBS and trehalose as described above. The SOPS containing the oocytes are hold in air for 5 seconds after they were taken out of the liquid nitrogen container. After 5 seconds by touching the end of the tip to the warming solution the oocytes are loaded out into the droplet by a reverse phenomenon of the capillarity due to the temperature differences between the two solutions. The oocytes are kept in each concentration for 5 minutes for equilibration. In this time the EG is sucked out and the oocytes are re-hydrated step by step. The gametes are placed then in a solution of PBS containing 0.4% BSA (bovine seric albumin) for at least 10 minutes.

Viability test for the thawed oocytes. A good and rapid way to test the viability of oocytes, that were previously cryopreserved, is fluorescent staining with fluorescein diacetate (FDA). The non-polar FDA molecules enter the cell, are hydrolyzed by cell esterases, and fluorescein is produced. This polar compound can not leave the cell because it is unable to pass through the intact cell membrane, and it therefore accumulates in the cytoplasm of the cell. Damaged cells however show a distinct loss of fluorescein through the cell membrane. With the aid of a fluorescence microscope, a photometer and a recorder, the amount of radiated light can be measured. If the display shows green fluorescent oocytes, it means that they are alive, if not it means that the esterases are not active anymore and the cells are dead. Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI is membrane impermeant and generally excluded from viable cells. The viable oocytes are non colored and those that are dead have a red color (Figure 1 C). PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques, so we used these two fluorescein stain together (FDA+PI) to be sure if the cells are alive or dead, for more accuracy. Groups of 10 oocytes were kept in 30 µL of PBS containing FDA and PI (5 µL and 2.5 µL respectively, in 1 mL PBS) for 10 minutes in the incubator at 37 ºC and 5% CO_2 in air. In this time the dye sticks to the cells and penetrates or not. After 10 minutes the cells were examined with a microscope with inversion (Olympus) with UV light.

Statistical analysis. The results were analyzed using GraphPad InStat 3, unpaired t test.

Results and Discussion. The stained oocytes were evaluated after incubation with fluorescent dyes and counted. The oocytes that appear green (FDA) or non colored (PI) in UV light were considered alive and those colored red (PI) or noncolored or a low fluorescent green are considered dead cells, as shown in Figure 1 (original photos).

