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Piglets produced from *in vivo* blastocysts vitrified using the Cryologic Vitrification Method (solid surface vitrification) and a sealed storage container

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Abstract

The objective was to develop a simple successful porcine cryopreservation protocol that prevented contact between embryos and liquid nitrogen, avoiding potential contamination risks. *In vivo*-derived blastocysts were collected surgically from donor pigs, and two porcine embryo vitrification protocols (one used centrifugation to polarize intracytoplasmic lipids, whereas the other did not) were compared using the Cryologic Vitrification Method (CVM), which used solid surface vitrification. The CVM allowed embryos to be vitrified, without any contact between embryos and liquid nitrogen. Both protocols resulted in similar *in vitro* survival rates (90% and 94%) and cell number (89 ± 5 and 99 ± 5) after 48 h *in vitro* culture of vitrified and warmed blastocysts. The protocol that did not use centrifugation was selected for continued use. To protect vitrified embryos from contact with liquid nitrogen and potential contamination during storage, a sealed outer container was developed. Use of this sealed outer container did not affect *in vitro* survival of cryopreserved blastocysts. *In vivo* blastocysts (n = 151) were collected, vitrified, and stored using the selected protocol and sealed container. These embryos were subsequently warmed and transferred to six recipients; five became pregnant and farrowed a total of 26 piglets. This embryo vitrification method allowed porcine embryos to be successfully vitrified and stored without any contact with liquid nitrogen.

Keywords: Pig; Blastocysts; Vitrification; Solid Surface Vitrification; Embryo Transfer; Piglets

1. Introduction

A reliable method of cryopreserving and storing porcine genetic material is becoming increasingly important, as it would facilitate storage of genetics of high-value pigs (e.g., transgenic pigs created for xenotransplantation), and protect these genetics from potential destruction due to a disease outbreak. Cryopreservation would also enable low-cost international transport of valuable pig genetics with minimum risk of disease transmission.

The first reported litter of piglets from transferred vitrified and warmed zona pellucida intact blastocysts used centrifugation to polarize lipid out of the blastomeres [1]. Since then, there have been numerous reports of piglets born from zona pellucida-intact embryos [2–9]. Farrowing rates of ~75% and *in vivo* survival rates of ~30% have been reported using both

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Meishan [10] and Large White × Landrace cross as recipients [8]. Almost all reported protocols used a method or technique to very rapidly cool the sample containing the embryos, as faster cooling rates were expected to improve embryo survival after warming. These methods include using open pulled straw technology [11], superfine open pulled straws [12], microdrops [7], metal mesh [9], and reducing the temperature of the liquid nitrogen in which the sample is to be plunged to below −204 °C [6,8]. A disadvantage of all these techniques is that direct contact between the sample and the liquid nitrogen occurred during vitrification, thereby creating a risk of contamination.

A technique that has very fast cooling rates and does not involve contact between the embryos and the liquid nitrogen is solid surface vitrification. With this technique, a microdrop (approximately 1 to 3 μL) containing the embryos is vitrified by touching it to a metal surface that has been precooled in liquid nitrogen [13]. A specific vitrification system developed that uses solid surface vitrification is the Cryologic Vitrification Method (CVM) [14]. The CVM provides a way of holding and storing the microdrop containing the embryos (using a nylon loop and handle; the CVM fibreplug; Fig. 1) and a metal block with a highly heat conductive surface on which to vitrify the microdrop. It has been successfully used to vitrify bovine IVP embryos [15], but its use with pig embryos has not yet been reported.

The aim of the present study was to develop a method by which porcine blastocysts could be successfully vitrified and stored without coming into contact with liquid nitrogen. Two protocols were tested with the CVM; one was selected, further modified, and used to cryopreserve blastocysts that were subsequently transferred into recipients.

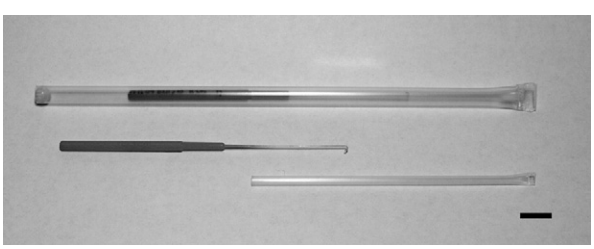

Fig. 1. CVM fibreplug and outer straw. This image shows (from top) an outer straw with a CVM fibreplug and sleeve sealed inside (5 mL cryostraw heat sealed at one end and sealed with a plastic ball at the other end), a CVM fibreplug (nylon hook with attached handle) and a sleeve for the fibreplug. Scale bar = 1 cm.

2. Materials and methods

2.1. Chemicals, media and in vitro culture

All chemicals were purchased from Sigma-Aldrich, Inc (St Louis, MO, USA) unless otherwise specified. Medium 199 was purchased from Gibco (Invitrogen, CA, USA). All media used for culture and handling of the embryos contained 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp, Carlsbad, CA, USA). The general embryo handling medium was a HEPES-buffered North Carolina State University medium 23, containing MEM essential and non-essential amino acids (both included at 1:100 dilution; mod-HNCSU23; Invitrogen). The media used to culture *in vivo* embryos was NCSU23 [16] containing MEM essential and non-essential amino acids (mod-NCSU23) [17]. All media also contained 4 mg/mL bovine serum albumin (BSA; Bovostar, Victoria, Australia). Long-term culture was conducted in 50 μL droplets of mod-NCSU23 under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2, balance N_2 at 38.5 °C. All media was warmed and equilibrated as appropriate for at least 2 h before use.

2.2. Donor sows and embryo collection

Animal experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004), as stipulated by the National Health and Medical Research Council and approved by the University of Adelaide Animal Ethics Committee. Embryos were collected from Large White × Landrace sows that were culled for age. They were given 1000 IU eCG (Folligon; Intervet Aust. Pty. Ltd.) on the morning of weaning, followed by 750 IU hCG (Chorulon; Intervet Aust. Pty. Ltd.) approximately 80 h later. The sows were artificially inseminated or naturally mated twice during the time of standing estrus, commencing approximately 24 h after hCG treatment. Embryos were collected surgically 5 d after the onset of standing estrus [18]. Uterine horns were flushed with 50 mL of Dulbecco's phosphate buffered saline containing 2% heat inactivated FBS and the flushings collected in a sterile 50 mL tube. The tube was emptied into a sterile 90 mm petridish, which was searched for embryos under a stereomicroscope. Apparently viable embryos were washed twice in mod-HNCSU23 medium, placed into 3 mL of mod-HNCSU23 containing 10% FBS in 5 mL tubes, and transported from the embryo collection location to the laboratory in a temperature controlled incubator set at 38.5 °C. Each donor's embryos were kept separate.