J Assist Reprod Genet (2015) 32:1197–1203
DOI 10.1007/s10815-015-0480-8

GAMETE BIOLOGY

Effect of different rehydration temperatures on the survival of human vitrified-warmed oocytes

Gao Shanshan[1,2,3,4,5] · **Li Mei**[1,2,3,4,5] · **Wu Keliang**[1,2,3,4,5] · **Sheng Yan**[1,2,3,4,5] · **Tang Rong**[1,2,3,4,5] · **Chen Zi-Jiang**[1,2,3,4,5]

Received: 9 October 2014 / Accepted: 13 April 2015 / Published online: 10 May 2015

Abstract
Purpose To evaluate the effect of different exposure temperatures during the dilution process on the survival rate of vitrified oocytes and following development.
Methods Patients were divided at random into two groups for different dilution temperature (20–22 °C, RT group; 37 °C, 37 °C group) according to computer-generated random numbers on the day of oocyte warming. The survival and fertilization rates of vitrified oocytes as well as the implantation and clinical pregnancy rates of the resulting embryos were recorded.
Results A total of 662 and 676 oocytes were warmed in the 37 °C group and RT group, respectively, and significant difference was observed in the survival rate between 37 °C group (88.37 %) and RT group (79.88 %) (P=0.0000). There was significant difference between the survival rate of 37 °Cgroup (87.27 %) and RT group (75.64 %) in nondonor patients (P=0.0001). Multiple linear regression analysis showed that dilution temperature (β=0.079, P=0.017) and clinical outcomes of fresh cycles (β=0.063, P=0.001) were significantly and independently associated with survival rate. No significant difference was found between the 37 °C group and RT group in: fertilization rate (66.67 versus 65.37 %), implantation rate (20.0 versus19.46 %), clinical pregnancy rate (37.5 versus 35.0 %).
Conclusions In conclusion, the results of this study give supportive evidence of the application of 37 °C in the dilution process, especially for oocytes of poor quality. Further studies with well-controlled experimental groups are needed to optimize protocols for human oocyte vitrification.

Keywords Oocyte · Vitrification · Dilution temperature · Rehydration

Capsule The temperature at which the oocytes are exposed to CPA solutions might have different osmotic and toxic consequences on cryosurvival and subsequent development. In this study, we aimed to compare the effect of different temperatures during rehydration on the cryosurvival and subsequent development of vitrified oocytes from infertile patients. The results of this study give supportive evidence of the application of 37 °C in the dilution process, especially for oocytes of poor quality.

✉ Chen Zi-Jiang
chenzijiang@hotmail.com

[1] Center for Reproductive Medicine, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, People's Republic of China

[2] National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Jinan, People's Republic of China

[3] Reproductive Medical Hospital Affiliated to Shandong University, Jinan, People's Republic of China

[4] The Key Laboratory for Reproductive Endocrinology of Ministry of Education, Jinan, People's Republic of China

[5] Shandong Provincial Key Laboratory of Reproductive Medicine, Jinan, People's Republic of China

Introduction

Oocyte cryopreservation has become an integral part of routine IVF procedure in recent years [1]. It provides flexibility in clinical scenarios such as the unavailability of sperm at the time of oocyte retrieval, oocyte donation programs, poor responders, cancer patients and surplus oocyte storage after controlled ovarian stimulation as a supplement to embryo cryopreservation. The clinical applications of oocyte cryopreservation are due mainly to the significant advances in implementing vitrification at ultra-rapid cooling rates in oocyte cryopreservation [2]. However, consistent results have not been achieved across different groups. The available

literature suggests that rates of vitrified donor oocytes are 92.5 to 96.9 % and 55.45 to 65.2 %, respectively [1, 3, 4]. In a meta-analysis of vitrified autologous (non-donor) oocytes, the survival rates ranged from 68.6 to 89.7 %, and the clinical pregnancy rates ranged from 10 to 43.3 % [5]. Oocyte quality is one of the major factors affecting the outcome of oocyte vitrification, which can be due to variations in female age, ovarian stimulation protocols and infertility factor. Therefore, it is essential to optimize vitrification protocols tailored to different infertile patient groups.