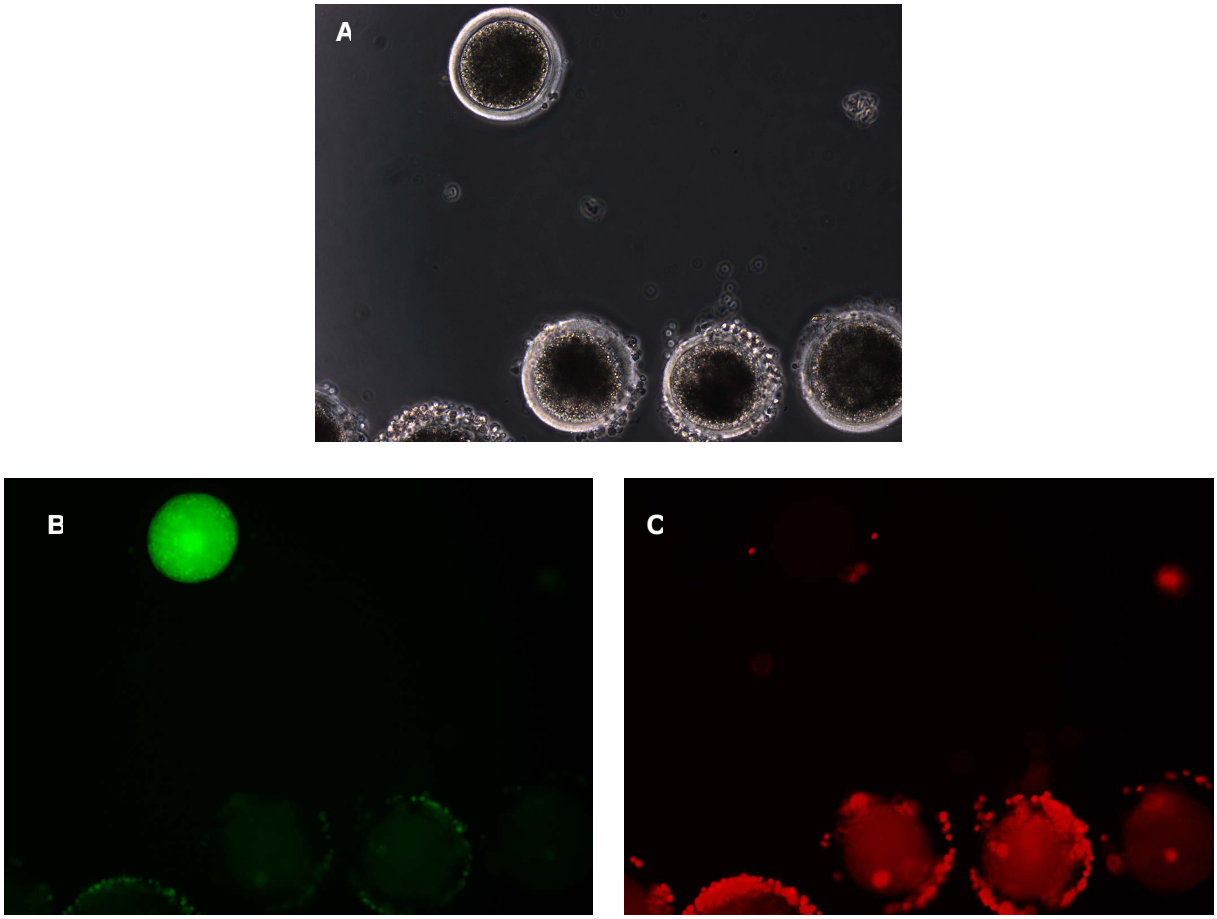

Figure 1. A – Phase contrast; B – FDA staining; C – PI staining.

The results of the two experiments, using different concentrations of cryoprotectants in the vitrification solution, are presented in Table 1.

Table 1

Number and percentage of viable oocytes.

Rep. no.	Experiment A		Experiment B	
	No. of oocytes /SOPS	No. of viable oocytes/Percentage	No of oocytes /SOPS	No. of viable oocytes/Percentage
1	132	46 (34.84%)	148	3 (4.05%)
2	124	40 (32.25%)	100	5 (5.00%)
3	136	46 (33.82%)	87	5 (5.74%)
4	120	42 (29.57%)	108	6 (5.55%)
5	108	37 (34.25%)	146	7 (4.79%)
6	102	38 (33.92%)	90	3 (3.33%)

From a total number of 722 oocytes from the first experiment, 249 were considered viable and the rest of 473 cells were dead. The percentage of viable oocytes was between 29.57% the lowest and 34.84% the highest. The average percentage of viable cells, for this experimental group A, was 33.10%. In the second experiment, with a higher concentration of cryoprotectant, the number of the dead oocytes was higher. From a total of 672 cells vitrified, 643 were dead and only 29 were counted viable. The average percentage for this experiment is 4.74%. The differences between the two experiments are extremely significant (Figure 2).

