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Survival of vitrified *in vitro*–produced bovine embryos after a one-step warming in-straw cryoprotectant dilution procedure

J.N. Caamaño, E. Gómez, B. Trigal, M. Muñoz, S. Carrocera, D. Martín, C. Díez*

Genética y Reproducción Animal, Centro de Biotecnología Animal, SERIDA, Gijón, Asturias, Spain

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ABSTRACT

Vitrification is an alternative to slow-rate freezing for cryopreserving bovine embryos. However, this technology requires simplification if it is to be used under field conditions. The main objective of this work was to develop a new system for the direct transfer of vitrified embryos to be used under farm conditions. For this, three objectives were set: (1) to compare the effect of vitrification, using the cryologic vitrification method (CVM), and slow-rate freezing on bovine embryo development and quality; (2) to develop a one-step warming procedure for bovine *in vitro*–produced (IVP) vitrified (by CVM) embryos; and (3) to assess the effects on embryo survival of a new method for the direct transfer of vitrified IVP bovine blastocysts. *In vitro*–produced blastocysts were initially either vitrified by CVM or subjected to slow freezing to compare embryo survival and quality (experiment 1). No differences were detected between these cryopreservation techniques in terms of the survival and quality variables at 24 hours or in terms of the proteins expressed. However, at 48 hours the vitrified embryos showed higher hatching rates, greater total cell numbers, and lower apoptotic indices ($P < 0.05$). In experiment 2, CVM-vitrified IVP blastocysts were warmed by the conventional two-step or one-step warming procedure by incubating them at 41 °C in 0.25 M sucrose for 10 minutes, 0.15 M sucrose for 10 minutes, or 0.25 M sucrose for 5 minutes. In addition, embryo transfer (ET) was performed using vitrified embryos warmed by the one-step procedure in 0.25 M sucrose solution for 5 minutes. As a control group, IVP fresh embryos were transferred to recipient females. No differences were observed in embryo survival or total cell number between any of the warming procedures. Moreover, no significant differences for pregnancy at 60 days were found between the ET groups. In experiment 3, expanded IVP blastocysts were then either vitrified using a conventional or a modified fiber plug designed to allow direct ET after in-straw cryoprotectant (CP) dilution. They were warmed using the one-step process (0.25 M sucrose, 5 minutes) in a 0.25 mL French straw. Embryo recovery associated with the modified fibreplug system was less reliable than with the conventional system. However, no differences were seen between the systems in terms of *in vitro* embryo survival among those finally recovered. Finally, IVP blastocysts were vitrified using conventional fibreplugs to maintain a high embryo recovery rate, and then warmed using the one-step warming in-straw CP dilution procedure, but using an adapter with a wider opening coupled to the French straw and a heated metal chamber to protect and keep the straw at 41 °C (experiment 4). No differences were seen in embryo survival rates between the two groups. The CVM combined with this new one-step warming in-straw CP dilution procedure could be used for direct ET under field conditions.

* Corresponding author. Tel.: +34 984 50 20 10; fax: +34 984 50 20 12.
E-mail address: mcdiez@serida.org (C. Díez).

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1. Introduction

Compared with *in vivo*–recovered bovine embryos, *in vitro*–produced (IVP) embryos show reduced viability and total cell numbers, as well as differences in metabolism and gene expression [1–6]. They also show poorer survival rates after cryopreservation [7–9]; their use is therefore associated with reduced pregnancy rates (PRs) [10,11]. Nonetheless, IVP embryos produced using oocytes collected from cows of high genetic merit by the ovum pickup method or from bovine ovaries at slaughterhouse are becoming ever more used in the laboratory [12–16]. If such embryos are to be used in commercial bovine reproduction, cryopreservation techniques must be improved to efficiently cryopreserve them [12–16].

Slow-rate freezing and vitrification have been used to cryopreserve IVP bovine embryos. The principles and methodologies for slow-rate freezing and vitrification have been described elsewhere [17,18]. One of the advantages of vitrification is that there is no need for expensive programmable freezers because samples are plunged directly into liquid nitrogen (LN). Furthermore, vitrification is simple and quick, making it a good option for IVP embryo cryopreservation. In terms of embryo survival after warming or thawing, the vitrification of IVP bovine embryos has been shown at least as efficient [11] or better [17,19,20] than slow-rate freezing under different experimental conditions. However, it has also been reported in a recent report [21] that slow-rate freezing is a good alternative to cryopreserve IVP bovine embryos. In this regard, it is controversial to select one methodology over the other. Each research group should decide which one adapts better under their laboratory conditions.

