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Birth of piglets from *in vitro*-produced, zona-intact porcine embryos vitrified in a closed system

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Abstract

As the importance of swine models in biomedical research increases, it is essential to develop low-cost, high-throughput systems to cryopreserve swine germplasm for maintenance of these models. However, porcine embryos are exceedingly sensitive to low temperature and successful cryopreservation is generally limited to the use of vitrification in open systems that allow direct contact of the embryos with liquid nitrogen (LN_2). This creates a high risk of pathogen transmission. Therefore, cryopreservation of porcine embryos in a "closed" system is of very high importance. In this study, *in vitro*-produced (IVP) porcine embryos were used to investigate cryosurvival and developmental potential of embryos cryopreserved in a closed system. Optimal centrifugal forces to completely disassociate intracellular lipids from blastomeres were investigated using Day-4 embryos. Cryosurvival of delipidated embryos was investigated by vitrifying the embryos immediately after centrifugation, or after development to blastocysts. In this study, centrifugation for 30 min at 13,000 g was adequate to completely delipidate the embryos; furthermore, these embryos were able to survive cryopreservation at a rate comparable to those centrifuged for only 12 min. When delipidated embryos were vitrified at the blastocyst stage, there was no difference in survival between embryos vitrified using OPS and 0.25 mL straws. Some embryos vitrified by each method developed to term. These experiments demonstrated that porcine embryos can be cryopreserved in a closed system after externalizing their intracellular lipids. This has important implications for banking swine models of human health and disease.
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Keywords: Porcine embryos; Delipidation; Vitrification; Closed system

1. Introduction

Germplasm cryopreservation offers a cost-effective alternative to maintain live animal models in biomedical research. This is especially important with the explosive increase of genetically modified animal models in recent years [1]. Sperm and embryo cryopreservation are routine procedures for banking of mouse and rat models. However, similar protocols for banking swine germplasm are currently inefficient, largely due to difficulties associated with cryopreservation of porcine sperm and embryos. Since the use of pigs in biomedical research has dramatically increased for use as disease models and potential organ donors in xenotransplantation [2,3], there is an increased need for effective methods to maintain swine models in the form of cryo-

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preserved embryos. Cryopreservation of genetically-modified swine models safeguards unique genotypes from several potential problems, including: (a) genetic drift, (b) genetic instability, (c) genetic contamination, and (d) loss due to disease or catastrophic disasters to housing facilities. Importantly, it also allows models to be maintained only as "cryopreserved lines," thereby reducing live animal housing requirements and costs. Embryo cryopreservation also facilitates global exchange of swine genetics, due to its cost effectiveness, as well as aiding in management of potential disease transmission and other health concerns.

Maintenance and storage of animal models in the form of cryopreserved germplasm requires that the cells are stored in a closed system, which can keep germplasm in a pathogen-free environment during cryopreservation and storage to protect it from potential contamination with pathogens from various sources, such as LN_2 [4–6]. The commonly used 0.25 mL mini-straws that can be sealed at both ends will serve this purpose. However, due to the exceeding sensitivity of porcine embryos to low temperatures, cryopreservation in conventional closed 0.25 mL mini-straws without any pretreatment is very difficult [7,8].

The sensitivity of porcine embryos to low temperature is mainly due to their high levels of intracellular lipids [9,10]. Removal of intracellular lipids through micromanipulation after lipid polarization by high speed centrifugation of the embryos allows porcine embryos to avoid chilling injury. The first live births from cryopreserved porcine embryos resulted from this approach [10]. Currently, this method is most effective for the cryopreservation of porcine embryos derived both *in vivo* and *in vitro* [10–13].

A second approach to reduce the chilling sensitivity of porcine embryos is based on the knowledge that chilling injury can be minimized by dramatically increasing the cooling rate so that embryos quickly pass through the temperature-sensitive zone during cooling [14,15]. Achieving high cooling rates, such as those used with vitrification, requires one to minimize the volume (and therefore the thermal mass) of the medium, as well as to maximize the surface area to volume ratio of the sample. To accomplish this, special devices, such as open pulled straws (OPS), superfine OPS, cryotop, microdroplet method, etc, have typically been used [12,16–27].

Unfortunately, the two approaches described above either damage the zona pellucidae of delipidated embryos or allow direct contact of the sample with LN_2, respectively, thus increasing the chances of pathogen transmission during embryo cryopreservation and storage [4–6]. Therefore, a protocol that allows the porcine embryos to be cryopreserved with an intact zona pellucida in a closed system is needed.

To retain intact zona pellucidae, a non-invasive version of the mechanical delipidation approach was developed and successfully used for generation of live piglets from cryopreserved porcine embryos [16,18–20]. However, complete delipidation of porcine oocytes is beneficial to maximize their cryotolerance [18,28,29], especially when a closed system is used (e.g., 0.25 mL mini straws) [18]. Non-invasive, complete separation of intracellular droplets from blastomeres was achieved by partial digestion of the zona pellucidae [30], or by using a hyperosmotic solution. Live births were derived from *in vitro*-generated embryos, using the latter approach [13]. However, both approaches used ultrarapid cooling rates which required direct contact between LN_2 and the embryo-containing solution.

In the present study, a series of experiments was conducted to test the hypotheses that: (1) complete separation of intracellular lipids from blastomeres of early stage embryos can be achieving using high-speed centrifugation for extended intervals; (2) cryosurvival of embryos delipidated using this approach is comparable to those delipidated using commonly used centrifugation time; (3) embryos delipidated by this approach could be successfully vitrified in both an open system (e.g., OPSs) and a closed system (e.g., 0.25 mL mini-straws); and (4) the embryos pretreated by this approach have the potential to develop full term *in vivo* after cryopreserved in either OPS or 0.25 mL mini-straws. We used IVP porcine embryos to test these hypotheses.

2. Material and methods

2.1. Oocyte maturation, fertilization and culture in vitro

Unless specifically stated, all chemicals were obtained from Sigma Aldrich Co (St. Louis, MO, USA). Immature porcine oocytes were obtained from a commercial source (ART Inc, Madison, WI, USA). The cumulus-oocyte complexes (COCs) were delivered overnight in groups of 50 oocytes/tube in plastic tubes containing 2 mL of TCM199-based Phase I maturation medium [31] in a commercial shipper capable of maintaining the temperature at 38.5 °C. Upon reception, tubes containing COCs were transferred into an incubator without opening the tubes. Phase II maturation

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