Effect of porcine immature oocyte vitrification on oocyte-cumulus cell gap junctional intercellular communication.

Fahiel Casillas (fahiel@xanum.uam.mx)  
Universidad Autonoma Metropolitana Iztapalapa  
https://orcid.org/0000-0002-9916-0586

Yvonne Ducolomb  
Universidad Autonoma Metropolitana Iztapalapa

Alma López  
Universidad Autonoma Metropolitana Iztapalapa

Miguel Betancourt  
Universidad autonoma metropolitan iztapalapa

Short communication

Keywords: vitrification, porcine, oocyte, viability, maturation, gap junctions

DOI: https://doi.org/10.21203/rs.3.rs-76723/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Vitrication may severely affect cumulus cells and oocyte morphology and viability, limiting their maturation and developmental potential. The aim of this study was to evaluate the gap junction intercellular communication (GJIC) integrity after the vitrication of porcine immature cumulus-oocyte complexes (COCs). Fresh COCs were randomly distributed in two groups: untreated (control) and vitrified (vitrification), then subjected to in vitro maturation (IVM). Oocyte viability and IVM were measured in both groups. The evaluation of GJIC was expressed as relative fluorescence intensity (RFI). Vitrification significantly decreased oocyte viability and maturation after 44 h of culture compared to control. Also, significantly reduced RFI was observed in vitrified COCs at 4 and 8 h of culture compared to control. This study demonstrates that porcine oocyte viability and maturation after 44 h of culture were compromised after vitrication. GJIC was also affected after the vitrication of GV oocytes, being the possible mechanism by which oocyte maturation decreased.

Introduction

Oocyte cryopreservation has enabled significant advances in fertility treatments in humans and allows the genetic improvement of livestock. It has been reported that vitrication may severely affect cumulus cells (CCs) and oocyte morphology and viability, limiting their maturation and developmental potential [2]. Premature meiotic progression [1], low maturation [4, 5], and embryo development rates [3, 14] have been reported after the vitrication of porcine germinal vesicle (GV) immature oocytes. So far, only one study has reported the birth of live offspring from vitried GV oocytes in pigs [15].

Oocyte growth is related to the functionality of the somatic cells surrounding the oocyte, which are known as granulosa cells. These cells provide the oocyte essential nutrients for later stages: maturation, fertilization, and embryo development. CCs primary function is to regulate the mechanism of oocyte meiotic arrest and resumption. Maintaining the viability of the CCs is critical for oocyte maturation after vitrication. It has been reported that CCs viability is affected after vitrication [4], reducing oocyte maturation. Different types of cell communication have been described, such as tight junctions, desmosomes, cell adhesion molecules, and gap junction intercellular communication (GJIC). The latter establishes communication between CCs-oocytes. GJIC consists of arrays of intracellular channels that allow the exchange by passive diffusion of compounds < 1 KDa as metabolites, ions, sugars, second messengers, and water [13]. GJIC consists of membrane hemicannels called connexons, each of them composed of six transmembrane protein subunits called connexins. GJIC is directly involved in oocyte meiotic arrest and resumption. GJIC provide a direct communication by which cGMP enters the oocyte, inhibiting phosphodiesterase A, maintaining high levels of cAMP, and the meiotic arrest. For resumption, LH receptor activation in the CCs reduces the influx of cGMP by causing the GJIC closure and meiotic resumption [8]. The knowledge of the possible mechanisms involved in reducing GV oocyte maturation after vitrication is important for the development of more efficient vitrification protocols. Therefore, the aim of this study was to evaluate the GJIC integrity after the vitrication of immature porcine cumulus-oocyte complexes (COCs).
Experimental Design

Fresh COCs were randomly distributed in two groups: untreated (control) and vitrified (vitrification), then subjected to in vitro maturation (IVM). Before and after IVM, at 0 h and 44 h, oocyte viability was measured in both groups. For viability, at least three replicates were performed with n = 162 control and n = 128 vitrification, evaluated oocytes. For maturation, seven experiments were performed with n = 250 control and n = 175 vitrification, evaluated oocytes. GJIC integrity was evaluated at 4, 8, 22, and 44 h of culture. Experiments were carried out in triplicate with n = 80 control, n = 80 carbenoxolone, and n = 80 vitrification, evaluated oocytes.

