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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.

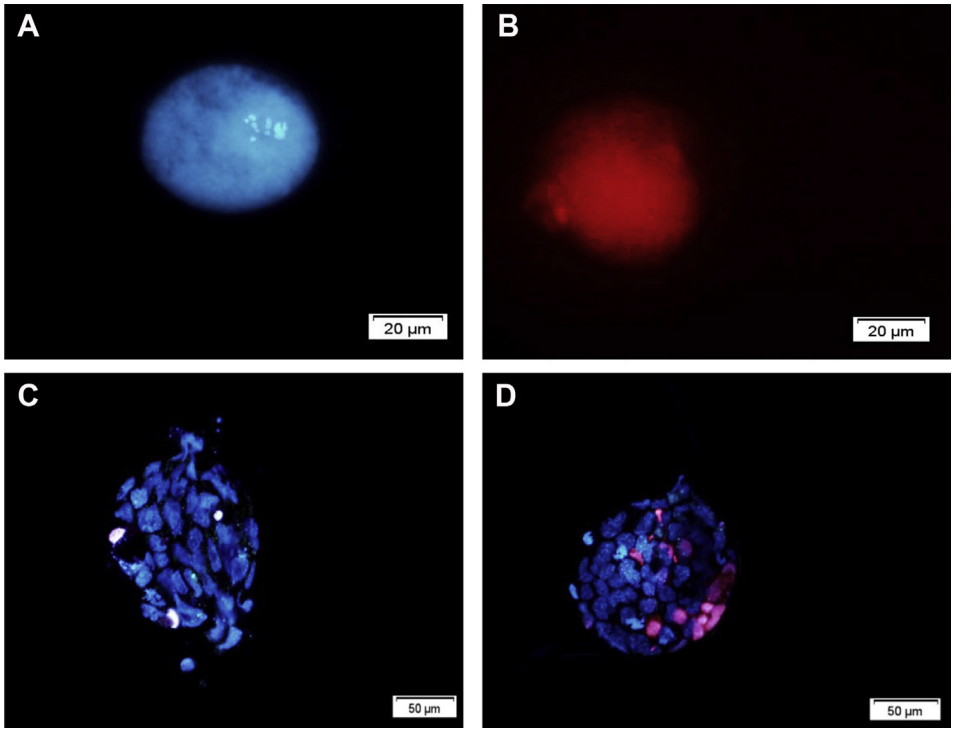

Figure 1. (A, B) MII oocyte and (C, D) blastocysts after staining with Hoechst/propidium iodide. Viable cell (blue fluorescence), dead cell (red fluorescence).

Statistical analysis

The analyses were performed using SPSS version 20 for windows (SPSS Inc., Chicago, IL, USA). The data were analyzed using the Chi-square test. A p value < 0.05 was considered significant.

Results

After thawing, there was an insignificant difference in oocyte degeneration rate between the control and experimental groups ($p > 0.99$, Table 1). Moreover, the most parthenogenesis and arrest rates were in Experimental II (16% and 21%, respectively) that was supplemented with 1μM paclitaxel compared with control (6% and 5%, respectively) and Experimental I (5% and 3%, respectively). Also, the fertilization rate was statistically not different between the three groups, although there were more positive effects at Experimental I than Experimental II (Table 2). However, the cleavage rate (55%) in Experimental II was significantly lower compared to the Experimental I and control groups (88% and 83%, $p < 0.001$) as well as a significant difference between the Experimental II and control groups ($p = 0.001$). However, 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, $p < 0.001$; Table 2). Also, our data showed that there was a significant decrease in viability rate of oocytes in Experimental II compared to Experimental I ($p = 0.007$) and control ($p < 0.001$). Regarding the rate of blastocysts viability, there was a decreasing trend in the Experimental II group, but the difference was not significant compared to other groups (Table 3).

Table 1
Oocyte qualification based on morphology after thawing in control and experimental groups.

Oocyte variables (%)	Control ($n = 100$)	Exp. I ($n = 100$)	Exp. II ($n = 100$)	p
Degeneration	10	9	10	$> 0.99^{a,b,c}$
Parthenogenesis	6	5	16	0.01^{a} $< 0.99^{b}$ 0.04^{c}
Arrest	5	3	21	$< 0.99^{a}$ 0.7^{b} 0.001^{c}
Normal morphology	79	83	53	$< 0.99^{a,c}$ 0.58^{b}

Exp. = Experimental.
[a] Difference between Exp. I and Exp. II.
[b] Difference between Exp. I and control.
[c] Difference between Exp. II and control.

Discussion

According to the reports from Lee et al [11] and Park et al [12], we used two different concentrations of paclitaxel during vitrification of mouse MII oocytes. Because of disadvantages of oocyte cryopreservation at the GV stage, such as higher sensitivity of the

Table 2
Fertilization, cleavage, and blastocyst rates following *in vitro* fertilization (IVF) protocol in mice.