Oocyte vitrification uses high concentrations of cryoprotective agents (CPAs) to avoid intracellular crystal formation [6]. However, the high concentration of CPAs in these vitrification solutions increase the risk of cell damage by the elevated toxic effect as well as osmotic stress. Both the cytotoxicity and osmotic stress are influenced by temperature, which is not being employed consistently in various protocols used by different groups. For example, the first live birth from vitrified human oocytes was conducted at a physiological temperature (37 °C) for the entire procedure including equilibration, vitrification, warming and dilution steps [7]. The concern regarding the potentially increased cytotoxicity of high concentrations of CPAs at physiological temperature has resulted in modified protocols employing lower, non-physiological temperatures during equilibration, vitrification and rehydration processes with variable success [8–11]. The theory that lowering the temperature during exposure to CPAs reduces cytotoxicity is somewhat misleading, since these protocols typically require longer incubation periods to allow permeation of CPAs and adequate dehydration. The temperature at which the cells are exposed to CPA solutions might have different osmotic and toxic consequences on cryosurvival and subsequent development, due to temperature-dependent hydraulic conductivity and CPA permeability; therefore, the general consensus is to carry out warming at 37C. The subsequent serial dilutions are then either conducted at RT [9–13] or at 37 °C [7, 14–17].

The quality of oocytes varied due to differences in patient ages, ovary stimulation regimes and infertility factors. Since oocyte quality has a significant effect on cryosurvival and subsequent development, optimized protocols tailored to different patients may be advantageous. In this study, we aimed to compare the effect of different temperatures during rehydration on the cryosurvival and subsequent development of vitrified oocytes from infertile patients undergoing in vitro fertilization (IVF)-embryo transfer (ET) cycles. The hypothesis of this study was that rehydration at physiological temperature would improve cryosurvival of oocytes and their subsequent development as compared to rehydration at room temperature. The survival and fertilization rates, as well as implantation and clinical pregnancy rates, of the resulting embryos were compared.

Materials and methods

All chemicals were obtained from Vitrolife (Sweden) company unless otherwise indicated.

Source of oocytes

An institutional review board at Shandong Provincial Hospital approved this clinical application of oocyte vitrification. Patients with more than 20 oocytes retrieved were given the option to vitrify their supernumerary oocytes for future use. Some patients chose oocytes vitrification because of unavailability of sperm at the time of oocyte retrieval. We estimate that at least 324 oocytes per group would be required to show a 10 % difference in fertilization rate between the two groups, assuming a statistical power of 80 % at an alpha level of 0.05. All of the patients gave their written informed consents. The participants were blinded to the protocol used.

Donor oocytes were from patients who volunteered to donate their surplus cryopreserved oocytes. The patients were screened by trained personnel using a detailed questionnaire about patient's medical history and their family medical history, coupled with a pertinent physical examination, chromosomal analysis and serum virus detection.

Nondonor oocytes were from patients who failed to have a live birth with their fresh IVF-ET and frozen embryo transfer (FET) cycles and returned for ET using embryos generated from their vitrified-warmed oocytes (Table 1).

Oocyte vitrification and warming

Mature oocytes were equilibrated in equilibration medium (Medicult, Jyllinge, Denmark) containing 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) 1,2-propanediol (PROH) for 5 min at room temperature, and then transferred to vitrification medium (Medicult) containing 15 % (v/v) EG, 15 % (v/v) PROH and 0.5 M sucrose at room temperature for 45 to 60 s. Oocytes in a group of 2–3 were then loaded on Cryoloop, and plunged immediately into liquid nitrogen (LN_2) for storage [8]. Warming of oocytes were conducted by direct immersion of Cryoloop into warming medium (Medicult) containing 1.0 M sucrose for 1 min at 37 °C. Oocytes from donors or nondonors were then randomly assigned into two treatment groups using a computer-generated randomization model for rehydration. Specifically, oocytes were randomly transferred into diluent medium-I (Medicult) containing 0.5 M sucrose for 3 min and then into diluent medium-II (Medicult) containing 0.25 M sucrose for 3 min at two different temperatures (20–22 °C, RT group; 37 °C, 37 °C group). All diluting solutions at 37 °C were covered with mineral oil pre-heated to 37 °C to prevent evaporation. Oocytes were then washed twice in washing medium (Medicult) for 3 min each at 37 °C for both groups. Oocytes

Table 1 Patients' characteristics of oocytes-retrieval-cycles and laboratory, clinical outcomes of fresh oocytes