Methods

Unless otherwise mentioned, all chemicals were acquired from Sigma Chemical Co. (St. Louis, MO, USA). For oocyte collection and IVM, ovaries were obtained from pre-pubertal Landrace gilts at a local slaughterhouse “Los Arcos”, located in Texcoco, State of Mexico (animal health federal law authorization 6265375) and transported to the laboratory in a 0.9% NaCl solution at 25 °C within 2 h after collection. Ovarian follicles between 3–6 mm in diameter were punctured to obtain COCs. For COCs collection and washing, Tyrode modified medium supplemented with 10 mM sodium lactate, 10 mM HEPES and 1 mg/mL polyvinyl alcohol (PVA) (TL-HEPES-PVA) was used. Only oocytes with uniform cytoplasm surrounded by a two-four-layer compact mass of CCs were selected. COCs were washed three times in 500 µL drops of maturation medium composed of TCM-199 with Earle’s salts and 26.2 mM sodium bicarbonate (In Vitro, Mexico City) and supplemented with 0.1% PVA, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine and 10 ng/mL EGF. For IVM, groups from 30 to 50 COCs were placed in each well of a four-well dish (Thermo-Scientific Nunc, Rochester NY) containing 500 µL of maturation medium supplemented with 0.5 µg/mL LH and 0.5 µg/mL FSH for 44 h [3]. Oocyte culture was performed under mineral oil and incubated at 38.5 °C in an atmosphere of 5% CO₂ in air and humidity at saturation.

For COCs vitrification and warming, the medium used was TCM-199-HEPES supplemented with 0.5 mM L-glutamine and 0.1% PVA (VW medium). For vitrification, oocytes were washed twice in VW medium and sequentially equilibrated in the first vitrification solution containing 7.5% dimethylsulphoxide (Me₂SO) + 7.5% ethylene glycol (EG) for 3 min, and 1 min in a second vitrification solution containing 16% Me₂SO, 16% EG and 0.4 M sucrose. Then, groups of seven oocytes were placed in a 2 µL drop of the second vitrification solution and loaded into the Cryolock device (Importadora Mexicana de Materiales para Reproduccion Asistida S. A. de C.V., Mexico) in less than 1 min. Then, the Cryolock device was immediately plunged horizontally into liquid nitrogen and stored during 30 min [3]. Warming was performed by the one-step method [10]. For oocyte recovery, the Cryolock device was submerged vertically in a four-well dish containing 800 µL of VW medium supplemented with 0.13 M sucrose. Finally, oocytes were incubated in VW medium for 5 min, then recovered and transferred to maturation medium.

Oocyte viability was measured at T 0 h = immediately after COCs collection or vitrification and at T 44 h = after IVM. Oocytes were stained with 100 µL of 0.5 mg/mL Thiazolyl blue (MTT) diluted in PBS. After 1 h,
Oocytes were analyzed under a light microscope (Zeiss Axiostar) and classified as viable (purple-stained) and non-viable (colorless). To evaluate IVM, oocytes were stained with 10 µg/mL bisbenzimide (Hoechst 33342) diluted in PBS for 45 min and evaluated using an epifluorescence microscope (Zeiss Axiostar) at 40 X. Oocytes showing a GV, or in the metaphase I (MI) were considered immature, and those in the metaphase II (MII) with the first polar body as matured. After COCs vitrification, GJIC transfer between CCs-oocyte was evaluated using the acetoxyethylmethyl (AM) ester derivative of the fluorescent indicator calcein (calcein-AM; Molecular Probes, Eugene, OR) method [12]. After 4, 8, 22 and 44 h of culture, COCs were exposed to calcein-AM for 15 min, then were transferred to calcein-AM free media and cultured for 25 min, allowing the dye exchange between CCs-oocyte. Then, COCs were washed once in 0.01% (w/v) PVA and PBS and were analyzed under a confocal epifluorescence microscope (Zeiss, LSM T-PMT). Fluorescence intensity (FI) profiles of calcein in CCs-oocytes were obtained using the Zen blue lite 2.3 software. Carbenoxolone, a gap junction blocker, was used at 100 µM as a positive control [17].

