Theriogenology 82 (2014) 599–604

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

First pregnancy and live birth from vitrified rabbit oocytes after intraoviductal transfer and *in vivo* fertilization

E. Jiménez-Trigos, J.S. Vicente, F. Marco-Jiménez*

Institute of Science and Animal Technology, Laboratorio de Biotecnología de la Reproducción, Universidad Politécnica de Valencia, Valencia, Spain

ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 22 May 2014
Accepted 24 May 2014

Keywords:
Vitrification
Slow-freezing
Offspring
In vivo fertilization
Oocyte transfer

ABSTRACT

Intraoviductal oocyte transfer in combination with *in vivo* fertilization has arisen as an alternative method to induce pregnancies from cryopreserved oocytes in rabbits. In this study, offspring were obtained for the first time from vitrified rabbit oocytes using this technique. In all the experiments, recipients were artificially inseminated 9 hours before oocyte transfer. Cryopreserved (vitrified and slow-frozen) and noncryopreserved (fresh) oocytes were transferred into both oviducts, which were immediately closed using cyanoacrylate tissue adhesive to block the entry of the recipient's own oocytes. Three transferred group females that received vitrified oocytes became pregnant and delivered a total of nine live young naturally. The results revealed that there were no differences in the live birth rate between vitrified and slow-frozen oocytes (5.5% and 4.4%, respectively). When fresh oocytes were transferred, this rate increased to 19.2%, whereas in the control females (nontransferred) the rate of offspring obtained was 71.4%. This is the first reported result of the development to term of vitrified rabbit oocytes and suggests that an *in vivo* environment could help improve the results of oocyte cryopreservation.

1. Introduction

Cryopreservation of female genetics can be achieved through the preservation of oocytes, embryos, or ovarian tissue [1]. Current knowledge suggests that cryopreservation of unfertilized oocytes plays an essential role in different assisted reproductive technologies [2]. In livestock, it permits the preservation of valuable genetic lines until the female and the appropriate male express their genetic merit and suitable mating can be accomplished [3,4].

Because Whittingham [5] successfully froze mouse oocytes, cryopreservation methodology and materials have progressed and live birth has been obtained in different species [6]. Nevertheless, developmental rates are compromised and lower than those yielded by fresh oocytes [7–10]. In rabbits, to our knowledge, only four studies using slow-freezing oocytes obtained live young. Two of them were carried out in 1989 and revealed that the rate of live births per oocyte transferred was reported to be 7.5% (4 of 53) [11] and 8.6% (9 of 105) [12], but in unborn offspring at Day 25 of gestation. The other two studies were carried out in our laboratory and revealed that this rate ranged from 3.3% (4 of 121) to 13.2% (10 of 76) ([6] and personal contribution). However, to our knowledge, to date there are no reports of offspring obtained from vitrified oocytes.

Vitrification has emerged as an optimal procedure for oocyte and embryo cryopreservation [13]. This method avoids intracellular ice crystallization by supercooling the solution and transforming it into a "vitreous," state [14], which could reduce oocyte damage, increasing survival rates after warming [15]. However, a critical concentration of cryoprotectants is required for this process, which contributes to the damages associated after warming [16,17]. Rabbit oocytes are particularly sensitive to high levels of

* Corresponding author. Tel.: +34 96 38794335; fax: +34 96 3877439.
E-mail address: fmarco@dca.upv.es (F. Marco-Jiménez).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.05.029

cryoprotectants [18,19], and as a result, blastocyst rate after vitrification is compromised [18–23].

To date, different technologies to assess *in vitro* developmental ability of cryopreserved rabbit oocytes have been used, such as parthenogenetic activation [18,19,22,23], *in vitro* fertilization [11], and intracytoplasmic sperm injection [20,21,23]. However, they have not been successfully developed in this species [20,24,25], and offspring were only obtained from slow-frozen oocytes using *in vivo* [6,12] and *in vitro* [11] fertilization.

Intraoviductal oocyte transfer has emerged as a minimally invasive method to induce pregnancies in rabbits [6] and mares [26,27]. It has also been a good alternative to evaluate the capacity of cryopreserved oocytes to generate viable offspring.

In this study, *in vivo* fertilization after intraoviductal oocyte transfer was used to obtain live offspring from vitrified rabbit oocytes for the first time.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated.