Fresh Cycles	group 37 °C (nondonor)	group RT (nondonor)	group 37 °C (donor)	group RT (donor)	*P* value	
No. of patients	35	37	35	34	–	
Age(±SD)	30.17±3.85	29.89±3.73	28.37±3.46	28.50±3.61	0.7549	0.8791
No. of MII oocytes retrivaled	25.46±8.26	22.08±8.46	30.26±11.68	30.76±9.65	0.0910	0.8471
No. of patients freezing for supernumerary oocytes	23	23	32	32	–	
No. of patients freezing for unavailability of sperm	9	12	0	0	–	
No. of MII oocytes fresh fertilized	470(18.80±3.50)	455(16.85±5.90)	–	–	0.0948	–
Normal Fertilization (2PN) rates(%)	262(55.74)	238(52.31)	–	–	0.2943	–
Clinical pregnancy cycles(%)	4(11.43)	2(5.41)	22(62.86)	21(61.76)	0.4230	0.9254
Live birth cycles	0	0	17(48.57)	20(58.82)	–	0.3932

with intact zona pellucida and plasma membrane were considered to be viable and were cultured at 37 °C in 5 % CO_2 in air for 2–3 h before fertilization by intracytoplasmic sperm injection (ICSI).

Intracytoplasmic sperm injection, embryo culture, and embryo transfer

All viable oocytes were inseminated by ICSI (Day 0). Fertilization was evaluated 16–18 h after ICSI and embryos with two pronuclei were regarded as normal fertilized embryos (Day 1). Embryos were graded on Day 2 and 3 by the number and size of blastomeres. Embryos with 3–5 blastomeres of uniform size on Day 2 (40–42 h after injection) and more than 7 blastomeres of uniform size on Day 3 (64–66 h after injection) were regarded as normal developing embryos. Embryos were classified as high quality embryos when there were more than 7 blastomeres and less than 20 % fragments on Day 3. Embryo transfer was carried out on Day 2 or Day 3 depending on embryo quality or quantity. No more than 3 embryos were transferred per patient per transfer. The supernumerary high quality embryos were then vitrified for future use.

Endometrial preparation

The endometrium was prepared using 4 mg of oral oestradiol supplementation (oestradiol valerate) daily for 5 days starting on day 2–3 of the menstrual cycle, and then followed by 6 mg oral oestradiol for an additional 5 days. Serum oestradiol levels and the endometrial thickness were measured. If endometrial thickness was no more than 7 mm at this time, the dose of oestradiol was increased to 8 mg for an additional 4–5 days. Daily injection of progesterone (60 mg/day) was initiated on the day of oocyte warming.

Pregnancy assessment

Biochemical pregnancies were assessed 14 days after embryo transfer by measuring the levels of hCG-beta subunit in blood. Clinical pregnancies were detected with the confirmation of gestational sacs by transvaginal sonography. Progesterone supplementation was continued until the 12th week of pregnancy. Obstetric and perinatal outcomes, as well as incidence of congenital malformations, were measured.

Statistical analysis

The difference in means and prevalence among the groups were analyzed by Student's *t*-test for continuous data and Chi square for categorical data. The relationship between age, donor/nondonor, number of collected oocytes, clinical outcomes of fresh cycles and dilution temperature (as independent variables) and survival rate (as dependent variables) were analyzed using stepwise linear regression analysis. A p value <0.05 was considered statistically significant.

Results

Fresh cycle characteristics of donors and nondonors

Seventy-two patients (nondonors) who failed to have a live birth with their fresh IVF-ET and FET cycles returned for ET using their vitrified-warmed oocytes. Most of these patients had poor laboratory and clinical outcomes because of fertilization failure, poor embryo quality or low endometrial receptivity. The fertilization rates were 55.74 and 52.31 % in patients assigned to the 37C group and RT group, respectively. Sixty-nine patients (donors) donated their vitrified oocytes, and most of them donated after their clinical pregnancy or healthy live birth delivery (Table 1). Sixty nine recipients ranging from 25 to 50 years old (the mean age was 37.1± 7.47 years) accepted donated vitrified oocytes.