**Statistical Analysis**

The percentage of oocyte viability, maturation, and FI in control and vitrified groups were compared by the Student’s T-Test with a confidence level of $P<0.05$. Data are presented as mean ± SD.

**Results**

Oocyte viability at T 0 h was not statistically different from control ($P>0.05$). However, vitrification significantly decreased oocyte viability after 44 h of culture compared to control ($P<0.05$), as shown in Fig. 1A. After vitrification, the MI stage was not affected compared to control. However, a significantly high percentage of GV oocytes was observed after vitrification compared to control. Consequently, the percentage of MII oocytes significantly decreased after vitrification compared to control (Fig. 1B). The evaluation of GJIC was performed after 4, 8, 22, and 44 h of culture, and it was expressed as relative fluorescence intensity (RFI), as shown in Fig. 2. Carbenoxolone was used as a positive control of GJIC blockage. In control, significantly high RFI was observed at 4 and 8 h, which was decreased at 22 and 44 h. After vitrification, significantly reduced RFI was observed in COCs at 4 and 8 h compared to control. At 22 and 44 h, reduced RFI was observed in all groups (Fig. 2). Representative images of GJIC evaluation after vitrification are shown in Fig. 3. CCs-oocyte GJIC was measured by the quantitative FI profiles of calcein in COCs (Fig. 3A). In control, results showed that high FI is observed in the CCs-oocyte at 4 and 8 h (red lines). In the carbenoxolone group, high FI was observed in the CCs but not in the oocyte at 4 and 8 h (red crossed lines). In the vitrification group, reduced FI was observed in the CCs-oocyte at 4 and 8 h. Also, in control and vitrification groups, reduced FI was observed in CCs-oocyte at 22 and 44 h (Fig. 3A). For all groups, FI profiles were measured in COCs using the confocal microscope software, as shown in Fig. 3B.

**Discussion And Conclusion**
The present study demonstrates that porcine immature oocyte viability was not affected immediately after vitrification, but it was decreased after 44 of culture. These results confirm those previously reported [6], where the viability of pig oocytes decreases after IVM. This could be explained by the fact that during in vitro culture, the levels of \( O_2 \) and reactive oxygen species increase, affecting cell viability. Likewise, in the GV stage, oocytes are highly cryo-sensitive during the vitrification process compared to other developmental stages. Porcine oocytes vitrified in the GV stage, present a high intracellular lipid content, which prevents the permeability and protection capacity of intracellular cryoprotectants. Therefore, a high amount of intracellular lipids affects glutathione levels in vitrified oocytes, which increases the production of \( H_2O_2 \) and the generation of oxidative stress [16]. Also, GV oocyte meiotic spindle organization, chromosome and actin filaments distribution has been reported to be extremely sensitive to low temperatures, compared with MII oocytes [9]. Our results demonstrate that oocyte maturation was significantly affected after vitrification. GJIC is directly involved in oocyte meiotic arrest and resumption. In the present study, we evaluated the integrity of GJIC after vitrification using the calcein-AM transfer assay. As far as we know, information about the integrity of GJIC after the vitrification of porcine GV oocytes is limited. In porcine, using transmission electron microscopy, a study reported that gap junctions and microvilli were ruptured after GV oocyte vitrification [18]. Also, in feline GV oocytes, GJIC was evaluated by the lucifer yellow dye microinjection. They reported that vitrification by slow freezing or vitrification impairs intercellular junctions, which resulted in low oocyte maturation (32.5 and 14.1%, respectively) [7]. Our results showed that vitrification impairs GJIC between the CCs-oocyte, reducing the IVM rates. GJIC functionality was observed in control oocytes at 4 and 8 h, in order to promote meiotic resumption. It was reported that GJIC functionality is critical during the first 4.5 h of IVM for meiotic resumption [11]. However, GJIC was significantly compromised after vitrification compared to control. Therefore, in the present study, it was demonstrated that GJIC was affected after vitrification, and as a consequence, oocyte IVM decreased. This study demonstrates that porcine oocyte viability and maturation after 44 h of culture were compromised after vitrification. GJIC was also affected after the vitrification of GV oocytes, being the possible mechanism by which oocyte maturation decreased.

