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Original Article

Vitrification of mouse MII oocytes: Developmental competency using paclitaxel

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ABSTRACT

Objective: Oocyte cryopreservation provides an important alternative for fertility preservation for women who will be treated with cytotoxic drugs. However, it can cause spindle disorganization of microtubules, putting the zygote at risk for aneuploidy. Paclitaxel is known to stabilize the microtubules that constitute the spindle. The aim of this study was to investigate the suitable concentration of paclitaxel for adding to the vitrification media to improve the developmental potential of post-thawed mature oocytes to blastocyst formation in mice.

Materials and Methods: A total of 300 MII oocytes were retrieved from superovulated mice, and were divided into three groups of control, Experimental I, and Experimental II. Oocytes in Experimental I and Experimental II were cryopreserved in the presence of 0.5μM or 1μM of paclitaxel in vitrification media, respectively. After thawing, all oocytes were incubated in G-IVF medium for 1 hour. From each group,12 oocytes were selected for viability evaluation by Hoechst/propidium iodide nuclear staining. Standard *in vitro* fertilization was performed on the rest of the oocytes and embryo development was followed to the blastocyst stage.

Results: Fertilization rate was not significantly different between the three groups. However, the cleavage rate (55%) in Experimental II group was significantly lower compared to Experimental I (88%) and control groups (83%). There was a detectable difference between the three groups at the blastocyst rate (Experimental I and control groups, $p = 0.004$; Experimental II vs. control and Experimental I, $p < 0.001$). The highest rates of parthenogenesis and arrest were in Experimental II (16% and 21%, respectively) compared with control (6% and 5%, respectively) and Experimental I (5% and 3%, respectively). There was also a significant decrease in viability rate of oocytes in Experimental II compared to the other groups.

Conclusion: A high concentration of paclitaxel, an anticancer drug, interrupted the mouse oocyte competency when supplemented to vitrification media. Consequently, the optimal concentration of this cytoskeleton stabilizer may improve the post-thawed developmental abilities of oocytes.

Introduction

Oocyte cryopreservation provides an important alternative for fertility preservation for women who might lose their ovarian function because of surgery, chemotherapy, radiotherapy, failed *in vitro* fertilization (IVF) cycle, ovarian hyperstimulation syndrome, or inability to obtain sperm on fertilization day. Moreover, oocyte cryopreservation can be a choice for pregnant women to maintain their gametes even after the physiological effects of age, or women who are not married and still want to preserve their fertility [1,2]. However, unlike embryos, the freezing protocols used for oocytes is not ideal and clinically has had little success because of the differences in oocyte and embryo membrane permeability, the meiotic spindles, and ultimately, special physiological oocyte conditions [3,4].

It was recently shown that there was no difference in the survival rate of human oocytes vitrified at the germinal vesicle (GV) stage or MII stage, although the maturation ability of GV oocytes

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was reduced [5]. Many studies have reported that vitrification is an efficient cryotechnology for oocytes storage [6–8]. However, it must be considered that various factors in vitrification, such as high concentrations of cryoprotectants, cooling, and osmotic stress may contribute to the initiation of apoptosis that later threatens viability and development competency [9]. Vitrification of MII oocytes can cause spindle disorganization and/or depolymerization of microtubules, putting the zygote at risk for aneuploidy. As a result, it causes low fertilization and embryo development rates from MII oocytes after thawing [4].

Paclitaxel is known to stabilize the microtubules that constitute the spindle [10]. It is an anticancer drug that in certain concentrations may cause cell toxicity. Paclitaxel was used in different mammalian species with less consideration on its toxicity and the probable effects on oocyte viability. In addition, literature review shows that only specific dose of paclitaxel were applied, while different doses should be evaluated on oocytes survival [11,12]. It seems necessary to obtain an optimal concentration of paclitaxel for favorable impact as a cytoskeleton stabilizer on post-thawed oocytes for future possible clinical use. Therefore, the aim was to obtain a suitable concentration of paclitaxel for adding to the vitrification media to improve the developmental potential and viability of post-thawed MII oocytes, with consequent embryo developmental ability, to blastocysts in mice.

Materials and methods

Animals

In the present study, 6–12-week-old adult female Naval Medical Research Institute (NMRI) mice were maintained under controlled temperature (25 ± 38°C), proper humidity (50 ± 5%), and a 12-hour light/dark cycle. Also, this study was approved by the Ethics Committee of our institution.

Chemicals

All culture media were purchased from Vitrolife (Kungsbacka, Sweden). Paclitaxel was purchased from Sigma Chemical (St Louis, MO, USA).