Table 2 Comparison of survival, fertilization, pregnancy, and implantation rates of vitrified human oocytes

groups	group 37 °C		group RT		*P* value	
	Nondonor oocytes	Donors oocytes	Nondonor oocytes	Donor oocytes	Nondonor oocytes	Donor oocytes
No. of cycles	70		71		–	
	35	35	37	34	–	–
No. of vitrified–thawed oocytes	662		676		–	
	330	332	349	327	–	–
Survival (%)	585(88.37)		540(79.88)		0.0000	
	288(87.27)	297(89.42)	264(75.64)	276(84.40)	0.0001	0.0541
No. of fertilized (2PN) zygotes (%)	390(66.67)		353(65.37)		0.6464	
	194(67.36)	196(65.99)	181(68.56)	172(62.32)	0.7629	0.3592
No. of ET/FET cycles	64		60		–	
	28	36	31	29	–	–
No. of clinical pregnancies (%)	24(37.5)		21(35.0)		0.7723	
	8(28.57)	16(44.44)	8(25.81)	13(44.83)	0.8115	0.9754
No. of implantations (%)	33(20)		29(19.46)		0.9050	
	11(14.86)	22(24.18)	10(13.33)	19(25.68)	0.7882	0.8245
Miscarriage (%)	5(16.67) a		4(19.05)		0.6970	
No. of deliveries	19		17		–	
No. of live births	23		23		–	
	7	16	9	14	–	–
Percentage of oocytes to achieve a live birth %	3.47		3.40		0.9444	
	2.12	4.82	2.58	4.28	0.7012	0.7516
Singleton/Multiple pregnancies	15/4		12/5		–	
No. of deliveries at <37 weeks (%)	5(26.32)		4(23.53)		0.7166	
No. of low birth weight (%)	6(26.09)		3(13.04)		0.2295	
Congenital anomalies	0		0		–	

a: including one abortion of twin pregnancy at 24 weeks

Fertilization rate = No. of 2PN zygotes/ No. of survival oocytes

Implantation rate = No. of gestational sacs/No. of embyos transferred

Clinical pregnancy rate = No. of patients with ultrasound evidence of a gestational sac/No. of embyos transferred cycles

Survival, fertilization, and subsequent development of vitrified human oocytes

A total of 662 (330 nondonor oocytes and 332 donor oocytes) and 676 (349 nondonor oocytes and 327 donor oocytes) oocytes were warmed in the 37 °C group and RT group respectively (Table 2). Significant difference (P=0.0000) was observed in the survival rates between 37 °C group (88.37 %) and RT group (79.88 %) in all oocytes examined. By comparing the survival rates within a patient group, the results showed that there was significant difference (P=0.0001) in survival rates between 37 °C group (87.27 %) and RT group (75.64 %) in nondonor patients, while no significant difference (P=0.0541) was found between the two groups (89.46 versus 84.40 %) in donor patients. No significant differences were found between the 37 °C group and RT group in fertilization rates (66.67 versus 65.37 %), implantation rates (20.0 versus 19.46 %), and clinical pregnancy rates (37.5 versus 35.0 %).

Stepwise multiple linear regression analysis

Multiple linear regression analysis showed that dilution temperature (β=0.079, P=0.017) and clinical outcomes of fresh cycles (β=0.063, P=0.001) were significantly and independently associated with survival rates (Table 3). The regression equation is: survival rate=0.685+0.079 (dilution temperature)+0.063 (clinical outcomes of fresh cycles).

Discussion

The present study investigated the effect of different rehydration temperatures (37 °C vs. RT) on the survival, fertilization and subsequent development of vitrified-warmed oocytes. Our results demonstrated that there was significant difference in survival rate between the 37 °C group and RT group (P= 0.0000). The multiple linear regression analysis also confirmed that dilution temperatures (β=0.079, P=0.017) were significantly and independently associated with survival rates. Specifically, there was significant difference between 37 °C group (87.27 %) and RT group (75.64 %) in nondonor patients (P=0.0001), while an improved trend of survival but no significant difference was observed between the two groups (89.46 versus 84.40 %) in donor patients (P=0.0541).

Table 3 Stepwise multiple linear regression analysis for the effect of independent variables on survival rate in the 141 warming cycles

Model (adjusted R^2=0.086)		β	t	p
1	(constant)	0.685	15.116	0.000
	Clinical outcomes of fresh cycles	0.063	3.250	0.001
	Dilution temperature	0.079	2.409	0.017

Survival rate=0.685+0.079(Dilution temperature)+0.063(Clinical outcomes of fresh cycles)

Because of the temperature-dependent nature of hydraulic conductivity and CPA permeability, the levels of osmotic stress and toxicity on an oocyte's survival will depend on the temperatures at which they are exposed to cryopreservation as well as rehydration solutions. It is difficult to reach the optimal balance between reducing CPAs toxicity and raising hydraulic conductivity/CPAs permeability. It is generally accepted that exposure to cryopreservation and rehydration solutions at lower temperature will reduce the toxicity of CPAs [18]. However, exposure to anisosmotic solutions at lower temperature has been confirmed to result in larger cell volume change in oocytes because of relatively lower hydraulic conductivity/CPAs permeability [19], and consequently impair survival and subsequent developmental potential [20]. On the other hand, warming and rehydration at higher temperature, such as physiological temperature, may be beneficial to oocyte survival and subsequent development due to increased permeability of CPAs and, consequently, reduced exposure time of oocytes to high concentration of CPAs [21].

