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Vitrification of porcine immature oocytes: Association of equilibration manners with warming procedures, and permeating cryoprotectants effects under two temperatures

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ABSTRACT

The aim of this study was to evaluate the association of equilibration manners with warming procedures, and the different permeating cryoprotectants (pCPAs) effects under two temperatures, in terms of survival, maturation and subsequent parthenogenetic development of porcine immature oocytes after Cryotop vitrification. In Experiment 1, oocytes were equilibrated by exposure to 5% (v/v) ethylene glycol (EG) for 10 min (EM1) or stepwise to 7.5% (v/v) and 15% (v/v) EG for 2.5 min respectively (EM2). Warming procedures were performed in 1.0 M sucrose for 1 min, then in 0.5 and 0.25 M sucrose for 2.5 min respectively (WP1), or in 0.5, 0.25 and 0.125 M sucrose each step for 2 min (WP2), or in 0.25, 0.125 and 0.063 M sucrose each step for 2 min (WP3). After 2 h of warming, the survival rate of oocytes treated by EM1 and WP1 was significantly higher ($P < 0.05$) than that of the other groups. Moreover, a similar proportion of survival and nuclear maturation in all vitrified groups was obtained after completion of the IVM. No significant difference in blastocyst development was observed among vitrified groups except the group treated by EM2 and WP3. In Experiment 2, oocytes were vitrified by using EG alone, EG combined with dimethyl sulphoxide (EG + DMSO) or propylene glycol (EG + PROH) as pCPAs under 25 °C and 39 °C. The percentages of cryosurvival and nuclear maturation were similar in all vitrified groups. Under 25 °C, the embryo development and total cell numbers of blastocysts were not significantly different among EG, EG + DMSO and EG + PROH groups. However, the application of EG + PROH at 39 °C resulted in significantly decreased both cleavage and blastocyst formation rates. In conclusion, our data showed that equilibration manner and warming procedure affect the cryosurvival of porcine immature oocytes, and the combination of pCPAs cannot give a better cryopreservation outcome whether 25 °C or 39 °C. Notably, the Cryotop vitrification accompanied by our modified strategy for porcine immature oocytes could achieve high survival and respectable blastocyst production.

1. Introduction

In pigs, successful cryopreservation of oocytes has a great importance in genetic resources preservation and assisted reproductive technologies [42]. Because the porcine oocyte is the most sensitive to low temperature, it has been difficult to cryopreserve as compared to the oocyte of other domestic animals [26]. Fortunately, with progress in the techniques of vitrification, the efficacy of porcine oocyte cryopreservation has improved dramatically in recent years. This is particularly true for embryo development of porcine oocytes vitrified at the immature germinal vesicle (GV) stage being significantly better than that of those vitrified at the mature metaphase II (MII) stage [12,40], and live piglets have also been generated from vitrified GV oocytes [35]. Nevertheless, there is no standard vitrification procedure applied to porcine GV oocyte and the cryosurvival currently available is unstable from laboratory to laboratory [37]. Moreover, blastocyst production of porcine GV oocytes was still severely compromised by the vitrification [34], and continued efforts must be made to optimize the vitrification

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procedure.

Factors which affect the oocyte cryopreservation are associated with maturational stages, vitrification solution components, equilibration manners and warming procedures, etc. [1,3,39]. Often the equilibration manner was performed by single-step or gradual stepwise addition of the permeating cryoprotectants (pCPAs). It has been recently shown that cryosurvival of porcine GV oocytes is also influenced by the concentration of pCPAs during equilibration [37]. On the other hand, the warming procedure is a critical factor for survival of vitrified oocytes. The initial concentration of sugars during the dilution of pCPAs is inconsistent, but few studies have been conducted to compare their difference in performance. Moreover, no reports are currently available on the relationship between equilibration manner and warming procedure.

It is generally agreed that the two main contributory factors are necessary to successfully achieve vitrification, one of which is rapid cooling and warming rates and the other a high concentration of CPAs [21]. Therefore, a variety of vitrification carriers have been developed especially for the improvement of cooling and warming rates, and corresponding vitrification methods are also produced based on the carriers such as open pulled straw, Cryotop, Cryoloop, Cryolock, spatula, solid surface vitrification (SSV) methods, etc. [30]. Also, these methods differ in the process operation and performance or the using of vitrification solution, even with carrier of the same type. The vitrification solution contains different types and concentrations of both pCPAs and non-permeating CPAs. Ethylene glycol (EG), dimethyl sulphoxide (DMSO) and propylene glycol (PROH) are the three major conventional pCPAs extensively used in vitrification. The EG is a key component of vitrification solution and has been the most commonly utilized alone or in combination with either DMSO or PROH, as it shows low toxicity and high permeability [5]. Nevertheless, these pCPAs inevitably cause both toxic and osmotic damage to the oocytes, which is strongly associated with their concentration and temperature of use [2]. In pigs, the temperature condition at which oocytes are exposed to equilibration solution and vitrification solution is usually set at room temperature (about 25 °C) or physiological temperature (about 39 °C). However, it still remains unclear for vitrifying porcine GV oocytes, which of pCPAs components shows stronger effectiveness under different temperature conditions.