2.2. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (Official Spanish State Gazette [BOE], 2013). Ethical approval for this study was obtained from the Universidad Politécnica de Valencia Ethics Committee. New Zealand white females (n = 60), 5-month old, were used as oocyte donors and recipients. The animals used came from the experimental farm of the Universidad Politécnica de Valencia. The rabbits were kept in conventional housing (with an alternating cycle of 16 light hours and 8 dark hours, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 °C and 25.5 °C, respectively). All rabbits had free access to fresh food and water.

2.3. Oocyte collection

Cumulus oocyte complexes at the metaphase II stage were collected from donor females induced to ovulate by an intramuscular dose of 1 μg of buserelin acetate (Suprefact; Hoechst Marion Roussel, S.A., Madrid, Spain). Cumulus oocyte complexes were collected 14 to 15 hours after ovulation induction by flushing each oviduct with Dulbecco's phosphate-buffered saline (DPBS) without calcium chloride supplemented with 0.1% (wt/vol) of BSA. Cumulus cells were removed and oocytes were incubated for 15 minutes at room temperature with 0.1% (wt/vol) hyaluronidase.

2.4. Vitrification procedure

The vitrification protocol with Cryotop device (Kitazato BioPharma Co, Japan) and solution was described by Kuwayama, et al. [28]. Oocytes were first exposed for 3 minutes to equilibration solution containing 3.75% (wt/vol) ethylene glycol (EG) and 3.75% (wt/vol) DMSO in base medium (BM: Tissue Culture Medium 199 (TCM199) + 25 mM HEPES + 20% [v:v] serum substitute supplement, SSS [Irvine Scientific, County Wicklow, Ireland]). Then, the oocytes were exposed for 3 minutes to solution containing 5% (wt/vol) EG and 5% (wt/vol) DMSO in BM, after which the oocytes were placed for 9 minutes in a solution containing 7% (wt/vol) EG and 7% (wt/vol) DMSO in BM. Finally, the oocytes were transferred to vitrification solution composed of 15% (wt/vol) EG, 15% (wt/vol) DMSO, and 0.5 M sucrose in BM before being loaded onto Cryotop devices and directly plunged into liquid nitrogen (LN2) within 1 minute. For warming, oocytes were transferred stepwise into decreasing sucrose solutions (1 M for 1 minute and 0.5 M for 3 minutes) and then washed twice in BM for 5 minutes. After warming, the oocytes were incubated for 2 hours in TCM199 medium containing 20% (v:v) fetal bovine serum (FBS) at 38.5 °C and 5% CO_2 in humidified atmosphere.

2.5. Slow-freezing procedure

The slow-freezing procedure was adapted from previously described methods [29]. Briefly, oocytes were incubated for 15 minutes at room temperature in a solution containing 1.5 M of 1,2-propanediol in DPBS and 20% FBS. Oocytes were then placed for 10 minutes in the freezing solution composed of 1.5 M of 1,2-propanediol and 0.2 M of sucrose in DPBS and 20% FBS and mounted between two air bubbles on 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 (CL-8800; CryoLogic) 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, straws were directly plunged into LN2 and stored for later use.

For thawing, the straws were taken out of the LN2 into ambient temperature for 10 to 15 seconds and plunged into a 20 °C water bath. Oocytes were transferred stepwise into decreasing sucrose solutions (0.5, 0.3, and 0.1 M sucrose in TCM199 with 20% FBS) for 5 minutes before being equilibrated for 10 minutes in TCM199 containing 20% (v:v) FBS. After that, oocytes were incubated for 2 hours in TCM199 medium containing 20% (v:v) FBS at 38.5 °C and 5% CO_2 in humidified atmosphere.

2.6. In vivo fertilization

Recipient females were artificially inseminated 9 hours before oocyte transfer with 0.5 mL of fresh heterospermic pool semen at a rate of 40×10^6 spermatozoa per milliliter in Tris-citric-glucose extender [30]. Motility was examined at room temperature under a microscope with phase contrast optics at 40× magnitude. Only ejaculates with greater than 70% motile sperm were pooled. Immediately after insemination, ovulation was induced by an intramuscular injection of 1 μg of buserelin acetate.

The intraoviductal oocyte transfer procedure was adapted from a previously described technique used in rabbit [31]. The equipment used was a Hopkins Laparoscope,