**Declarations**

**Acknowledgements**

The authors wish to thank M.Sc. Alejandro Domínguez for technical assistance, and the slaughterhouse “Los Arcos”, State of Mexico for the donation of porcine ovaries.

**Authors contributions**

All authors contributed to the design of the study. FC did the experimental work, statistical analysis, analysis of the results, and wrote the manuscript. YD, AL, and MB analyzed the results, revised, corrected and approved the manuscript.

**Ethics approval and consent to participate**
This study was performed under the Ethics Committee regulations for the care and use of animals; Metropolitan Autonomous University-Iztapalapa campus.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests to declare

References

[1] Appeltant R, Somfai T, Santos ECS, Dang-Nguyen TQ, Nagai T, Kikuchi K. Effects of vitrification of cumulus-enclosed porcine oocytes at the germinal vesicle stage on cumulus expansion, nuclear progression and cytoplasmic maturation. Reprod Fertil Dev. 2017;29:2419–2429.

[2] Brambillasca F, Guglielmo MC, Coticchio G, Renzini MM, Del Canto M, Fadini R. The current challenges to efficient immature oocyte cryopreservation. J Assist Reprod Genet. 2013;30:1531–1539.

[3] Casillas F, Betancourt M, Cuello C, Ducolomb Y, López A, Juárez-Rojas L, et al. An efficiency comparison of different in vitro fertilization methods: IVF, ICSI and PICSI for embryo development to the blastocyst stage from vitrified porcine immature oocytes. Porcine Health Manag. 2018;4:16.

[4] Casillas F, Teteltitla-Silvestre M, Ducolomb Y, Lemus AE, Salazar Z, Casas E, et al. Co-culture with granulosa cells improve the in vitro maturation ability of porcine immature oocytes vitrified with Cryolock. Cryobiology. 2014;69:299–304.

[5] Casillas F, Ducolomb Y, Lemus AE, Cuello C, Betancourt M. Porcine embryo production following in vitro fertilization and intracytoplasmic sperm injection from vitrified immature oocytes matured with a granulosa cell co-culture system. Cryobiology. 2015;71:299–305.

[6] Fernández-Reyes F, Ducolomb Y, Romo S, Casas E, Salazar Z, Betancourt M. Viability, maturation and embryo development in vitro of vitrified immature and porcine oocytes. Cryobiology. 2012;64:261-266.

[7] Luciano AM, Chigioni S, Lodde V, Franciosi F, Luvoni GC, Modina SC. Effect of different cryopreservation protocols on cytoskeleton and gap junction mediated communication integrity in feline germinal vesicle stage oocytes. Cryobiology. 2009;59 90-95.

[8] Norris RP, Freudzon M, Mehlmann LM, Cowan AE, Simon AM, Paul DL, et al. Luteinizing hormone causes MAPK-dependent phosphorylation and closure of Cx43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development. 2008;135:3229–3238.
[9] Rojas C, Palom MJ, Albarracin JL, Mogas T. Vitrification of immature and in vitro matured pig oocytes: study of distribution of chromosomes, microtubules, and actin microfilaments. Cryobiology. 2004;49:211–20.

