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Effects of two combinations of cryoprotectants on the *in vitro* developmental capacity of vitrified immature porcine oocytes

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ABSTRACT

This study evaluated two cryoprotectant (CPA) combinations, ethylene glycol (EG) + DMSO and EG + propylene glycol (PG), used for the vitrification of germinal vesicle (GV) porcine oocytes. In experiment 1, the equilibration of GV with the two CPA combinations increased ($P < 0.05$) the percentage of oocytes that degenerated after IVM (18.1 ± 2.3% and 19.4 ± 2.6% for EG + DMSO and EG + PG groups, respectively) compared with control oocytes (7.6 ± 1.3%). However, CPAs did not affect the fertilization or developmental parameters of the embryos. In experiment 2, the percentages of live vitrified–warmed GV oocytes at 2 hours after warming (EG + DMSO: 67.0 ± 2.3% and EG + PG: 57.6 ± 2.3%) were lower than those of fresh control GV oocytes (97.3 ± 0.7%). The percentage of degenerated oocytes after IVM was higher ($P < 0.001$) in vitrified–warmed oocytes (EG + DMSO: 59.8 ± 2.3% and EG + PG: 56.2 ± 2.6%) than in the control (1.6 ± 1.3). Fertilization efficiency was higher ($P < 0.05$) in the EG + PG (39.6 ± 2.4%) and control (42.0 ± 2.2%) groups than in the EG + DMSO (26.3 ± 7.7%) group. The cleavage and blastocyst formation rates of the EG + DMSO (25.9 ± 3.5% and 6.6 ± 2.5%, respectively) and EG + PG (20.2 ± 5.4% and 4.7 ± 1.6%, respectively) vitrification groups were lower ($P < 0.001$) than those observed in the control oocytes (53.4 ± 2.7% and 31.9 ± 1.7%, respectively). In conclusion, in the absence of vitrification, the toxic effects of both CPA combinations on the GV oocytes were minimal. Vitrification resulted in important losses in viability at each step of the *in vitro* embryo production procedure. However, the surviving oocytes were able to mature and be fertilized, although the fertilization efficiency in the EG + DMSO group was lower. Blastocysts formation was similar for both CPA combinations.

1. Introduction

Successful cryopreservation of pig oocytes is crucial for the preservation and management of valuable genetic resources. In addition, oocyte cryopreservation could be important for the application of reproductive technologies, such as *in vitro* embryo production, genetic engineering, and nuclear transfer [1]. Unfortunately, oocyte cryopreservation in pigs is very difficult compared with other domestic species [2]. The greater hypothermic sensitivity of porcine oocytes has been ascribed to the large amount of cytoplasmic lipids [3]; fatty acid quantity in immature porcine oocytes is twice that in bovines and even threefold greater than in sheep [4].

Successful cryopreservation of porcine oocytes was first achieved by Isachenko et al. [5], and since then, much research has been focused on improving the cryopreservation protocols for this species. Currently, vitrification

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appears to be the best alternative for cryopreserving oocytes; ultrarapid vitrification procedures result in high cooling rates during vitrification, which reduces the adverse effects associated with high concentrations of cryoprotectants (CPAs) and decreases injury from chilling [3]. Development to the blastocyst stage has been achieved in mature and immature porcine oocytes that have been vitrified using the solid-surface vitrification [1,6], open pulled straw [7], and Cryotop [8] methods. The meiotic stage highly influences the ability of porcine oocytes to be vitrified, and metaphase II (MII) oocytes are traditionally preferred for vitrification. Although the survival of oocytes matured *in vitro* after vitrification is relatively high, their fertilization and developmental competence are seriously compromised [9,10]. Previous investigations have revealed a failure in male pronuclear formation in vitrified MII oocytes after fertilization [11]. Vitrification at the MII stage also induces parthenogenesis and reduces cytoplasmic glutathione levels [11].

In contrast, oocytes vitrified as cumulus cell–oocyte complexes (COCs) at the germinal vesicle (GV) stage display low survival rates but maintain their capacity to undergo normal fertilization [9]. In addition, vitrification at this stage may avoid spindle damage [10], which is one of the most common alterations observed in vitrified MII oocytes [12]. Although live offspring have been obtained from vitrified immature oocytes [13], the total yield of blastocyst stage embryos remains very low.

