



ELSEVIER

www.sciencedirect.com  
www.rbmonline.com



ARTICLE

# Rapid warming increases survival of slow-frozen sibling oocytes: a step towards a single warming procedure irrespective of the freezing protocol?




Lodovico Parmegiani <sup>a,\*</sup>, Carla Tatone <sup>b</sup>, Graciela Estela Cognigni <sup>a</sup>, Silvia Bernardi <sup>a</sup>, Enzo Troilo <sup>a</sup>, Alessandra Arnone <sup>a</sup>, Antonio Manuel Maccarini <sup>a</sup>, Giovanna Di Emidio <sup>b</sup>, Maurizio Vitti <sup>b</sup>, Marco Filicori <sup>a</sup>

<sup>a</sup> Reproductive Medicine Unit, GynePro Medical Centers, Bologna, Italy; <sup>b</sup> Department of Life, Health, and Environmental Sciences, University of L'Aquila, L'Aquila, Italy

\* Corresponding author. E-mail address: [l.parmegiani@gynepro.it](mailto:l.parmegiani@gynepro.it) (L Parmegiani).



After completing a Master's degree in biology in 1996 at the University of Bologna, Italy, Lodovico Parmegiani completed his postgraduate specialization in biochemistry and clinical chemistry in 2000 at the University of Modena and Reggio Emilia, Italy. In 2008, he received certification as a senior clinical embryologist from the European Society for Human Reproduction and Embryology. In his career he has developed and optimized devices and methods for clinical embryology. His current research interests are cryobiology, gamete selection and micromanipulation. He is currently the Laboratory Director at the Reproductive Medicine Unit, GynePro Medical Centers in Bologna, Italy.

**Abstract** Nowadays, human oocytes/embryos are cryopreserved via slow freezing or vitrification. The aim of this study was to evaluate a rapid warming protocol for slow-frozen human oocytes based on the standard warming procedure for vitrification. This was a prospective study on 216 sibling oocytes randomized for either conventional rapid thawing or rapid warming with vitrification warming solution. The primary endpoint was morphological assessment of survival at 2 h. Surviving oocytes were divided into two subgroups: (i) parthenogenetically activated; and (ii) fixed and observed for spindle/chromosome configuration. Secondary endpoints were parthenogenetic development and spindle/metaphase configuration. Survival rate with rapid warming was higher (92/102, 90.2%) than with rapid thawing (85/114, 74.6%;  $P = 0.005$ ), and after 3 d of culture the rapidly warmed parthenotes had more blastomeres compared with those rapidly thawed ( $P = 0.042$ ). Meiotic spindle and chromosomal configuration were not significantly influenced by rapid warming or rapid thawing. The finding of this study allows IVF centres to increase the efficiency of oocyte slow freezing, enabling survival rates comparable to vitrification protocols, and potentially to optimize costs by using the same warming protocol for both slow-frozen and vitrified reproductive cells. 

© 2014, Reproductive Healthcare Ltd. Published by Elsevier Ltd. All rights reserved.

**KEYWORDS:** oocyte slow freezing, oocyte vitrification, parthenogenetic activation, rapid thawing, universal warming protocol, warming

<http://dx.doi.org/10.1016/j.rbmo.2014.01.015>

1472-6483/© 2014, Reproductive Healthcare Ltd. Published by Elsevier Ltd. All rights reserved.

## Introduction

Nowadays, in assisted reproduction laboratories, human oocytes and embryos are cryopreserved by two main methods: vitrification or slow freezing.

During vitrification, the cells are converted to a glassy state without ice-crystal formation by using a viscous medium with a very high concentration of cryoprotectants (Rall and Fahy, 1985; Yavin and Arav, 2007). However, during slow freezing due to the progressive permeation of some cryoprotectants as the ice crystals form in the cryopreservation solution, a glassy/vitrified state is also obtained within the cell throughout the cooling procedure (Vanderzwalmen et al., 2013). For this reason, since both vitrified and slow-frozen oocytes have a vitrified cytoplasm, it can be postulated that the same warming protocol can be used for both. Furthermore, since for any given concentration of cryoprotectant, the warming rates are much faster than the critical cooling rates (Fahy et al., 1987), the minimal concentration of cryoprotectant to prevent crystallization during warming must be higher than during cooling (Vanderzwalmen et al., 2012). In fact, in most vitrification protocols or commercial kits, the cryoprotectant concentration in the first warming solution is approximately 1 mol/l (The Alpha Consensus Meeting, 2012), which is higher than the cryoprotectant concentration in both the freezing solution and the first thawing solution for rapid thawing of slow-frozen oocytes or embryos (e.g. for oocytes: 0.2–0.3 mol/l in freezing solution, 0.3–0.5 mol/l in thawing solution; Bianchi et al., 2007; Boldt et al., 2006; Fabbri et al., 2001; Parmegiani et al., 2008a). This suggests that vitrification warming solution could be used for thawing slow-frozen oocytes/embryos.

