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Successful vitrification of bovine blastocysts on paper container

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Abstract

Cryopreservation of bovine embryos can be performed by a variety of methods with variable degree of success. Here, we report a new, easy to perform, simple, inexpensive, and successful method for vitrification of bovine blastocysts. *In vitro* produced bovine blastocysts were exposed to vitrification solution (5.5 M ethylene glycol, 10% serum and 1% sucrose) in one single step for 20 s, loaded on a paper container prepared from commonly available non-slippery, absorbent writing paper, and then were directly plunged into liquid nitrogen for storage. Vitrified blastocysts were warmed by serial rinsing in 0.5, 0.25 and 0.125 M sucrose solution for 1 min each. Results showed that one step exposure of bovine blastocysts to cryoprotective agents was sufficient to achieve successful cryopreservation. Under these conditions, more than 95% of blastocysts survived the vitrification-warming on paper containers which was significantly higher than those obtained from other containers, such as electron microscope (EM) grid (78.1%), open pulled straw (OPS; 80.2%), cryoloop (76.2%) or plastic straw (73.9%). Embryo transfer of blastocysts vitrified-warmed on paper container resulted in successful conception (19.3%) and full-term live birth of offspring (12.3%) which were lower ($P < 0.05$) than those obtained from non-vitrified blastocysts (38.0 and 32.7%) but were comparable ($P > 0.05$) to those obtained from blastocysts vitrified-warmed on EM grid (23.3 and 14.2%). Our results, therefore, suggest that paper may be an inexpensive and useful container for the cryopreservation of animal embryos.

Keywords: Vitrification; Bovine embryo; Paper container; Embryo cryopreservation

1. Introduction

Cryopreservation of gamete and embryos has become almost essential for the cost-effective, long-term conservation and widespread dispersion of animal genetic resources. It also has applications in the management of infertility in both human and animals. However, despite millions of offspring being born from cryopreserved gamete and embryos of more than 20 species, including human, a perfect cryopreservation

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protocol for oocyte and embryos has not yet been established [1–2].

Among different cryopreservation strategies, vitrification is considered an important methodology for long-term storage of embryos in a glass-like amorphous vitreous state without the formation of ice crystals. Since its first reported application for embryo cryopreservation in mouse [3] and cattle [4], tremendous improvements have been made or been suggested to improve the post-warming survival of vitrified-warmed embryos that included the application of stress, such as hydrostatic pressure [5] or the use of exogenous additives, such as plant-derived vegetal peptones [6], Rho-associated coiled-coil kinase inhibitors [7], antioxidants [8], linoleic acid-albumin [9], serum replacer [10], trans-10 cis-12 conjugated linoleic acid [11], cytoskeleton stabilizers [12], fatty acid synthesis inhibitors [13], etc. into the vitrification, warming, or culture media. However, the most dramatic improvement has probably been achieved by the use of newer vitrification containers that aimed to minimize the volume of vitrification solution surrounding the oocyte/embryo (0.1–1.0 μL) and thereby increase the speed of cooling and warming (up to 20 000°C/min) by facilitating the rapid transfer of heat to and from liquid nitrogen (LN2). Such containers included modified plastic straws [14], electron microscope (EM) grid [15], open pulled straw (OPS) [16], cryoloop [17], cryotop [9] and their various derivatives (See [18–19] for detailed review). Unfortunately, most of these containers can provide physical support to hold a limited number of embryos per container (~10–15 embryos per container). Matsumoto, et al. [20] reported the use of a nylon mesh which can hold more than 50 bovine oocytes per container and therefore is suitable for simultaneous vitrification of large number of oocytes/embryos, such as those matured/fertilized *in vitro* from abattoir-derived ovaries. Aluminum foil has also been used as a solid substrate for solid surface vitrification (SSV) of large numbers of oocytes and embryos, which are then transferred as microdroplets (without the aluminum foil) into cryotubes or cryovials for storage in LN2 [8,12]. Aluminum foil, however, has not been used as a storage device [8,12]. We hypothesized that a good, easily available and cheap alternative, fulfilling all the above requirements, could be the use of a paper container that can be easily prepared from any non-slippery, absorbent writing paper. Because paper containers can be easily prepared and easily sterilized, they could potentially be useful in the vitrification of embryos at the farm level.

Vitrification of embryos requires their exposure to a highly concentrated cryoprotective agent (CPA) to prevent ice crystal formation [3]. Unfortunately, almost all permeating CPAs are embryotoxic at high concentrations, which is required to prevent ice crystal formation during vitrification, and may result in severe embryonic damage that increases with the increase in the duration of exposure [21]. However, if CPA exposure is too brief, its permeation inside the embryos may be inadequate and may result in intracellular ice crystal formation [21–22]. Consequently, in most embryo vitrification protocols, embryos are first equilibrated with a low concentration of CPA before being exposed to full strength CPA in the final vitrification solution [12,18]. Nonetheless, high post-warming survival has been obtained when mouse oocytes and embryos were directly exposed to the vitrification solution for very brief period (5–10 s) and no equilibration step was carried out [23–24].

This study was therefore, designed to investigate the feasibility of using paper container and one step CPA addition procedure for vitrification of embryos using bovine as a model system. The EM grid, plastic straw, OPS and cryoloop were used as comparison.

2. Materials and methods

All chemicals used were purchased from Sigma Aldrich, Co (St. Louis, MO) unless otherwise specifically indicated. All animal experiments were performed in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching, published by the Federation of Animal Science Societies (3rd Edition, 2010), and were approved by the Konkuk University.

2.1. Oocyte retrieval and in vitro maturation (IVM)

Oocytes were retrieved from abattoir-derived bovine ovaries and matured *in vitro* as described earlier [25–26] with partial modifications. Briefly, cumulus-oocyte-complexes were aspirated from follicles (2–8-mm diameter) using 10 mL syringe fitted with an 18 G needle, washed three times with HEPES-buffered Tyrode's lactate (TL-HEPES) medium and matured in groups of 50 in 500 μL of Tissue Culture Medium 199 with Earle's salts (TCM-199; Gibco BRL, Grand Island, NY) supplemented with 25 mM NaHCO3, 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 0.22 μg/mL sodium pyruvate, 25 μg mL^{-1} gentamicin, 1 μg/mL FSH (Folltropin V; Vetrepharm, Canada), and