One of the factors limiting the vitrification of immature oocytes is their lower permeability to CPAs compared with matured oocytes [14,15], which may result in low survival rates. Furthermore, the presence of cumulus cells affects permeability, decreasing the efficiency of vitrification protocols [16]. Denudation is not an option for addressing this problem because cumulus cells are important for the cytoplasmic maturation of oocytes [17,18] and contribute to mitochondrial functions [19]. Therefore, the selection of highly permeable combinations of CPAs should be the strategy used to overcome this limitation. Combining CPAs has the advantage of reducing the total CPA concentration needed for vitrification, which reduces the toxicity of the vitrification solution.

The permeating CPAs most commonly used for oocyte cryopreservation are ethylene glycol (EG), glycerol [20], DMSO [1], propylene glycol (PG) [13], and acetamide [21]. Among them, EG, which has high permeability and low toxicity, has been shown to be the most effective CPA for porcine oocytes [22,23] and has been used alone [23] or in combination with DMSO [24,25]. Although the combination of EG and DMSO has been shown to be very effective in the vitrification of porcine embryos [26], DMSO detrimentally affects the meiotic competence of GV stage porcine [1] and murine [27] oocytes. These investigations suggest that DMSO may not be adequate for oocyte vitrification; thus, PG could be an appropriate substitute for DMSO. This CPA is more permeable than DMSO and EG in both porcine oocytes and embryos [28]. The replacement of DMSO with PG would decrease toxicity and increase the permeability of vitrification solutions, which may improve the efficiency of the vitrification of immature porcine oocytes.

Therefore, this study aimed to assess the effectiveness of two combinations of CPAs, EG + DMSO and EG + PG, for the vitrification of GV stage porcine oocytes. We investigated the viability, fertilization, and developmental competence of oocytes vitrified using both combinations of CPAs. The vitrification study was preceded by a CPA toxicity experiment.

2. Materials and methods

All the experimental procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments and were reviewed and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain (research code: 638/2012).

2.1. Chemicals and media

All chemicals used in this study were purchased from Sigma–Aldrich Co. (Alcobendas, Madrid, Spain) unless indicated otherwise. The medium used to transport ovaries from the slaughterhouse to the laboratory was a 0.9% (wt/vol) NaCl saline solution containing 70 μg/mL of kanamycin. The collection and washing of COCs were performed using a modified Dulbecco's PBS medium (mDPBS) composed of 136.89-mM NaCl, 2.68-mM KCl, 8.1-mM Na_2HPO_4, and 1.46-mM $CaCl_2.2H_2O$, which was supplemented with 4 mg/mL of BSA, 0.34-mM sodium pyruvate, 5.4-mM D-glucose, and 70 μg/mL of kanamycin. The oocyte maturation medium was TCM-199 (Gibco Life Technologies S.A., Barcelona, Spain) supplemented with 0.57-mM cysteine, 0.1% (wt/vol) polyvinyl alcohol (PVA), 10 ng/mL of EGF, 75 mg/mL of penicillin G potassium, and 50 mg/mL of streptomycin sulfate. The basic medium used for IVF was the same as that used by Abeydeera and Day [29], which was designated as modified Tris-buffered medium and consisted of 113.1-mM NaCl, 3-mM KCl, 7.5-mM $CaCl_2.2H_2O$, 20-mM Tris (Trizma Base), 11-mM D-glucose, and 5-mM sodium pyruvate supplemented with 2-mM caffeine and 0.2% BSA. The embryo culture medium was the North Carolina State University (NCSU-23) medium [30] supplemented with 0.4% (wt/vol) BSA. All vitrification and warming solutions were chemically defined media. The basic medium for vitrification and warming (TL-PVA) was a modification of the protein-free Tyrode medium [31] composed of 124.3-mM NaCl, 3.2-mM KCl, 2-mM $NaHCO_3$, 0.34-mM KH_2PO_4, 10-mM sodium lactate, 0.5-mM $MgCl_2.6H_2O$, 2-mM $CaCl_2.2H_2O$, 10-mM HEPES, 0.2-mM sodium pyruvate, 12-mM sorbitol, 0.1% (wt/vol) PVA, 75 μg/mL of potassium penicillin G, and 50 μg/mL of streptomycin sulfate. The first vitrification medium (V1) was TL-PVA containing 7.5% (v:v) of each CPA, and the second vitrification medium (V2) was TL-PVA containing 16% (v:v) of each CPA and 0.4-M sucrose. The warming medium consisted of TL-PVA supplemented with 0.13-M sucrose.

2.2. Oocyte collection and oocyte in vitro maturation

Ovaries were obtained from prepubertal gilts at a local slaughterhouse (El Pozo S.A., Alhama, Murcia, Spain) and transported to the laboratory in medium at 35 °C. Oocytes