Cryobiology xxx (2015) 1–7

Contents lists available at ScienceDirect

Cryobiology

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

State of actin cytoskeleton and development of slow-frozen and vitrified rabbit pronuclear zygotes

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ARTICLE INFO

Article history:
Received 27 July 2015
Received in revised form
24 November 2015
Accepted 27 November 2015
Available online xxx

Keywords:
Pronuclear
Slow-freezing
Vitrification
Actin filaments
Development
Rabbit

ABSTRACT

This study was focused on the effect of cryopreservation on the state of actin cytoskeleton and development of rabbit pronuclear zygotes. Zygotes were collected from superovulated females and immediately used for 1) slow-freezing in a solution containing 1.5 M 1,2-propanediol and 0.2 M sucrose, or 2) vitrification in a solution containing 42.0% (v/v) of ethylene glycol, 18.0% (w/v) of dextran and 0.3 M sucrose as cryoprotectants. After thawing or warming, respectively, zygotes were evaluated for 1) actin distribution, 2) *in vitro* or 3) *in vivo* development to blastocyst. Comparing actin filaments distribution, a significantly higher number of vitrified zygotes with actin distributed in cell border was observed (55 ± 7.7 vs. 74 ± 6.1% for slow-frozen vs. vitrified, respectively). After 24 and 72 h of *in vitro* development, significant differences in the cleavage and morula rate among the groups were observed (9 ± 2.4 and 3 ± 1.3 vs. 44 ± 3.0 and 28 ± 2.7% for slow-frozen vs. vitrified, respectively). None of the slow-frozen zygotes reached the blastocyst stage, in contrast to the vitrified counterparts (11 ± 1.9%). Under *in vivo* culture conditions, a significant difference in blastocyst rate was observed between vitrified and fresh embryos (6 ± 1.5 vs. 35 ± 4.4% respectively). Our results showed that alterations in actin cytoskeleton and deteriorated development are more evident in slow-frozen than vitrified pronuclear zygotes. Vitrification method seems to be a more effective option for rabbit zygotes cryopreservation, although pronuclear zygotes manipulation *per se* resulted in a notable decrease in embryo development.

1. Introduction

Successful freezing of mammalian embryos, including rabbits, has been the subject of intensive research over many years [34]. It maintains the advantage of full genetic complement of sire and dam conservation, protecting species and population integrity as well as heterozygosity [42]. Moreover, it is widely used in assisted reproductive technologies (ARTs) in both laboratory and domestic animals [32]. Banks of genetic resources are a valuable tool in livestock improvement schemes, where population control is necessary to measure the current rate of genetic gain or to preserve the present selected lines [31]. It has been shown that long-term storage of embryos in cryobank entails the advantage of maintaining similar pregnancy rate, fertility and survival at birth for at least 15 years [44,31].

At present, conventional slow-freezing and vitrification are the two major methods of embryo cryopreservation [56,49], although there are still concerns regarding whether one technique is better than the other [1]. It has been suggested that vitrification might be the cryopreserving procedure of choice for rabbit embryos, as non-cellular investments (*zona pellucida* and mucin coat) are frequently damaged during conventional freezing/thawing and with the vitrification approach it may be possible to reduce the damage [28,50]. Moreover, there is evidence that vitrification provides better implantation and birth rates for rabbit embryos than slow-freezing [47].

As with other species, survival of cryopreserved rabbit embryos depends on the cryoprotective agent (CPA) and the embryonic stage of development [21,35]. In rabbits, morula stage embryos in

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http://dx.doi.org/10.1016/j.cryobiol.2015.11.009
0011-2240/

particular are commonly cryopreserved with generation of live offspring (ranging between 25% and 65%) using either slow-freezing [58,59,40,46,47] or vitrification [28,60,36,40,38,47]. However, both rabbit oocytes and pronuclear zygotes are completely different scenarios and there are only a few publications reporting live offspring after rabbit oocyte (3.3–13.2%) [4,25,26] and pronuclear zygote (3.7–36%) [21,22,35] cryopreservation. The difficulties throughout the studies on oocyte and zygote cryopreservation in rabbits might be due to singularities (low surface/volume ratio, not activated genome, sensitivity of microtubules and microfilaments to high CPA concentration) which make the early stages highly sensitive to the cryopreservation process [41,39,5,25,26]. Nevertheless, there are reasons which make the cryostorage of freshly fertilized rabbit ova interesting. Zygotes are important in transgenic animal production, as the pronuclear microinjection of exogenous DNA is the most conventional and reliable method for transgenesis [20]. Transgenic rabbits are suitable tools for protein production, such as human interleukin-2 [7], insulin-like growth factor-1 [62] or human clotting factor VIII [9]. However, the rabbit is a unique mammal in that its embryos have thick mucin coat deposited during oviductal passage. Therefore, rabbit embryos cultured from the 1-cell stage *in vitro* to the morula or blastocyst stage have no mucin coat and after transfer the lack of mucin coat significantly increases pregnancy failure rates [27].

The aim of this study was to compare the effect of slow-freezing and vitrification procedure on the actin cytoskeleton status and *in vitro* or *in vivo* development competence of rabbit pronuclear zygotes.

