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Cryotop vitrification of porcine parthenogenetic embryos at the early developmental stages

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ABSTRACT

The objective of this study was to evaluate the effects of early developmental stages at which Cryotop vitrification is performed on subsequent survival and *in vitro* development of porcine parthenogenetic activation embryos. The zygotes that were cultured for 4, 8, and 18 hours post electric activation (h.p.a.) and two- and four-cell embryos were vitrified, warmed, and continuously cultured for the remaining period. The zygotes vitrified at 4, 8, and 18 h.p.a. showed similar percentages of survival, cleavage, and blastocyst formation. No difference in viability was observed after vitrification of two- and four-cell embryos, but the embryos vitrified at the two-cell stage exhibited significantly higher blastocyst formation rate than those vitrified at the four-cell stage. However, vitrifying embryos resulted in significantly decreased survival and development rates, regardless of the developmental stage of the embryos. In addition, the final developmental stage, diameter, apoptotic index, and the number of inner cell mass, trophectoderm, and total cells of blastocysts derived from embryos vitrified at any stage of the early culture were similar to those of fresh blastocysts. In conclusion, our data indicate that the early-stage porcine parthenogenetically activated embryos including the zygote, two cells, and four cells have a high ability to survive cryopreservation; these viable embryos after vitrification can produce respectable development rates and good-quality blastocysts.

1. Introduction

Cryopreservation of porcine embryos is favorable for the utilization of valuable genetic resources. It is also considered as a possible way to preserve embryos derived from *in vitro* procedures such as genetic engineering and cloning, which would facilitate studies on biotechnology and biomedicine [1]. However, the extensive use of this technology faces the hindrance of the porcine embryos' high sensitivity to cooling and freezing [2]. Currently, vitrification (an "ice-free" method) has been regarded as the most effective cryopreservation method of porcine embryos [3]. To achieve this, many special techniques such as open pulled straw (OPS), superfine OPS, solid-surface vitrification, Cryotop, and so forth have typically been used [4]. These porcine embryo vitrification methods have resulted in acceptable survival rates and reliably produced piglets after transfer [5–10]. Nevertheless, it still requires further studies of the factors affecting embryo cryosurvival to improve the pregnancy and farrowing rates.

The stage of embryonic development seems to have been the critical factor in successful cryopreservation of porcine embryos, which may be linked to morphologic and physiological characteristics [11]. It is generally accepted that early embryos from domestic animals including pigs, cattle, and sheep are more sensitive to cryopreservation compared with the embryos at advanced stages of development [12]. In fact, the successful vitrification of porcine embryos derived

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in vivo or *in vitro* has also been reported mainly at the morula and blastocysts stages [13–19]. However, the cryopreservation research studies using porcine embryos at early stages of development rarely gained attention. Vitrification is reported to result in extremely low survival and blastocyst formation rates when untreated porcine embryos at the two- and four-cell stages are used, regardless of their origin (*in vivo* or *in vitro*) [20–22]. Recently, encouraging results have also been obtained with the superfine OPS and solid-surface vitrification methods for *in vivo* porcine two- and four-cell embryos, and their blastocyst formation rates can reach about 56% and 26%, respectively [23]. However, there are no reports on changes in the viability and developmental competence of *in vitro*–produced (IVP) two-cell embryos in response to vitrification. Moreover, it is a need to receive accurate data about the survival rates of IVP porcine two- and four-cell embryos after vitrification because of no related results from previous studies.

On the other hand, the zygote stage also seems to be very suitable for vitrification in pigs. It has been reported that vitrification of *in vivo*–derived porcine zygotes results in more than 40% blastocyst development without any pretreatment [24]. In addition, relatively high survival rates after vitrification have been achieved with IVP porcine zygotes at the pronuclear (PN) stage, regardless of whether they are centrifuged or not, but a few of them developed to the blastocyst stage [25–27]. So far, minimal research has analyzed the influence of the time period during IVC on IVP porcine zygotes' ability to survive vitrification.