In this experiment, warming was conducted by direct immersion of vitrified oocytes into warming solution (1.0 M sucrose solution) for 1–2 min at 37 °C right after they were taken out of LN_2. At physiological temperature, intracellular CPAs diffuse quickly out of oocytes during this short period of time [20]. Therefore, in dilution steps, there will be little CPAs remaining in the oocyte cytoplasm or in the surrounding solutions. At this moment, the CPAs toxicity should no longer be the crucial problem and a higher hydraulic conductivity at higher temperature might be more beneficial to oocyte survival. This may account for the improved survival rate in the 37 °C group in our study. This slight adjustment may help the oocytes avoid possible injury from temperature and osmotic pressure fluctuations. The adjustment may be especially beneficial to oocytes with poor quality, i.e., the nondonor oocytes, where survival was significantly increased when rehydrated at 37 °C in the study.

The metaphase spindle in mature oocytes are very sensitive to temperature changes [22]. There are several reports detailing the disappearance and eventual reappearance (normally within 3 h) of the oocyte meiotic spindle during cryopreservation [23–25]. Larman et al. found that, by carrying out the entire vitrification and warming procedures at 37 °C, the spindle was unaffected in both mouse and human MII oocytes [26]. Ciotti et al. found that a progressively significant fast cumulative recovery rate was observed in the three vitrification groups by increasing the number of phases at physiological temperature [27]. These reports support the application of physiological temperatures in vitrification-warming procedures. However, in the present study, no statistically significant increase in fertilization rate was observed in oocytes

rehydrated at the 37 °C compared to those rehydrated at RT. It might be explained that 2–3 h of recovery after warming could help the spindle fully repolymerization in both groups.

It is difficult to evaluate the efficiency of oocyte vitrification in clinical settings. Besides vitrification, several other factors, such as the quality of gametes as a result of female age, ovarian stimulation, and sperm source and the impact of any ex vivo manipulations (oocyte handling, fertilization technique, and in vitro embryo culture) may affect embryo development and clinical outcomes. In the present study, oocytes were all from infertile women. In the nondonor groups, only patients who did not achieve pregnancy or live birth during their fresh cycle underwent ET using vitrified-warmed oocytes. These nondonor oocytes resulted in relatively poor laboratory and clinical results in the present study. Multiple linear regression analysis also confirmed that clinical outcomes of fresh cycles (β=0.063, P=0.001) was a significant and independent factor associated with survival rates. Besides oocyte factor, some patients chose vitrified-warmed autologous oocytes because of sperm factor (quality and/or quantity), and this also had a negative effect on the fertilization rate before and after oocyte vitrification. Day 2 and day 3 embryo transfers were performed in this study, and the implantation rates were only 13.33 and 14.86 % in nondonor groups.

In conclusion, the results of this study give supportive evidence of the application of 37 °C in the dilution process, especially for oocytes of poor quality. Further studies with well-controlled experimental groups are needed to optimize protocols for human oocyte vitrification.

Acknowledgments We thank our colleagues in our reproductive center for all of their work toward the study and data collection.

