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Porcine embryo production following in vitro fertilization and intracytoplasmic sperm injection from vitrified immature oocytes matured with a granulosa cell co-culture system

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ABSTRACT

This study was designed to evaluate the capacity of vitrified–warmed porcine immature oocytes to mature and to be fertilized using in vitro fertilization or intracytoplasmic sperm injection, and to determine the subsequent embryo development. Immature oocytes were vitrified using ethylene glycol and dimethylsulphoxide as cryoprotectants and the Cryolock method. After warming oocytes were cultured 44 h for maturation. Oocytes were randomly distributed in three treatment groups and subjected to in vitro fertilization (Experiment 1) or intracytoplasmic sperm injection (Experiment 2) procedures. The results indicate that the embryo development was higher in denuded oocytes co-cultured with granulosa cells (NkO-CC group) fertilized by in vitro fertilization or intracytoplasmic sperm injection compared to cumulus-cell oocyte complexes (COCs group), showing no significant differences with control. Vitrified denuded oocytes matured with a co-culture system NkO-CC group, displayed higher cleavage rate and blastocyst production than vitrified COCs group. Blastocysts were successfully obtained after IVF and ICSI procedures; however, the development to the blastocyst stage was better after IVF. These results show that the vitrification–warming media, the employment of a granulosa cell co-culture system and the Cryolock method during vitrification, increased the nuclear and cytoplasmic maturation of vitrified porcine immature oocytes. Further experiments are required to enhance porcine embryo production after vitrification.

1. Introduction

The establishment of oocyte cryopreservation procedures is of great importance in human assisted reproduction techniques, animal production for fertility and genetic improvement, including endangered animal species [8,11]. Successful vitrification can be determined by the meiotic stage of the oocyte. The oocyte developmental stage is considered a critical factor for its survival and developmental competence after cryopreservation [40]. In comparison with other mammals, porcine oocytes have shown to be more difficult to cryopreserve in the germinal vesicle stage (GV) [26,32,45]. This fact has been attributed to their high lipid content [34], which decreases in the following developmental stages. Mature oocytes display higher survival rates after vitrification than GV oocytes, but they often show spindle abnormalities, reducing their fertilization and developmental competence [38]. Although GV oocytes have low survival rates, they have the ability to undergo nuclear and cytoplasmic maturation to continue embryo development (ED). In regard in vitro embryo vitrification, blastocysts have been successfully cryopreserved with high survival and re-expansion rates [6,33]. However, in vitro produced blastocysts present higher DNA fragmentation and a lower total

Abbreviations: BSA, bovine serum albumin; CC, cumulus cells; CPAs, cryoprotectant agents; COCs, cumulus-cell oocyte complexes; ED, embryo development; EG, ethylene glycol; EGF, epidermal growth factor; GV, germinal vesicle stage; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; IVM, in vitro maturation; Me_2SO, dimethylsulphoxide; mTBM, modified Tris-buffered-medium; MTT, thiazolyl blue; NkO-CC, oocytes vitrified without cumulus cells and matured in co-culture with granulosa cells; PB, polar body; PN, pronuclei; PVA, polyvinyl alcohol; PVP, polyvinylpyrrolidone; TCM-199, tissue culture medium 199; V–W medium, vitrification–warming medium; ZP, zona pellucida.

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cell number than in vivo generated blastocysts [31]. In the present study we highlight that the vitrification of GV oocytes has more advantages for assisted reproduction techniques than other stages.

In a previous study [5] it was demonstrated that vitrified porcine immature oocytes were able to preserve survival up to 90%, and 49% were able to mature in vitro. These results were obtained using a co-culture with homologous granulosa cells. It was also reported that granulosa cells in co-culture with the oocytes after vitrification, seem to recognize and surround oocytes improving in vitro maturation (IVM). Therefore, maintaining their viability is essential for IVM.

In vitro models are useful tools to understand the effects and mechanisms involved during vitrification. Also the porcine model was chosen because of its reproductive and endocrine systems are very similar to those of humans [10,12].