[10] Sánchez-Osorio J, Cuello C, Gil MA, Parrilla I, Maside C, Almiñana C, et al. Vitrification and warming of in vivo- derived porcine embryos in a chemically defined medium. Theriogenology. 2010;73:300–308.

[11] Santiquet NW, Develle Y, Laroche A, Robert C, Richard FJ. Regulation of gap-junctional communication between cumulus cells during in vitro maturation in swine, a gap-FRAP study. Biol Reprod. 2012;87:46.

[12] Sasseville M, Gagnon MC, Guillemette C, Sullivan R, Gilchrist RB, Richard FJ. Regulation of gap junctions in porcine cumulus-oocyte complexes: contributions of granulosa cell contact, gonadotropins, and lipid rafts. Mol Endocrinol. 2009;23:700-710.

[13] Sohl G, Willecke K. Gap junctions and the connexin protein family. Cardiov Res. 2004;62:228-232.

[14] Somfai T, Nguyen HT, Nguyen MT, Dang-Nguyen TQ, Kaneko H, Noguchi J, et al. Vitrification of porcine cumulus-oocyte complexes at the germinal vesicle stage does not trigger apoptosis in oocytes and early embryos, but activates anti-apoptotic Bcl-XL gene expression beyond the 4-cell stage. J Reprod Dev. 2020;66:115-123.

[15] Somfai T, Yoshioka K, Tanihara F, Kaneko H, Noguchi J, Kashiwazaki N, et al. Generation of live piglets from cryopreserved oocytes for the first time using a defined system for in vitro embryo production. PLoS One. 2014;9:e97731.

[16] Somfai T, Ozawa M, Noguchi J, Kaneko H, Karja NWK, Farhudin M, et al. Developmental competence of in vitro-fertilized porcine oocytes after in vitro maturation and solid surface vitrification: effect of cryopreservation on oocyte antioxidative system and cell cycle stage. Cryobiology. 2007;55:115–26.

[17] Thomas RE, Armstrong DT, Gilchrist RB. Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3’,5’-monophosphosphate levels. Biol Reprod. 2004;70:548-556.

[18] Wu C, Rui R, Dai J, Zhang C, Ju S, Xiao-Lu BX, et al. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. Mol Rep Dev. 2006;73:1454-1462.

**Figures**
Figure 1

Effect of vitrification on oocyte viability and in vitro maturation. (A) Percentage of oocyte viability at T 0 h (immediately after vitrification) and after 44 h of culture. Oocyte viability decreased after vitrification after 44 h of culture. (B) Percentage of oocytes in different developmental stages after 44 h of culture. Oocyte maturation (MII stage) decreased after vitrification. GV= germinal vesicle; MI= metaphase I; MII= metaphase 2. Bars show the mean SD. * Significant differences vs. control.

Figure 2

Effect of vitrification on oocyte viability and in vitro maturation. (A) Percentage of oocyte viability at T 0 h (immediately after vitrification) and after 44 h of culture. Oocyte viability decreased after vitrification after
44 h of culture. (B) Percentage of oocytes in different developmental stages after 44 h of culture. Oocyte maturation (MII stage) decreased after vitrification. GV= germinal vesicle; MI= metaphase I; MII= metaphase 2. Bars show the mean SD. * Significant differences vs. control.

Figure 3

Representative images of GJIC evaluation after COCs vitrification. (A) Cumulus cell-oocyte GJIC was measured by the quantitative FI profiles of calcein in COCs. High FI was observed in the CCs and oocyte at 4 and 8 h (red lines). Reduced FI was observed in carbenoxolone and vitrification groups after 4 and 8 h. Carbenoxolone was used as a positive control of GJIC blockage (red crossed lines). (B) Representative FI profiles in COCs from all groups. Graphs show the FI profiles of calcein (marked in green) transferred from the CCs to the oocyte. GJIC= gap junction intercellular communication; FI= fluorescence intensity. Scale bar= 30 µM.