Cryobiology 68 (2014) 459–466

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Birth of a domestic cat kitten produced by vitrification of lipid polarized *in vitro* matured oocytes ☆

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ARTICLE INFO

Article history:
Received 3 December 2013
Accepted 25 February 2014
Available online 11 March 2014

Keywords:
Lipid polarization
Vitrification
Oocytes
Domestic cat
Cryosurvival
In vitro fertilization
Embryo transfer

ABSTRACT

The ability to cryopreserve oocytes is an effective method to retain valuable genetic material of mammals, including that of endangered animals. Embryos of domestic cats are amenable to cryopreservation, whereas their oocytes are much less cryo-tolerant. The capability of oocytes to survive cryopreservation is affected by several factors, one of which has been hypothesized to be the high concentration of intracellular lipids. To test this hypothesis, in this study we polarized lipids of cat oocytes and tested their cooling and freezing sensitivity. We found that the sensitivity of oocytes to cooling and cryopreservation does appear to be related to their high intracellular lipid content, as indicated by higher cryosurvival and development into blastocysts when intracellular lipids of *in* vitro matured oocytes were polarized before vitrification. However, polarization of all intracellular lipids was detrimental to development of embryos. Cell numbers in blastocysts derived from fully polarized/vitrified oocytes were significantly lower than those of partially polarized/vitrified or non-vitrified/fresh oocytes. Although embryos derived from fully polarized/vitrified oocytes developed to the blastocyst stage at higher rates than those of partially polarized/vitrified or non-centrifuged/vitrified oocytes, their *in vivo* developmental competence was compromised. When embryos derived from fully polarized/vitrified oocytes were transferred, although two recipients became pregnant, all implanted embryos were reabsorbed. In contrast, when embryos derived from oocytes that were only partially lipid polarized before vitrification and then were transferred, one recipient did become pregnant and produced a live healthy kitten. The present results suggest that other approaches to altering intra-cellular lipid levels in cat oocytes should be evaluated to improve their functional survival after cryopreservation.

Introduction

Cryopreservation of oocytes is a method of retaining valuable genetic material including that from endangered felids. Storage of oocytes collected post-mortem or from infertile females will enable the establishment of oocyte and embryo cryo-banks for future use [1,15]. Embryos of domestic cats can be cryopreserved, whereas their oocytes are much less cryo-tolerant. Cleavage of vitrified mature domestic cat oocytes fertilized by *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was similar to the rate observed with non-frozen domestic cat oocytes fertilized by IVF [26], but few embryos derived from vitrified oocytes developed to the blastocyst stage as compared to embryos produced from non-frozen oocytes [17,21,26]. Even so, we produced live kittens after the transfer of ICSI-derived zygotes and two-cell embryos produced from vitrified domestic cat oocytes [26]. The capability of oocytes to survive cryopreservation is affected by several factors, but the greater sensitivity to cooling as compared to embryos may be due to the relative concentration of intracellular soluble macromolecules. For example, cryo-sensitivity of pig oocytes is due partially to their high level of intracellular lipids [22]. Domestic cat oocytes also have high intracellular lipid content [9]. Therefore, the low *in vitro* developmental competence of domestic cat embryos derived from vitrified oocytes may be due partly to their high lipid content.

Removal of intracellular lipid droplets before cryopreservation may eliminate a potential cytoplasmic element that causes disruption of cellular organelles [23], and lipid removal (delipidation) in pig oocytes improved their cryotolerance to vitrification [22].

☆ *Statement of funding*: This study was partially funded by a grant from the Research Advisory Board of ACRES and Louisiana State University System Projects.

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http://dx.doi.org/10.1016/j.cryobiol.2014.02.012
0011-2240/

Reduction of intracellular lipids can be accomplished by centrifugation and/or removal of the polarized lipids using a micromanipulator [11]. This approach has been successfully applied to domestic cat zygotes [12]; development to the blastocyst stage of delipidated cat zygotes was similar to that of sham and control embryos. Also, survival rate after slow freezing and thawing of blastocysts derived from delipidated zygotes was similar to that of controls [12]. The limitations of using micromanipulation for removing lipid droplets can be overcome by a modified method that consists of inducing lipid polarization by centrifuging oocytes in high osmolality culture medium [18]. With this method, 90% of pig embryos that were centrifuged for 20 min in hypertonic solution containing sucrose (350–400 mOsm) were completely lipid polarized and developed to the blastocyst stage at rates similar to that of non-polarized embryos [18]. Therefore, centrifugation in a hypertonic solution may be a good alternative for removal of intracytoplasmic lipids of cat oocytes prior to vitrification, so as to improve their cryosurvival.

The purpose of the present project was to optimize a vitrification protocol used previously in our laboratory for cryopreservation of domestic cat oocytes. Specifically, we evaluated whether (1) lipid polarization of *in vitro* matured domestic cat oocytes before vitrification enhances cryosurvival and, (2) embryos derived from lipid polarized/vitrified oocytes can successfully produce healthy kittens after transfer to recipient females.

Materials and methods

Subjects

Domestic short hair cats (DSH) used as embryo recipients were group-housed in environmentally controlled rooms with a 14 h: 10 h light: dark cycle at 20–26 °C at the Audubon Center for Research of Endangered Species (ACRES). The rooms were cleaned and cats fed once daily (Pro Plan Adult: Nestle Purina Pet Care Co. St. Louis, MO, USA). Fresh water was available at all times. All animal procedures were approved by the Institutional Animal Care and Use Committee of ACRES as required by the Health Research Extension Act of 1985 (Public Law 99-1580).