Funding This study supported by National Basic Research Program of China (973 Program) (2011CB944502; 2012CB944700) and Independent Innovation Funding of Reproductive Medical Hospital Affiliated to Shandong University(20141105).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Cobo A, Diaz C. Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials. Fertil Steril. 2011;96:277–85.
2. Practice Committees of American Society for Reproductive Medicine; Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: a guideline. Fertil Steril. 2013; 99: 37–43.
3. Cobo A, Kuwayama M, Pérez S, Ruiz A, Pellicer A, Remohí J. Comparison of oncomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. Fertil Steril. 2008;89:1657–64.
4. Cobo A, Meseguer M, Remohí J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. Hum Reprod. 2010;25:2239–46.
5. Cil AP, Bang H, Oktay K. Age-specific probability of live birth with oocyte cryopreservation: an individual patient data meta-analysis. Fertil Steril. 2013;100:492–9.
6. Vajta G, Kuwayama M. Improving cryopreservation systems. Theriogenology. 2006;65:236–44.
7. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes: case report. Hum Reprod. 1999;14:3077–9.
8. Cobo A, Vajta G, Remohí J. Vitrification of human mature oocytes in clinical practice. Reprod Biomed Online. 2009;19 Suppl 4:4385.
9. García JI, Noriega-Portella L, Noriega-Hoces L. Efficacy of oocyte vitrification combined with blastocyst stage transfer in an egg donation program. Hum Reprod. 2011;26:782–90.
10. Smith GD, Serafini PC, Fioravanti J, Yadid I, Coslovsky M, Hassun P, et al. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. Fertil Steril. 2010;94:2088–95.
11. Chian RC, Huang JY, Gilbert L, Son WY, Holzer H, Cui SJ, et al. Obstetric outcomes following vitrification of in vitro and in vivo matured oocytes. Fertil Steril. 2009;91:2391–8.
12. Selman H, Rinaldi L, Pacchiarotti A, Floccari A, El-Danasouri I. Pregnancies and deliveries after injection of vitrified-warmed oocytes with cryopreserved testicular sperm. Fertil Steril. 2010;94: 2927–9.
13. Trokoudes KM, Pavlides C, Zhang X. Comparison outcome of fresh and vitrified donor oocytes in an egg-sharing donation program. Fertil Steril. 2011;95:1996–2000.
14. Kim TJ, Laufer LR, Hong SW. Vitrification of oocytes produces high pregnancy rates when carried out in fertile women. Fertil Steril. 2010;93:467–74.
15. Selman H, Angelini A, Barnocchi N, Brusco GF, Pacchiarotti A, Aragona C. Ongoing pregnancies after vitrification of human oocytes using a combined solution of ethylene glycol and dimethyl sulfoxide. Fertil Steril. 2006;86:997–1000.
16. Kim MK, Lee DR, Han JE, Kim YS, Lee WS, Won HJ, et al. Live birth with vitrified-warmed oocytes of a chronic myeloid leukemia patient nine years after allogenic bone marrow transplantation. J Assist Reprod Genet. 2011;28:1167–70.
17. Yoon TK, Kim TJ, Park SE, Hong SW, Ko JJ, Chung HM, et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. Fertil Steril. 2003;79:1323–6.
18. Szurek EA, Eroglu A. Comparison and avoidance of toxicity of penetrating cryoprotectants. PLoS One. 2011;6, e27604.
19. Mullen SF, Li M, Li Y, Chen ZJ, Critser JK. Human oocyte vitrification: the permeability of metaphase II oocytes to water and ethylene glycol and the appliance toward vitrification. Fertil Steril. 2008;89:1812–25.
20. Agca Y, Liu J, Rutledge JJ, Critser ES, Critser JK. Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. Mol Reprod Dev. 2000;55:212–9.
21. Akhoondi M, Oldenhof H, Stoll C, Sieme H, Wolkers WF. Membrane hydraulic permeability changes during cooling of mammalian cells. Biochim Biophys Acta. 1808;2011:642–8.
22. Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. Hum Reprod. 2001;16:2374–8.
23. Rienzi L, Martinez F, Ubaldi F, Minasi MG, Iacobelli M, Tesarik J, et al. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. Hum Reprod. 2004;19:655–9.

24. Gao S, Li Y, Gao X, Hu J, Yang H, Chen ZJ. Spindle and chromosome changes of human MII oocytes during incubation after slow freezing/fast thawing procedures. Reprod Sci. 2009;16:391–6.
25. Chen CK, Wang CW, Tsai WJ, Hsieh LL, Wang HS, Soong YK. Evaluation of meiotic spindles in thawed oocytes after vitrification using polarized light microscopy. Fertil Steril. 2004;82:666–72.
26. Larman MG, Minasi MG, Rienzi L, Gardner DK. Maintenance of the meiotic spindle during vitrification in human and mouse oocytes. Reprod Biomed Online. 2007;15:692–700.
27. Ciotti PM, Porcu E, Notarangelo L, Magrini O, Bazzocchi A, Venturoli S. Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. Fertil Steril. 2009;91: 2399–407.