Furthermore, a practical approach for warming of vitrified embryos is necessary to be considered as an option for working under field conditions. One-step procedures for the direct transfer of frozen-thawed embryos into recipients have been widely adopted by the embryo transfer (ET) industry. However, the warming of embryos cryopreserved by vitrification requires the removal of the cryoprotectant (CP) agent *via* successive dilution steps. The trouble this causes when working under farm conditions has led to efforts to develop vitrification procedures that make direct ET less problematic [11,22–25]. We have previously reported the use of the cryologic vitrification method (CVM) for vitrifying IVP bovine blastocysts in fibreplugs to be associated with promising survival and PRs [19,26].

Given this background, the aim of the present study was to develop new system for the direct transfer of vitrified embryos that could be used under farm conditions. We assessed systematically different procedures and steps that allowed us to select and define the best conditions to achieve our main goal. For this reason, three objectives were set: (1) to compare the effect of vitrification and slow-rate freezing on bovine embryo developmental potential and quality (*via* morphologic evaluation, differential cell counting, cell viability testing, the determination of apoptotic index (AI) and necrotic index (NI), and the assessment of expression of specific proteins involved in metabolism and blastocyst formation [8,27–30]); (2) to assess a simple, one-step warming procedure for IVP bovine embryos vitrified by the CVM; and (3) to assess the effects on embryo survival of a new warming method for the direct transfer of vitrified IVP bovine blastocysts.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma (Madrid, Spain), unless otherwise indicated.

2.2. In vitro embryo production

Cumulus-oocyte complexes (COCs) from slaughterhouse ovaries were aspirated from 3 to 8 mm visible follicles and rinsed three times in a holding medium (HM) consisting of tissue culture medium (TCM) 199 (Invitrogen, Barcelona, Spain), 25 mM HEPES, and BSA 0.4 g/L. Oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected and washed twice in maturation medium, which consisted of bicarbonate-buffered TCM199, porcine FSH-LH (1:5 μg/mL) (Stimufol; ULg FMV, Liège, Belgium), 17β-estradiol (1 μg/mL), and 10% fetal calf serum (FCS). Maturation was performed by culturing approximately 50 COCs in 500 μL of maturation medium in four-well dishes at 39 °C in air with 5% CO_2 at high humidity for 22 to 24 hours.

For IVF, sperm separation was performed following a swim-up procedure [31]. Briefly, semen from one frozen straw (sperm from two bulls was used for the entire work) was thawed in a water bath and added to a polystyrene tube containing 1 mL of pre-equilibrated Sperm-tyrode, albumin, lactate, pyruvate. After 1 hour of incubation, the upper layer of supernatant containing motile sperm was removed. The spermatozoa were centrifuged for 7 minutes at 200 × *g* and the supernatant aspirated to leave a pellet containing sperm cells, the concentration of which was determined using a hemocytometer. After washing the COCs in HM, they were rinsed twice in fertilization medium (Fert-tyrode, albumin, lactate, pyruvate) and placed in four-well culture dishes containing the same medium plus 10 μg/mL heparin (Calbiochem, La Jolla, CA, USA). Spermatozoa were added at a concentration of 2×10^6 cells/mL in 500 μL of medium per well, each containing a maximum 100 COCs (Day 0). *In vitro* fertilization was accomplished by incubating the oocytes and sperm cells together for 18 to 20 hours at 39 °C in air with 5% CO_2 at high humidity.

For *in vitro* culture, cumulus cells were detached using a vortex, and presumptive zygotes cultured in synthetic oviduct fluid containing amino acids, citrate, and myo-inositol (modified Synthetic Oviduct Fluid)—as described by Holm et al. [32]—plus 6 g/L of BSA. Droplets (1–2 μL per embryo) were covered by a mineral oil layer and the embryos cultured in groups of 35 to 45 at 39 °C in a 5% CO_2/5% O_2 atmosphere at high humidity. The culture medium was renewed on Days 3 and 6 by transferring the embryos to fresh droplets. Embryo development was recorded on Days 3 and 6 to 8.

2.3. Embryo vitrification and freezing

Expanded Day 7 and Day 8 blastocysts were subjected to either vitrification or slow-rate freezing. Vitrification was