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

In vitro oocyte maturation

To produce *in vitro* matured (IVM) oocytes, domestic cat ovaries were obtained after ovariohysterectomy from local veterinary clinics, and were transported to the laboratory within 3–4 h. The ovaries were minced and cumulus–oocyte complexes (COC) were cultured in TCM-199 supplemented with 1 IU/mL human chorionic gonadotropin (hCG; Pregnyl, Organon Inc., West Orange, NJ, USA), 0.5 IU/mL equine chorionic gonadotropin (eCG; G4788), 10 ng/mL epidermal growth factor (EGF), and 3 mg/mL bovine serum albumin (BSA; #81-068, EDM Millipore, Billercia, MA, USA) for 22-24 h in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 °C [8].

Oocyte delipidation and vitrification

Delipidation

After IVM, oocytes were denuded of cumulus and corona cells by vortexing in 1 mg/ml of hyaluronidase for 2 min, followed by gentle mechanical pipetting. Denuded mature oocytes, characterized by the extruded first polar body, were incubated in a Ca^{2+}-free and Mg^{2+}-free solution (0.2 g/L KCL, 8.0 g/L NaCl, 0.04 g/L NaH_2PO_4, 1.0 g/L D-glucose) supplemented with 1% MEM nonessential amino acids, 3 mg/mL BSA, 30 mM $NaHCO_3$, 0.36 mM pyruvate, 1 mM glutamine, 50 μg/mL gentamicin (centrifugation medium, CM) for 10–15 min and centrifuged in 100 μl of CM at 12,000*g* for 20 min at 20 °C.

Vitrification

Lipid polarized or non-centrifuged oocytes were cryopreserved by the Cryotop method as described previously [14,26] in a vitrification solution (VS) consisting of 15% (v/v) dimethyl sulfoxide (DMSO), 15% (v/v) ethylene glycol (EG), and 18% sucrose diluted in Hepes buffered 199 medium supplemented with 4 mg/ml dextran 70 and 10 mg/mL BSA (He199). Briefly, one to three oocytes were exposed to an equilibration solution (ES) consisting of He199 +7.5% (v/v) EG and 7.5% (v/v) DMSO at 22 °C for 12–15 min. Then, oocytes were transferred into VS. After 20 s, oocytes were loaded onto a Cryolock device (Biotech Inc. Cummings, GA, USA) and plunged immediately into liquid nitrogen. After storage, oocytes were warmed by immersion of the Cryolock directly into thawing solution (TS, 4 mL) that consisted of 36% sucrose in He199, at 38.5 °C for 1 min, transferred into dilution solution (DS, 18% sucrose in He199) for 3 min, and then into a wash solution (WS, He199). The warmed oocytes were cultured for 2 h in IVF medium at 38.5 °C, 5% CO_2 in air (see below) before IVF.

In vitro fertilization and embryo culture

For IVF, vitrified/warmed or non-vitrified (control) oocytes were co-incubated with epididymal sperm ($\approx 1 \times 10^6$ motile sperm/mL) in 50 μl droplets of Tyrode's balanced salt solution (T2397) supplemented with 15 mM $NaHCO_3$, 0.36 mM pyruvate, 1 mM glutamine, 2.2 mM calcium lactate, 50 μg/mL gentamicin and 6 mg/ml BSA (IVF medium) under mineral oil (4008-5, Sage, In vitro fertilization Inc., Trumbull, CT, USA) in a humidified atmosphere of 5% CO_2 in air at 38.5 °C. At 4 h post-insemination (PI), oocytes were rinsed and cultured with or without feeder layers of cat fetal fibroblast cells (CFF) in a three-step culture system [24,25] for: (1) 24–48 h before being transferred to oviducts of domestic cat recipients on day 1, or (2) cultured *in vitro* until day 8 at which time the number of embryos developing to the blastocyst stage was recorded.

Cat cell feeder layers were prepared by thawing a vial of CFF cells, plating $\approx 1 \times 10^5$ cells/well in a 4-well dish containing 500 μL of Glasgow Minimal Eagle's Medium (GMEM) supplemented with 12% foetal bovine serum (FBS), 2.4 mM L-glutamine, 2.4 mM sodium pyruvate, 2.2 mL DMEM, 10 U/mL penicillin, 10 μg/mL streptomycin and 50 μg/mL gentamicin and culturing in a humidified atmosphere of 5% CO_2 in air at 38.5 °C. When cells reached 50% confluence, GMEM medium was replaced with IVF medium containing 4 mg/mL BSA (instead of 6 mg/mL) and 2% non-essential amino acids (NEAA; IVC-1, see below).

The three-step embryo culture system consisted of culturing embryos in: (1) IVC-1 medium until day 2; (2) fresh IVC-1 medium containing 1% essential amino acids (EAA; IVC-1A) until day-5 and; (3) IVC-1A with 10% FBS instead of BSA (IVC-2) in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C until day 8 [24,25].

Embryo transfer and pregnancy detection

Vitrified/warmed presumptive zygotes and embryos were transferred by laparoscopy into the oviducts of gonadotropin-treated domestic cat recipients on day 1 after induction of ovulation or oocyte aspiration [6]. Recipients were examined by ultrasonography on day 21 after embryo transfer to determine pregnancy