Variable	Control	Exp. I	Exp. II	p
Fertilization (%)	60/79 (76)	68/83 (81)	36/53 (68)	0.051^{a} 0.49^{b} 0.27^{c}
Cleavage (%)	50/60 (83)	60/68 (88)	20/36 (55)	$< 0.99^{a,c}$ 0.42^{b}
Blastocyst (%)	27/60 (45)	45/68 (66.2)	6/36 (16.7)	$< 0.99^{a,c}$ 0.004^{b}

Exp. = Experimental.
[a] Difference between Exp. I and Exp. II.
[b] Difference between Exp. I and control.
[c] Difference between Exp. II and control.

Table 3
Viability rate of MII oocytes and blastocysts derived from *in vitro* fertilized oocytes.

Viability (%)	Control	Exp. I	Exp. II	*p*
MII oocyte	91.6	100	75	0.007[a] 0.002[b] < 0.99[c]
Blastocyst	93.1	92.7	81.8	> 0.99[a] 0.7[b,c]

Exp. = Experimental.
[a] Difference between Exp. I and Exp. II.
[b] Difference between Exp. I and control.
[c] Difference between Exp. II and control.

meiotic spindle, when the chromosomes were within the nuclear membrane with subsequent low IVF outcome, MII oocytes were examined as the first candidate in this investigation [19]. The optimal concentration of cytoskeleton stabilizers that was used by some investigators to pretreat different kinds of mammalian or human oocytes was 1μM paclitaxel. Also, 1μM and 0.5μM concentrations of paclitaxel were selected according to previous studies and our pilot works (data not shown). Our results showed significant differences in post-thaw oocyte survival rates between groups, in agreement with Lee et al [11]. Therefore, the results showed that low concentration of paclitaxel may improve the survival rate of MII oocytes after freezing and thawing. By contrast, a higher concentration of this chemical was harmful with elevation in rates of oocytes arrest and parthenogenesis after thaw. Also, there were no significant differences in fertilization rate of intact oocytes between the three groups, although there was a positive trend in Experimental Group I (81%),in comparison with the Experimental II and control groups, respectively (68% and 76%). Park et al [12] showed similar results in ICR mouse oocytes. They showed an improvement in post-thawed preimplantation embryo development after vitrification by adding 1μM paclitaxel without significant difference in other parameters, such as fertilization [12]. However, there was incoherence in our finding in fertilization, blastocyst rate, and blastocyst quality at 1μM paclitaxel use in comparison with the control group. One study used 1μM paclitaxel for vitrification of *in vitro* matured oocytes in porcine. They indicated an improvement of blastocyst rate in comparison with untreated oocytes, but with significant difference with intact oocytes (73% vs. 18.3%) and indifferent results on blastocyst cell numbers (50.3 ± 10.3 vs. 56 ± 8) [10]. By contrast, Jiménez-Trigos et al [20] demonstrated that normal spindle chromosome configuration, and blastocyst development of *in vivo* matured rabbit oocytes were damaged during vitrification, which was not improved by Taxol pretreatment before vitrification by utilizing 1μM concentration.

However, animal species, quality of oocytes and differences in vitrification method and timing of pretreatment [21] can all affect the generated results, causing our different findings at 1μM paclitaxel. Recently, higher survival rates have been achieved with efficient vitrification compared with the traditional slow freezing method [22]. However, there is still room in the vitrification procedure for enhancement, allowing complete retrieval of developmental competence after thawing. This possibility is mostly available through protection of the oocyte cytoskeleton system, which is involved in meiotic division and embryo development from injury [23]. Our main goal in this study was to improve vitrification conditions by increasing the oocyte retrieval and developmental ability of derived embryos, using safe and nontoxic concentrations of stabilizer. Because, the stabilizer increases the rate of polymerization by reducing the critical concentration of tubulin. It is noticeable that tubulin is a main component for polymerization mitotic spindles. In addition, stabilized MII oocytes will undergo more frequent cytoskeleton rearrangement after thawing, preparing for further treatment. Paclitaxel is able to stabilize the cytoskeleton and interferes with spindle formation during the cryopreservation procedure, ensuring that post-thaw oocytes proceed to future development [24].High doses of paclitaxel, however, stabilize microtubules by causing a tighter linkage between tubulin dimers and by enhancing microtubular cross-linking after changes in the conformation and binding of high molecular weight microtubule-associated proteins [15]. Like all drugs, paclitaxel exhibits concentration-dependent effects, and utilized concentration at vitrification media referred to mechanism of its action on mitosis and cell death. Higher concentrations of paclitaxel were originally believed to be responsible for its efficacy in cancer therapy [25]. Therefore, only concentration may improve the developmental embryo competency and post-thawed viability that prevent mitotic spindles of MII oocytes during vitrification.

However, the best condition for application of these stabilizers for clinic is still controversial and needs further investigations using serial experiments on defining the optimal dose of paclitaxel. There are only a few studies on following developmental ability, viability, and pregnancy rates in oocytes treated with the abovementioned drug. Moreover, one of the limitations of our work was lack of chromosomal and spindle detection assays, which should be discussed at later investigations.

In conclusion, paclitaxel, as an anticancer drug, can preserve oocyte integrity when used at low concentrations in a vitrification program. Consequently, the optimal concentration of these cytoskeleton stabilizers may improve the post-thaw developmental abilities of mature oocytes without causing irreversible harm.

Conflict of interest

The authors have no conflicts of interest relevant to this article.

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