The aim of the present study was therefore to evaluate the association between equilibration manners and warming procedures and the different pCPAs effects under two temperatures, in terms of survival, maturation and subsequent development of porcine GV oocytes submitted to vitrification by Cryotop method.

2. Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) except for those specifically mentioned. Cryotop was purchased from Kitazato Biopharma (Shizuoka, Japan).

2.1. Oocyte collection and in vitro maturation

Pre-pubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory within 2 h in 0.9% NaCl (w/v) containing 75 mg/L penicillin G potassium and 50 mg/L streptomycin sulphate at 35–37 °C. The ovaries were washed twice with this solution and kept at 36 °C. Follicles (3–8 mm in diameter) were punctured using a disposable syringe with 18 gauge needle and follicular contents were allowed to sediment in a 50 mL conical tube. The precipitate was re-suspended with Tyrode's lactate-HEPES-polyvinyl alcohol medium (TLH-PVA) [16] and then observed under a stereomicroscope (Olympus, Tokyo, Japan) for cumulus-oocyte complexes (COCs) collection. Only oocytes with uniform cytoplasm and compact cumulus cells were selected and washed three times in *in vitro* maturation (IVM) medium. The IVM medium was TCM199 supplemented with 10% (v/v) porcine follicular fluid (pFF), 3.05 mM D-glucose, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/mL epidermal growth factor, 0.01 units/mL each follicle-stimulating hormone and luteinizing hormone (Sioux Biochemicals, Sioux City, IA, USA). Groups of 50–70 COCs were placed in each well of a 24-well plates (Costar, Corning, NY) containing 500 μL of IVM medium and incubated for 42–44 h at 39 °C in an atmosphere of 5% CO_2 with saturated humidity.

2.2. Vitrification and warming of oocytes

Porcine GV oocytes were subjected to vitrification using the Cryotop method as previously described [22] with some modifications, in a laboratory maintained at 25 ± 1 °C. All solutions for vitrification and warming were prepared using Dulbecco's phosphate buffered saline (DPBS) supplemented with 20% (v/v) synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) as the basal medium (BM). For vitrification, oocytes were incubated in BM for 3 min and then equilibrated according to the experimental design. Thereafter, the equilibrated oocytes in groups of 10–15 were exposed to vitrification solution containing 0.6 M sucrose, 50 mg/mL polyvinylpyrrolidone and 35% (v/v) pCPAs (35% EG, 17.5% EG + 17.5% DMSO, or 17.5% EG + 17.5% PROH, depending on the experimental design) in BM for 20–30 s and then were loaded onto the tip of the Cryotop with the minimum volume of vitrification solution. The Cryotop was immediately plunged into liquid nitrogen, protected with a plastic cap, and stored for at least 1 week. For warming, after removal of the plastic cap, the Cryotop tip with loaded oocytes were rapidly dipped into 42 °C warming solution at a 42 °C hot plate. The oocytes were warmed by the stepwise dilution method, after which they were kept for 5 min in BM at 39 °C. The concentration gradient of sucrose was carried out as described in the experimental design. Finally, the vitrified oocytes were washed three times with IVM medium and cultured in the same solution under conditions described earlier.

2.3. Assessment of oocytes cryosurvival and nuclear maturation

Survival of the vitrified oocytes was assessed under a stereomicroscope, based on normal morphology. After 2 h of warming, the oocytes with disappeared vitelline membrane and altered cytoplasm have been confirmed to be definitely dead after maturation in our preliminary experiment, and removed (Fig. 1.). After maturation, oocytes were mechanically denuded from cumulus cells by repeated pipetting in TLH-PVA containing 0.1% (w/v) hyaluronidase. Similarly, yellowish-brown discolouration of the cytoplasm and disappearance of the vitelline membrane were observed in nonviable oocytes, and the oocytes exhibiting normal volume, smooth vitelline membrane and evenly granuled dark cytoplasm were regarded as viable (Fig. 1.). These live oocytes were moved around by a pipette under a stereomicroscope to find out the first polar body (PB1). The presence or absence of PB1 in remaining oocytes were further determined following staining with Hoechst 33342 (referred to as nuclear staining of blastocyst described below). The oocytes presenting a PB1 were regarded as matured oocytes (MII stage) and the maturation rate was recorded from each group.

2.4. Parthenogenetic activation and in vitro embryo culture

For parthenogenetic activation (PA), oocytes with a PB1 were washed three times in activation medium (0.3 M mannitol, 0.1 mM