For a number of reasons, but most of all for the guaranteed high survival rate, vitrification is increasingly used worldwide, especially for oocytes (Edgar and Gook, 2012). Nevertheless, to date, perhaps millions of slow-frozen oocytes/embryos have already been stored in IVF cryobanks. Furthermore, IVF centres that have completely switched their cryopreservation programme from slow freezing to vitrification may still receive slow-frozen oocytes/embryos from other centres, cryopreserved by various different protocols. Nowadays, regulations recommend the use of FDA/CE-marked thawing media approved for human IVF; because the shelf life of these media is usually short (some months), it becomes expensive to keep available the appropriate thawing solution for any slow freezing protocol. In this scenario, the possibility of using a 'universal medium' to thaw any cell/tissue irrespective of the freezing protocol may simplify the management of thawing procedures. For all these reasons, the aim of this study is to evaluate a rapid warming protocol for slow-frozen human oocytes based on the standard warming procedure for vitrification, to optimize the survival rate and reduce costs by using the same solutions for both slow freezing and vitrification warming. The rapid warming protocol proposed in this study is potentially applicable also to slow-frozen embryos at any stage of cleavage.

## Materials and methods

### Study population

Since April 2004, all patients undergoing an IVF treatment at the GynePro Medical Centres, Bologna, Italy have had the option of cryopreserving their surplus oocytes not inseminated during the fresh cycle. This study was performed between December 2012 and January 2013 on 216 sibling oocytes obtained from 40 patients and cryopreserved via slow freezing from April 2004 to November 2008. Patient age (mean  $\pm$  standard error) at time of freezing was  $33.70 \pm 0.77$  years. The number of oocytes thawed/warmed per patient was  $5.40 \pm 0.53$ .

### Ethical approval

All the women included in this cryopreservation programme were informed of the procedure and written consent was obtained from each at the time of oocyte freezing. The written consent included the option of oocyte donation for research purposes before destruction, should the patient decide to discontinue the cryostorage. A further agreement was obtained from any patient before starting the study. This study was approved by the Institutional Review Board of the clinic (reference no. 15.10.2012, approved 3 December 2012).

### Ovarian stimulation, oocyte retrieval and selection, study design, and endpoints

Ovarian stimulation and oocyte retrieval and selection before cryopreservation were performed as previously described (Parmegiani et al., 2008a). For this study, slow-frozen oocytes were randomized for either conventional rapid thawing or rapid warming via vitrification warming solutions. Randomization was performed by a different embryologist to the operator who performed oocyte thawing/warming using a specific software tool (<http://www.randomizer.org>): for example, for six straws containing slow-frozen oocytes, a randomized set, 1, 2, 6, was generated, so oocytes in straws 1, 2 and 6 were thawed by conventional rapid thawing and oocytes in straws 3, 4 and 5 were rapidly warmed. The primary outcome measure was morphological assessment of survival at 2 h. Secondary outcome measures were parthenogenetic development and spindle/metaphase configuration.

### Cryopreservation

The cryopreservation protocol consisted of a slow-cooling method (Fabbri et al., 2001). Oocyte freezing solutions (OocyteFreeze; Origio, Denmark) contained Dulbecco's phosphate-buffered saline (PBS) supplemented with human serum albumin, alpha- and beta-globulins and 1,2-propanediol (PROH) and sucrose as cryoprotectants.

After washing in a PBS solution (Vial 1—OocyteFreeze; Origio), oocytes were equilibrated for 10 min at room temperature in 1.5 mol/l 1,2-PROH (Vial 2—OocyteFreeze,

Origio) and then transferred into the loading solution of 1.5 mol/l 1,2-PROH and 0.3 mol/l sucrose (Vial 3—OocyteFreeze; rigio). Between one and three oocytes were loaded in plastic straws (Paillette Cristal 133 mm; Cryo Bio System, France) and transferred into an automated biological vertical freezer (Kryo 360—1.7; Planer, UK). The cooling process was initiated, reducing the chamber temperature from 20°C to -7°C at a rate of 2°C/min. Ice nucleation was induced manually at -7°C (seeding). After a hold time of 10 min at -7°C, the straws were cooled slowly to -30°C at a rate of 0.3°C/min and then rapidly to -150°C at a rate of 50°C/min. After 10–12 min at stabilization temperature, the straws were transferred into liquid nitrogen and stored for later use.

### Rapid thawing

The straws were air-warmed for 30 s and then immersed in a 30°C water bath for 40 s. The cryoprotectant was removed at room temperature by stepwise dilution of PROH in the thawing solutions: the contents of the straws were expelled in 1.0 mol/l 1,2-PROH and 0.3 mol/l sucrose solution (Vial 1—OocyteThaw) and the oocytes were equilibrated for 5 min. The oocytes were then transferred into 0.5 mol/l 1,2-PROH and 0.3 mol/l sucrose solution (Vial 2—OocyteThaw) for 5 min and then into 0.3 mol/l sucrose solution (Vial 3—OocyteThaw) for 10 min before final dilution in PBS solution (Vial 4—OocyteThaw) for 20 min (10 min at room temperature and 10 min at 37°C). The oocytes were finally cultured at 37°C in ISM1 medium (Origio) in an atmosphere of 6% CO<sub>2</sub> in air for 2 h before assessment of survival.