Ovarian stimulation and oocyte preparation

Superovulation was induced by intraperitoneal injections of 10 IU pregnant mare's serum gonadotropin (PMSG; 80056-608; VWR Scientific Inc.) and 10 IU human chorionic gonadotropin (hCG, CG-10; Sigma) administered 48–50 hours apart. The oviducts were excised 15 h after hCG injection and oocytes were collected by the flushing method. Immediately, cumulus-oocyte complexes were placed in G-MOPS media (Vitrolife). Then, cumulus-oocyte complexes were denuded of their cumulus and corona cells by exposure to HEPES-buffered medium (Vitrolife) containing 80 IU ml^{-1} hyaluronidase, and by pipetting with pasture pipettes. The oocytes maturity was assessed by visualization of the first polar body under a stereomicroscope. Only MII oocytes with good morphology were selected for vitrification [13,14].

Vitrification and thawing of MII oocytes

MII oocytes were divided into three groups of control, Experimental I, and Experimental II. In the control group, oocytes were placed in equilibration solution containing 7.5% ethylene glycol (Merck Co, Germany), 7.5% dimethyl sulfoxide (Merck Co) in Ham's F10 media (Vitrolife) supplemented with 20% human serum albumin (HSA: Plasbumin Co., USA) for 10 minutes at room temperature (RT). Then, oocytes were removed and placed into vitrification solution containing 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.5M sucrose (Sigma Co., USA) in Ham's F10 medium supplemented with 20 % HSA for 45–60 seconds at RT. The samples were then loaded onto cryotops (Kitazato BioPharma Co., Japan), at a volume > 1 μL and immediately transferred to liquid nitrogen for 1 month. In Experimental Group I and Experimental Group II, the vitrification procedure was done as well as in the control group, but equilibration solution and vitrification solution were supplemented with 0.5μM and 1μM paclitaxel, respectively [15].

Thawing of the oocytes was performed by placing the cryotops in thawing solution in three stages: (1) thawing solution (Ham's F10 supplemented with 20% HSA and 1M sucrose) for 60 seconds; (2) Dilution Solution 1 (Ham's F10 supplemented with 20% HSA and 0.5M sucrose) for 3–5 minutes; (3) Dilution Solution 2 (Ham's F10 supplemented with 20% HSA and 0.25M sucrose) for 3–5 minutes. The thawed oocytes were then rinsed three to five times in washing solution (Ham's F-10 + 20% HSA). After thawing, the oocytes were transferred into G-IVF medium (Vitrolife) at 37°C in an incubator with 5% CO_2 and 95% air with high humidity. One hour after incubation, the oocytes from each group were collected for IVF or staining protocols [16].

Viability evaluation of oocytes and embryos

A total of 12 oocytes from each group were assessed for viability based on oolemma integrity with Hoechst/propidium iodide (PI) nuclear staining [17]. Supravital immunostaining was prepared with 20 μg/mL of Hoechst 33342 (Sigma, USA), and 20 μg/mL of PI (Sigma, USA) added to Ham's F10 medium supplemented with 10% HSA. For viability evaluation, a few oocytes were kept in a dish with a few drops of stain. They were incubated for 15 minutes in the Hoechst/PI blended stain. After that, the oocytes were washed and observed under a fluorescence microscope (Olympus; Tokyo, Japan). The dead cells showed red fluorescence stain (PI-positive) for disruption of cellular membrane, while, the viable cells showed blue fluorescence (PI-negative) for the intact cell membrane (Figure 1).

Embryo viability was assessed according to procedure of Hosseini et al [18]. Expanded blastocysts were first washed twice in prewarmed Ca_2^+ and Mg_2^+ free phosphate buffer saline, then incubated in the freshly prepared PI (P 4127; 300 μg/mL) and Hoechst (H33342: 5μg/mL) for 20 minutes at the optimum incubation conditions of the embryos. Blastocysts were then washed three times by warm phosphate buffer saline and embryos were quickly fixed in 2.5% glutaraldehyde for at least 5 minutes at RT. Fixed blastocysts were mounted in a drop of glycerol. A coverslip was placed on top of the blastocysts and gently pressed until the embryos were slightly flattened. Prepared samples were examined under a fluorescent inverted microscope (Olympus BX51). The late apoptotic and early to completely necrosed cells appeared as red, while alive cells with intact cell membrane appeared as blue.

IVF and embryo assessment

For IVF, the spermatozoa were collected from 8–12-week-old mice. The sperms were released into the medium and dispersed for 15 minutes at 37°C. After dispersion, the sperm concentration was determined to achieve a final concentration of 1×10^6 sperm/mL. The insemination dishes were then incubated for 1–2 hours before addition of oocytes. The thawed oocytes from each group were separately transferred to 100 μL droplets of G-IVF medium. After 5 hours of incubation with spermatozoa, the oocytes were washed and cultured in G1 medium. Fertilization was determined by the presence of two pronuclei. The progression of embryonic development was monitored every 24 hours for 3 days until the blastocyst stage.