The effective cryopreservation of porcine embryos at early developmental stages may limit the potential disadvantages of prolonged *in vitro* embryo culture and improve the *in vivo* developmental ability of embryos after transfer. Moreover, there is an increase in demand of reliable cryopreservation protocols for IVP porcine early stage embryos because transgenic or cloned embryos are generally transferred to recipients at the stages of zygote and early cleavage. Currently, the bank of porcine parthenogenetic embryos has been considered to be established owing to their great value to application and research. Li et al. [17] have already examined the cryosurvival of porcine parthenogenetic embryos at different development times (morula at Day 4; blastocyst at Days 4, 5, and 6). In accordance with the aforementioned case, we used porcine embryos after parthenogenetic activation (PA) of oocytes as a model, to systematically evaluate the effects of early developmental stages including zygote and cleavage at which vitrification is performed on subsequent survival and development of PA embryos.

2. Materials and methods

Unless otherwise stated, all chemicals used were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Tissue culture medium 199, fetal bovine serum, and Dulbecco's PBS (DPBS) were obtained from Life technologies (Grand Island, NY, USA).

2.1. Porcine oocyte collection and in vitro maturation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory within 2 hours in 0.9% NaCl (wt/vol) containing 75 mg/L of penicillin G potassium and 50 mg/L of streptomycin sulfate at 35 °C to 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 an 18-ga needle, and follicular contents were allowed to sediment in a 50-mL conical tube. The precipitate was resuspended with Tyrode's lactate–HEPES–polyvinyl alcohol medium (TLH-PVA) and then observed under a stereomicroscope (Olympus, Tokyo, Japan) for cumulus–oocyte complexes collection. Only oocytes with uniform cytoplasm and compact cumulus cells were selected and washed three times in IVM medium. The IVM medium was tissue culture medium 199 supplemented with 10% (v:v) porcine follicular fluid, 3.05-mM D-glucose, 0.57-mM cysteine, 0.91-mM sodium pyruvate, 10 ng/mL of EGF, 0.01 units/mL each FSH and LH (Sioux Biochemicals, Sioux City, IA, USA). Groups of 50 to 70 cumulus–oocyte complexes were placed in each well of a four-well dish (Nunclon, Roskilde, Denmark) containing 500 μL of IVM medium and incubated for 42 to 44 hours at 39 °C in an atmosphere of 5% CO_2 with saturated humidity.

2.2. Production and culture of porcine parthenogenetic embryos

After 44 hours of IVM, cumulus cells were mechanically removed from the oocytes by repeated pipetting in TLH-PVA containing 0.1% (wt/vol) hyaluronidase. Oocytes with intact cytoplasm and a first polar body were selected and washed three times in activation medium (0.3-M mannitol, 0.1-mM $MgCl_2$, 0.05-mM $CaCl_2$, and 0.1% BSA) before electrical stimulation. The oocytes were next placed between two wires of a microslide 0.5-mm fusion chamber (model 450; BTX, SanDiego, CA, USA) covered with fusion medium and stimulated with a direct current pulse of 1.3 kV/cm for 80 μs using a BLS CF-150/B cell fusion machine (BLS, Budapest, Hungary). Thereafter, they were washed three times with porcine zygote medium 3 (PZM-3) supplemented with 5 μg/mL of cytochalasin B and 10 μg/mL of cycloheximide and transferred to the same medium for 4 hours at 39 °C in an atmosphere of 5% CO_2 with saturated humidity. Finally, the presumptive PA embryos were washed three times in PZM-3 before IVC in the same medium under conditions described previously.

2.3. Vitrification with Cryotop and warming

We used the Cryotop (Kitazato Biopharma, Shizuoka, Japan) method for vitrification of porcine PA embryos at the stages of zygote and cleavage in a laboratory maintained at 25 ± 1 °C. All solutions for vitrification and warming were prepared using DPBS supplemented with 20% (v:v) fetal bovine serum as the basal medium (BM). For vitrification, embryos were first washed three times in BM and then pre-equilibrated stepwise with 7.5% (v:v) and 15% (v:v) ethylene glycol in BM at room temperature for 3 minutes, respectively. The equilibrated embryos in groups of 5 to 10 were exposed to vitrification solution containing 35% ethylene glycol (v:v), 0.6-M sucrose, and 50 mg/mL of polyvinylpyrrolidone (PVP) in BM at room temperature for