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Co-culture with granulosa cells improve the *in vitro* maturation ability of porcine immature oocytes vitrified with cryolock ☆

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ABSTRACT

This study was designed to evaluate the efficiency of two oocyte vitrification–warming procedures using two different devices: Superfine Open Pulled Straws (SOPS) and Cryolock, as well as the effect of the co-culture of vitrified immature oocytes with fresh granulosa cells to improve *in vitro* maturation (IVM). Immature oocytes were vitrified with two procedures: A) Oocytes were exposed to an increasing concentration of ethylene glycol (EG) from 4% to 35% with 0.5 M trehalose. They then, were loaded in SOPS or Cryolock. For warming, oocytes were exposed to decreasing concentrations of trehalose 0.3, 0.2 and 0.1 M for IVM. B) Oocytes were exposed to two mixtures of EG and dimethylsulfoxide (Me_2SO), at 7.5% and 16%, both with 0.4 M of sucrose and then loaded in SOPS or Cryolock and stored in liquid nitrogen. For warming, oocytes were exposed to a single concentration of sucrose 0.5 M. After warming, viability was determined; and after 44 h of IVM both viability and meiotic stages were evaluated. The results indicate no significant differences between procedures A and B with SOPS in all maturation stages, reaching a maximum maturation rate of 21%. As to Cryolock, significant differences were observed between both procedures, being procedure B, more efficient with a yield of 38% in MII stage and increased to 49% due to the co-culture with fresh granulosa cells. In conclusion, viability and maturation rates were improved with Cryolock and procedure B with the co-culture system in vitrified immature oocytes.

Introduction

Vitrification is a process that turns liquid into a vitreous solid state excluding ice crystal formation. In 1985, the first study achieving ice-free cryopreservation by vitrification of mammalian cells was performed [28].

Vitrification of immature, mature oocytes and embryos has been performed in different mammalian species. Up to now, many studies have been performed to increase the oocytes and embryos survival rate in order to obtain live offspring. Immature oocytes vitrification continues to be a wide area of research because one of the greatest challenges to reproductive cryobiologists is to establish a vitrification-warming procedure that allows the banking of oocytes and the improvement of assisted reproductive technologies [23,29].

For oocyte cryopreservation the main factors affecting survival are the nuclear stage of the cell, cryoprotectant agents (CPAs)

Abbreviations: BES, beveled edge open straw; COCs, cumulus cell-oocyte-complexes; CPAs, cryoprotectant agents; EG, ethylene glycol; EGF, epidermal growth factor; Egf-L, LH dependent epidermal growth factor; FCS, fetal calf serum; FSH, follicle stimulating hormone; GV, germinal vesicle; IVM, *in vitro* maturation; LH, luteinizing hormone; Me_2SO, dimethylsulphoxide; MI, metaphase I; MII, metaphase II; mTBM, modified Tris-buffered-medium; MTT, Thiazolyl blue; NkO-CC, oocytes vitrified without cumulus cells and matured in co-culture with granulosa cells; OPS, open pulled straw; PVA, polyvinyl alcohol; GVB, germinal vesicle breakdown; SOPS, superfine open pulled straw; SSV, solid surface vitrification; TCM-199, tissue culture medium 199.

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viscosity, volume and exposure time, device cooling/warming rates and embryologist ability. Immature oocytes are more sensitive than those matured or embryos [34]. This impact is due to the structural changes in the membrane of mammalian oocytes through maturation, affecting the membrane lipid phase-transition temperature. It is known that vitrification of porcine immature oocytes is more difficult than those of ovine [13], mice, hamster [43] and cattle [41] because of their lipid content [21]. Most studies on lipid content in oocytes or embryos concern about its damaging influence during cryopreservation [16,19,46]. Immature oocytes have lower permeability to CPAs and a higher one to water, compared with matured oocytes. This suggests that the low survival rates could be due to the insufficient permeation of the CPAs [2].

Cryopreservation procedures have used additional chemicals to avoid cell damage. These chemicals are classified in two categories, permeating and non-permeating CPAs. Permeating CPAs form hydrogen bonds with water to prevent ice crystals formation. The most commonly used are propylene glycol (PROH), propanediol, glycerol, dimethylsulfoxide (Me_2SO) and ethylene glycol (EG), whose main function is to dehydrate cells by diluting their remaining electrolytes. The cryoprotective properties of Me_2SO were described by Lovelock in 1959 [18]. Since then, several studies indicate that the permeating speed of Me_2SO and PROH is higher than EG. However, Me_2SO and PROH have been found to be highly toxic and detrimental to the meiotic competence of immature porcine oocytes [25]. Non-permeating CPAs act by drawing free water from within the cell, dehydrating the intracellular space. They are commonly used in combination with permeating CPAs to increase dehydration and the incorporation of permeating agents. Also, non-permeating agents play an important role during warming reducing the osmotic shock that occurs if the permeable agents do not diffuse out quickly to prevent the excessive influx of free water inducing cell rupture [18]. The most commonly used for oocyte cryopreservation are sucrose and trehalose [5,10]. Previous reports suggest that the combination of different CPAs can improve vitrification procedures by reducing CPAs toxicity [35,36]. Furthermore, the presence of cumulus cells in immature oocytes decreases the CPAs intake, contributing to the ice crystal formation, which damages human [17] and mouse [20] oocytes. However, studies in horses [38] and goats [27], suggest that the presence of cumulus cells protects oocytes against vitrification-induced damage more than denuded oocytes [38]. In spite of this, the presence of cumulus cells is necessary to maintain the gap junction communications between cumulus cells and oocytes to acquire developmental competence and mitochondrial functions during maturation. Besides, antral granulosa cells provide a hormonal environment during folliculogenesis through gap junctions for oocyte maturation [42]. Nowadays, several vitrification procedures have been performed; but results in viability and maturation still remain low. This is why in the present study the efficiency of two vitrification procedures was tested.

