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Simplified cryopreservation of porcine cloned blastocysts ☆

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Abstract

Recently, a non-invasive delipation (lipid removal) method combined with ultrarapid vitrification has been used successfully for *in vitro* produced (IVP) porcine embryos. In the present study, this method was combined with parthenogenesis and a recent form of somatic cell nuclear transfer (SCNT)—handmade cloning (HMC)—to establish a simplified and efficient cryopreservation system for porcine cloned embryos. In Experiment 1, zonae pellucidae of oocytes were partially digested with pronase, followed by centrifugation to polarize lipid particles. Ninety percent (173/192) oocytes were successfully delipated in this way. Parthenogenetic activation (PA) after complete removal of zona resulted in similar blastocyst rates in delipated vs. control oocytes ($28 \pm 7\%$ vs. $28 \pm 5\%$, respectively). Subsequent vitrification of produced blastocysts with the Cryotop technique resulted in higher survival rates in the delipated group compared to the control group ($85 \pm 6\%$ vs. $32 \pm 7\%$, respectively; $P < 0.01$). In Experiment 2, delipated oocytes were used for HMC with normal oocytes as control. Partial zona digestion was further applied before enucleation both in delipated and control groups, to bisect oocyte successfully. Although the blastocyst rate of reconstructed embryos was similar between groups derived from delipated vs. control oocytes ($21 \pm 6\%$ and $23 \pm 6\%$, respectively), after vitrification higher survival rates were achieved in the delipated groups than in controls ($79 \pm 6\%$ vs. $32 \pm 8\%$, respectively). Our results prove that porcine embryos produced from delipated oocytes by PA or HMC can be cryopreserved effectively by ultrarapid vitrification. Further experiments are required to assess the *in vivo* developmental competence of the cloned-vitrified embryos.

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Introduction

Although cryopreservation of *in vivo*-derived embryos has been successfully established in several species [22], the efficiency for pig embryos is still far from satisfactory. The main reason for the differences is the high chilling sensitivity of porcine embryos due predominantly to the high cytoplasmic lipid content [23]. Accordingly, delipation of embryos with centrifugation and subsequent removal of polarized lipid droplets has improved survival rates both after slow-rate freezing and vitrification [1,21]. Recently, offspring were also reported with transfer of *in vivo*-derived, non-delipated blastocysts vitrified after cytoskeleton stabilization treatment [6] or without any pretreatment using ultrarapid vitrification methods (e.g., [3,5,1,20]).

IVP porcine embryos are even more sensitive to cryopreservation than their *in vivo*-derived counterparts since higher levels of intracellular lipids are accumulated under suboptimal culture conditions [24]. In addition, even unfrozen IVP porcine embryos are developmentally handicapped by high percentage of fragmentation, less total cell number and a lower inner cell mass to trophectoderm ratio in blastocysts [30]. So far, very limited *in vitro* survival rates of IVP porcine embryos were achieved after open pulled straw (OPS) vitrification [19]. Although piglets were born from SCNT embryos after oocyte delipation very recently, two rounds of micromanipulation both for SCNT and delipation is technically demanding and time-consuming [17].

A non-invasive method for delipation has been published recently [8]. Zonae pellucidae of embryos were slightly digested before centrifugation. In this way, IVP porcine morulae and blastocysts were successfully vitrified by using Cryotop method. This new delipation method provides a unique opportunity for producing delipated cloned embryos when HMC is applied for SCNT, since partial zona digestion is a crucial and prerequisite step for successful oocyte enucleation in HMC [7]. A combination of HMC and non-invasive delipation could produce cryotolerant cloned embryos in a simple and efficient way.

The purpose of the present study was to investigate how oocyte delipation influences subsequent embryo development after PA or HMC, measure the survival rates of derived blastocysts after vitrification and warming and further establish a simple cryopreservation system for cloned porcine embryos.

Materials and methods

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) except where otherwise indicated.

Oocyte collection and in vitro maturation (IVM)

Cumulus-oocyte complexes were aspirated from 2 to 6 mm follicles from slaughterhouse-derived sow ovaries and matured in groups of 50 in 400 μl IVM medium consisting of bicarbonate-buffered TCM-199 (GIBCO BRL) supplemented with 10% (v/v) cattle serum (CS), 10% (v/v) pig follicular fluid, 10 IU/ml eCG, 5 IU/ml hCG (Suigonan Vet; Skovlunde, Denmark) at 38.5 °C in 5% CO_2 in humidified air for 41–44 h.

Oocyte delipation

Cumulus investment was removed by repeated pipetting in 1 mg/ml hyaluronidase dissolved in T0 (T for Hepes-buffered TCM-199; the number means percentage (v/v) of cattle serum supplementation, here 0%). Delipation of oocytes was performed by a modification of the technique originally established for early embryos by Esaki et al. [8]. Zonae pellucidae were partially digested at 38.5 °C with 1 mg/ml pronase in T50 for 3 min, and oocytes were washed three times for 15 s in T20 (Fig. 1a). Subsequently 40–50 oocytes were centrifuged at room temperature ($12{,}000 \times$ rpm, 20 min) in T2 supplemented with 7.5 μg/ml cytochalasin B (Fig. 1b). After centrifugation, delipated oocytes were recovered in IVM medium for 1 h before any manipulation.

PA of oocytes

Zonae pellucidae of oocytes (both delipated and control group) used for PA were removed completely with further digestion in 3.3 mg/ml pronase solution for 15 s. The zona free oocytes were equilibrated for 10 s in activation medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.01% PVA) and transferred to a fusion chamber (BTX microslide 0.5 mm fusion chamber, model 450; BTX, SanDiego, CA, USA), where they were activated by a single direct current (DC) pulse of 0.85 kV/cm for 80 μs generated by a BLS CF-150/B cell fusion machine (BLS, Budapest, Hungary). The culture conditions for the activated PA oocytes were the same as for HMC reconstructed embryos.

HMC

Except where otherwise indicated, all manipulations were performed on a heated stage adjusted to 39°C, and all drops used for handling oocytes were 20 μl covered with mineral oil. For SCNT, the HMC technique described in our previous work [7] was applied. Both delipated and control oocytes were enucleated by bisection as described below.