2. Materials and methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma–Aldrich Química S.A (Madrid, Spain).

2.1. Animals

Five-month-old rabbit does belonging to the New Zealand White breed from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV) were used as donors and recipients. All experimental procedures involving animals were approved by the Research Ethics Committee of the UPV and licensed by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

2.2. Pronuclear stage zygote recovery

Eighteen New Zealand White females were superovulated by administration of 5 doses (7 IU each 12 h) of porcine follicle-stimulating hormone (pFSH, FOLLTROPINTM, BionicheTeoranta, Galway,Ireland) and 0.7UI of recombinant human luteinizing hormone (rhLH, Luveris®, Serono, MW, London, UK). The does were artificially inseminated with pooled semen at a density of 40×10^6 spermatozoa/mL in Tris-citric-glucose extender [61] (0.5 mL per doe) and induced to ovulate by intramuscular injection of 1 μg of Buserelin acetate 12 h after the last superovulation dose. Presumptive pronuclear stage zygotes were recovered from the oviducts 19 h after AI by flushing of each oviduct with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.2% of bovine serum albumin (BSA), 0.132 g/L of calcium chloride ($CaCl_2$) and antibiotics (sodium penicillin G300.000 UI, procaine G, penicillin 700.000 UI and dihydrostreptomycin sulphate 1250 mg, Penivet1, Divasa Farmavic, Barcelona, Spain).

2.3. Cryopreservation procedures

The slow-freezing procedure was adapted from previously described methods [51]. After recovery, zygotes were incubated for 10 min in a solution containing 1.5 M 1,2-propanediol (PROH) in a base medium (BM: DPBS + 20% foetal bovine serum, FBS). Zygotes were then placed into the freezing solution composed of 1.5 M PROH and 0.2 M sucrose in BM for 5 min and then mounted between two air bubbles in 0.25 mL sterile French mini straws (IMV Technologies. L'Aigle, France) sealed by a sterile plug. The straws were then placed in a programmable freezer (Cryologic, CL-8800) for the freezing process. Temperature was lowered from 20 °C to −7 °C at a rate of −2 °C/min. Manual seeding was performed at −7 °C. Temperature was then lowered to −30 °C at a rate of −0.3 °C/min. Finally, the straws were plunged directly into liquid nitrogen (LN_2) and stored until later use. For thawing, the straws were held at ambient temperature for 10–15 s and plunged into a water bath (20 °C). Zygotes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3 and 0.1 M sucrose in BM) for 5 min before being equilibrated for 10 min in TCM-199 containing 10% FBS. Afterwards, the zygotes were cultured in TCM-199 medium supplemented with 10% of FBS at 38.5 °C and 5% of CO_2 in humidified atmosphere.

The vitrification procedure was performed following the method of minimum essential volume (MEV), using Cryotop® as a device [30]. After recovery, zygotes were placed into equilibration solution containing 20% (v/v) of ethylene glycol (EG) in DPBS at 20–25 °C for 3 min, and then transferred into the vitrification solution composed of 42.5% EG (v/v), 18.0% (w/v) dextran and 0.3 M sucrose in DPBS. Three to five zygotes were placed in a cryotop in 2 μl of vitrification solution and plunged directly into LN_2 within 1 min. After storage in LN_2, the zygotes were warmed by immersing the cryotop into the warming solution composed of 0.5 M sucrose in DPBS. After 3 min, zygotes were washed three times in a solution composed of 0.25 M sucrose in DPBS. Finally, zygotes were washed in DPBS and equilibrated for 10 min in TCM-199 containing 10% FBS. After warming, the zygotes were cultured as stated above.

2.4. Actin cytoskeleton staining

Evaluation of cytoskeletal actin filaments was performed in the three experimental groups: slow-frozen, vitrified and fresh zygotes. Zygotes were washed in DPBS and then fixed in 4% (w/v) paraformaldehyde in DPBS (pH 7.2–7.4) for 45 min at 38.5 °C. After washing in DPBS, permeabilization was performed by 50 min incubation of zygotes in 0.5% Triton X-100 in DPBS. Afterwards, the zygotes were washed three times in DPBS-PVP solution (DPBS with 4 mg/ml polyvinylpyrrolidone; Sigma–Aldrich Chemie, Steinheim, Germany) and were then placed in the solution of phallodine-TRITC conjugate (Chemicon International; stock solution was diluted in DPBS at 1:500) for labelling of actin filaments, for 45 min. Thereafter the zygotes were transferred onto a microscopic slide and covered with 5 μl of Vectashield anti-fade mounting medium, containing nuclear DAPI stain (Vector Laboratories, Burlingame, CA, USA). The coverslip was attached to the microslide using nail polish. All treatments were performed at ambient temperature.

Stained zygotes were evaluated using a laser scanning microscope (LSM 700; ZEISS) equipped with an Axio Imager Z2 scanning unit. Phallodine-TRITC and DAPI fluorescence signals were excited using 546 and 405 nm laser, respectively. The images were acquired and processed using ZEN software. The actin cytoskeleton was classified as belonging to the grades according to [37] on the basis of appearance of actin filaments in rabbit morula stage embryos. We adapted the methodology for pronuclear stage zygotes, and classified Grade I as best (sharply stained actin filaments with

Please cite this article in press as: B. Kulíková, et al., State of actin cytoskeleton and development of slow-frozen and vitrified rabbit pronuclear zygotes, Cryobiology (2015), http://dx.doi.org/10.1016/j.cryobiol.2015.11.009