For assisted reproduction technologies, in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) were developed in order to improve fertilization and ED. The IVF technique was developed in 1940. But it was not until 1978 when Steptoe and Edwards established greater details of the IVF process, resulting in the first human IVF baby born [46]. After this, several studies have shown fertilization and outcome success in different species [9,17,25,27,37,44]. However, in some domestic species its use has currently decreased since it has the disadvantage of not excluding events as polyspermy, which causes aberrant development of the early embryo and difficulty completing gestation [24]. In swine, polyspermy by in vitro fertilization occurs in high incidence (50%), whereas in vivo is between 30% and 40% [50], reducing the percentages of monospermy and the possibility to obtain live offspring. In 1992, ICSI arose to solve this issue [35]. However, by ICSI, results have not been as expected because procedure failures in the oocyte activation, sperm head decondensation, sperm ejection, and zona pellucida (ZP) rupture have been reported [7,14,23,29,43]. One of the main vitrification problems is the hardening of the ZP and cytoplasmic membrane [49] affecting the sperm-oocyte recognition during IVF. For this reason we hypothesized that the ICSI procedure could solve some of the vitrification problems. Therefore, the aim of the present study was to evaluate the capacity of vitrified-warmed porcine immature oocytes to mature and to be fertilized using IVF or ICSI and to determine the subsequent ED.

2. Materials and methods

2.1. Experimental design

2.1.1. Experiment 1: embryo development obtained with vitrified immature oocytes after IVM and IVF

Nine replicates were performed to evaluate the ED after IVF. After collection, COCs were randomly allocated in the following treatment groups: (1) non-vitrified fresh oocytes (control group, $n = 373$); (2) COCs vitrified–warmed (COCs group, $n = 288$); (3) COCs denuded before vitrification, vitrified–warmed and matured in the granulosa cell co-culture system. (NkO-CC group, $n = 327$). After IVM matured oocytes derived from each experimental group were subjected to conventional IVF and cultured as described below.

2.1.2. Experiment 2: embryo development obtained with vitrified immature oocytes after IVM and ICSI

Five replicates were performed. After collection, COCs were randomly allocated in the following treatment groups: (1) non-vitrified fresh oocytes (control group, $n = 161$); (2) COCs vitrified–warmed (COCs group, $n = 125$); (3) COCs denuded before vitrification, vitrified–warmed and matured in the granulosa cell co-culture system (NkO-CC group, $n = 136$). After IVM, matured oocytes derived from each experimental group were subjected to ICSI and cultured as described below.

2.2. Chemicals

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

2.3. Culture conditions

Oocytes maturation, gametes co-incubation and embryos cultured were performed under mineral oil and incubated at 38.5 °C in an atmosphere of 5% CO_2 in air and humidity at saturation.

2.4. Oocyte collection and IVM

Ovaries were obtained from pre-pubertal gilts at a local slaughterhouse (“Los Arcos”, Edo. de Mexico) and transported to the laboratory in 0.9% NaCl solution at 25 °C within 2 h after collection. Ovarian follicles between 3 and 6 mm in diameter were aspirated using an 18-gauge needle fixed to a 10 mL disposable syringe to obtain the cumulus-cell oocyte complexes (COCs). Tyrode's modified medium supplemented with 10 mM sodium lactate, 10 mM HEPES and 1 mg/mL polyvinyl alcohol (PVA) (TL-HEPES-PVA) was used for COCs collection and washing [2]. Oocytes with uniform cytoplasm surrounded by a two-four layer compact mass of cumulus cells (CC) were selected for culture. Then, COCs were washed three times in 500 μL drops of maturation medium composed of TCM-199 with Earlés salts and 26.2 mM sodium bicarbonate (in vitro, Mexico City) 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 maturation, groups from 30 to 40 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 [2].

2.5. Vitrification and warming

The basic medium for vitrification and warming (V–W medium) was TCM-199-HEPES supplemented with 0.5 mM L-glutamine and 0.1% PVA. For vitrification, oocytes were washed twice in V–W medium and sequentially equilibrated in the first vitrification solution containing 7.5% dimethylsulphoxide (Me_2SO) and 7.5% ethylene glycol (EG) for 3 and 1 min in a second vitrification solution containing 16% Me_2SO, 16% EG and 0.4 M sucrose. Groups of six to seven oocytes were placed in a 1.5–2 μL drop of the second solution and loaded into Cryolock (Importadora Mexicana de Materiales para Reproduccion Asistida S. A. de C.V., Mexico City) in less than 1 min; then they were immediately plunged horizontally into liquid nitrogen and stored during 30 min (Cryolock method) [5].

Oocytes were warmed using the one-step method [41]. Briefly, the Cryolock was submerged vertically in a four-well dish containing 800 μL of V–W medium supplemented with 0.4 M sucrose to recover the oocytes. The warmed oocytes were incubated in this medium for 5 min and then recovered and transferred to IVM medium [40,41].

After 44 h of IVM control and vitrified oocytes were assessed by stereomicroscopy. Oocytes displaying signs of degeneration, such as lysed cytoplasmic membrane, were discarded and were not used for IVF procedures.

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