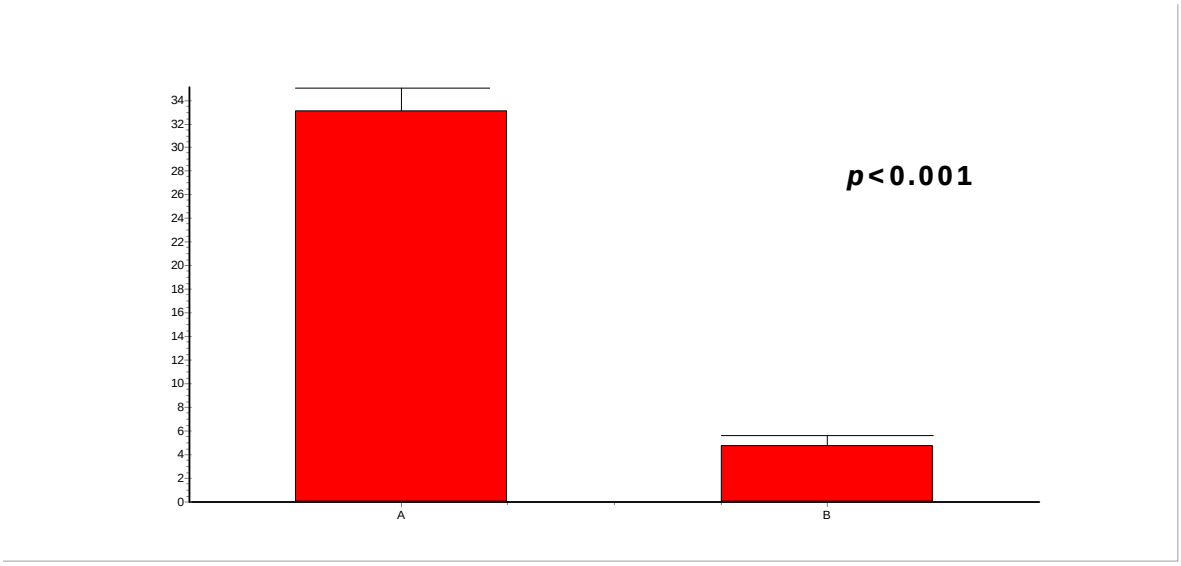

Figure 2. The means and standard deviations of the two experiments (percentage of viable oocytes)

As mentioned in the introduction, vitrification needs high concentrations of cryoprotectant agents to prevent ice crystal formation, to increase the intracellular viscosity and to turn into a glass-like state during vitrification without any irreversible damages to the cytoplasm or cell membrane. The present research shows that a higher concentration of cryoprotectant combined with a lower equilibration time does not increase the percentage of the viable oocytes following vitrification and warming, moreover this increasing of the CPA leads to a significantly lower viability rate of the oocytes after warming. This low rate using higher concentration can be explained either by the fact that the exposure time to pre-vitrification and/or vitrification solution was too long and the osmotic stress occured damaging the cells irreversible. It is known that the overpass of exposure/equilibration time of the oocytes with the concentrated cryoprotectant can be devastating, causing lipid phase-changing in the membrane lipids and damaging the cytoplasmic organelles. It is needed to reduce the exposure time lower than our times (pre-vitrification 2 minutes and vitrification 20 second) especially the pre-vitrification time.

Another theory which could explain the significantly differences ($p<0.001$) between the experiments is the low trehalose concentration. It seams that the concentration used was not enough to help prevent the chemical toxicity of the high concentration of the ethylene glycol. The addition of a sugar (sucrose, glucose, fructose, sorbitol, saccharose, trehalose, or raffinose) to an EG-based vitrification solution influenced the overall properties of the solution (Shaw et al 1995) so the properties of the sugar in the establishment or modification of a vitrification solution needs to be taken into consideration. Additives with large molecular weights, such as disaccharides, like sucrose or trehalose, do not penetrate the cell membrane, but they can significantly reduce the amount of cryoprotectant required, as well as the toxicity of EG, by decreasing the concentration required to achieve a successful cryopreservation of human oocytes and embryos. The incorporation of non-permeating compounds into the vitrification solution and the incubation of the cells in this solution before any vitrification help to withdraw more water from the cells and lessen the exposure time of the cells to the toxic effects of the cryoprotectants (Liebermann et al 2002).

Conclusions. The results show that the increased concentration of cryoprotectants did not increase the rate of viable oocytes. The differences between two experiments are extremely significant ($p<0.001$).

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Received: 15 May 2012. Accepted: 30 May 2012. Published online: 28 June 2012.

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How to cite this article:
Hettig A., Botha M., Miclea V., Zăhan M., Roman I., Miclea I., Varo-Ghiuru F., Orlovschi D., 2012 Approaches in swine germplasm preservation: the effect of different cryoprotectant concentrations on immature oocyte vitrification. Porc Res 2(1): 4-10.