For cell vitrification, the use of devices is required to store oocytes or embryos in liquid nitrogen. Several devices such as: open pulled straws (OPS), superfine open pulled straws (SOPS), solid surface vitrification (SSV), Cryotop, Cryoloop and beveled edge open straws (BES) have been used. The results on viability and maturation are different for each device. Several studies suggest that Cryotop is better than OPS, SOPS, SSV and BES, since it provides high cooling/warming rates (>23,000 °C/min) in cryopreservation solutions with a minimum volume [7,8,13,39,40]. Recently the Cryolock device has been manufactured to be used for assisted reproduction in humans.

In the present study, the use of the Cryolock in porcine oocyte vitrification was studied. It includes a secure cap to avoid liquid nitrogen storage contamination and also provides an easy handling for a safety cryopreservation and high cooling/warming rates (22,000 °C/min) allowing volumes <0.1 μL.

Pigs are important experimental animals; they have been currently used as a human model for organ xenotransplantation and regenerative medicine [46]. It is important to underline that in this species, vitrification procedures are still not optimal and reproducible. Therefore, porcine oocyte vitrification remains as a challenge.

Since the cumulus cells decreases CPAs permeability, if immature oocytes denudation is performed before vitrification, and afterwards they are co-cultured with granulosa cells for IVM, viability and maturation rates can be improved. The aim of the present study was: 1) to assess the viability and maturation of vitrified immature porcine oocytes, 2) to compare the efficiency of two vitrification procedures in two different devices: SOPS and Cryolock, 3) to determine viability and maturation in vitrified–warmed immature porcine oocytes in co-culture with fresh granulosa cells.

Materials and methods

Experimental design

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

At least nine experiments were performed. Viability and maturation were evaluated in different samples. The nuclear maturation stage was determined in all groups. Immature oocytes with uniform cytoplasm surrounded by cumulus cells were collected and randomly distributed in three treatment groups: 1) non-vitrified oocytes were subjected to IVM during 44 h. Viability was assessed immediately after COCs selection (0 h) and at the end of IVM (44 h) (Control, $n = 303$ viability, $n = 547$ maturation); 2) immature COCs were vitrified with two vitrification–warming procedures (A and B), and devices: SOPS (Minitube, México) and Cryolock (Importadora Mexicana de Materiales para Reproducción Asistida S.A. de C.V., México) described below for IVM. Viability was assessed immediately after warming (0 h) and at the end of IVM (44 h) (COCs group, $n = 444$ viability, $n = 336$ maturation); 3) According to results, immature oocytes were denuded and vitrified–warmed only with procedure B and Cryolock. Vitrified oocytes were placed in a 4-well dish (Nunc, Denmark) containing a fresh granulosa cell co-culture for IVM. Viability was assessed immediately after warming (0 h) and at the end of IVM (44 h) (NkO-CC group, $n = 60$ viability and $n = 87$ maturation).

Oocyte collection and IVM

Ovaries were collected from pre-pubertal female pigs at the slaughterhouse "Los Arcos" (Edo de México) and transported to the laboratory in 0.9% NaCl solution at 25 °C in less than 2 h. Ovarian follicles between 3 and 6 mm in diameter were punctured using an 18 gauge needle to obtain the follicular fluid. Follicular contents were left to sediment and washed twice with Tyrode modified medium supplemented with 10 mM sodium lactate, 10 mM HEPES and 1 mg/mL PVA (TL–HEPES–PVA) at pH 7.3–7.4 for cumulus-oocyte-complexes (COCs) collection. Oocytes with uniform cytoplasm surrounded by a two–four layer compact mass of cumulus cells were selected. COCs were washed three times in 500 μL drops of maturation medium: TCM-199 with Earle's salts and 26.2 mM sodium bicarbonate (In Vitro, México) supplemented with 0.1% polyvinyl alcohol (PVA), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine and 10 ng/mL EGF, 0.5 μg/mL luteinizing hormone (LH), and 0.5 μg/mL follicle stimulating hormone (FSH). Oocytes were placed in each well of a four-well dish containing 500 μL of maturation medium covered with mineral oil and incubated at 38.5 °C with 5% CO_2 in air and humidity at saturation for 44 h for IVM [1].