### Rapid warming

The straws were air-warmed for 10 s and then immersed in a 30°C water bath until the external ice had melted (but not for longer than 10 s because this is a critical step). Each straw was opened and the content was immediately expelled in 4 ml warming solution containing 1 mol/l sucrose at 37°C (Sucrose Warming Solution; Sage, USA). Then, the oocytes were incubated at room temperature for 3 min first in 0.5 mol/l Sucrose Warming Solution and subsequently in 0.25 mol/l of 1:1 (v/v) of 0.5 mol/l Sucrose Warming Solution and MOPS Solution (Sage) and finally washed for 4 min in basic medium (MOPS Solution) before culture. The oocytes were finally cultured at 37°C in ISM1 medium in an atmosphere of 6% CO<sub>2</sub> in air for 2 h before assessment of survival.

### Survival evaluation and oocyte destiny

After a 2-h post-thaw/post-warming culture, survival was evaluated by inverted microscopy with Hoffman modulation contrast. Oocytes were considered to have survived in the absence of negative characteristics: dark or contracted ooplasm, vacuolization, cytoplasmic leakage, abnormal perivitelline space, cracked zona pellucida. Some of these surviving oocytes were then studied either for their developmental competence or for their spindle/metaphase configuration, whereas 33 sibling oocytes taken from seven patients were observed only for survival. The remaining

oocytes were randomly assigned to parthenogenesis or spindle study by a sealed envelope procedure: 96 sibling oocytes taken from 26 patients were parthenogenetically activated and 48 sibling oocytes taken from seven patients were fixed and observed for spindle/chromosome configuration.

### Parthenogenetic activation

Two hours after thawing/warming, oocytes were exposed to calcium-ionophore (GM508 Cult Active Medium; Gynemed, Germany) for 15 min at 37°C with 6% CO<sub>2</sub> in air then washed twice and subsequently incubated in 2 mmol/l 6-dimethylaminopurine (Sigma—Aldrich, Italy) in ISM1 medium for 3 h at 37°C with 6% CO<sub>2</sub> in air. Oocytes were then washed in fresh ISM1 medium, placed in 50- $\mu$ l microdrops of the same medium under oil (Ferticult Mineral oil; Fertipro, Belgium) and cultured at 37°C with 6% CO<sub>2</sub> in air. After 18–20 h (with exposure to calcium-ionophore as time 0) oocytes were evaluated for signs of activation. Oocytes showing one enlarged pronucleus and no extrusion of the second polar body were considered activated (Loi et al., 1998). Parthenotes were kept in culture in ISM1 for 3 d, followed by a further 2 d of culture in Blast Assist Medium (Origio). Parthenote development was assessed as previously described (Paffoni et al., 2007), by counting the number of blastomeres at 42–44 h (day 2) and at 66–68 h (day 3) after activation; blastocyst formation was evaluated at 118–120 h (day 5) after activation.

### Assessment of meiotic spindle and chromosome configuration

Two hours after thawing/warming, surviving oocytes were fixed in 4% formaldehyde and used for the study of spindle and chromatin organization as previously described (Antczak and Van Blerkom, 1997; Cobo et al., 2008a; Messinger and Albertini, 1991) but with a slightly modified procedure. Briefly, the oocytes were washed with a solution of PBS (Life Technologies, USA) with 3 mg/ml polyvinylpyrrolidone (Sigma—Aldrich) and 0.01% Tween 20 (Bio-Rad Laboratories, USA) and was successively treated with pronase type XIV (Sigma—Aldrich) for 20 min at 37°C for zona pellucida removal. Permeabilization was performed with a PBS/PVP solution supplemented with 0.5% Triton-X-100 (Sigma—Aldrich) for 1 h at 37°C, followed by washing in blocking solution consisting of PBS/PVP solution supplemented with 1.5% bovine serum albumin (Sigma—Aldrich) for 2 h at 37°C. Oocytes were further processed for immunostaining through overnight incubation with a monoclonal primary anti- $\alpha$  tubulin antibody (Santa Cruz Biotechnology, USA) diluted 1:100 in blocking solution, followed by incubation for 2 h at room temperature with secondary antibodies conjugated with Alexa 488 (Life Technologies) diluted 1:500 in blocking solution; after each antibody incubation, oocytes were washed in blocking solution. Chromatin staining was performed by using 5  $\mu$ g/ml Hoechst 33342 (Sigma—Aldrich) for 8 min at room temperature. After washing, the oocytes were mounted with a mixture of glycerol/PBS (1:1, v/v) and analysed by confocal microscopy (TCS SP5 II; Leica Microsystems, Germany).

Meiotic spindle configurations of the oocytes were classified as follows: (i) normal, if microtubules formed two opposite poles; (ii) slightly aberrant, if microtubule structures displayed reduced dimensions of the spindle and lost normal poles; (iii) aberrant, if disorganized microtubule patterns were observed; and (iv) absent, if the spindle was not found. Based on chromosomal distribution in the metaphase plate, oocytes were classified as follows: (i) normal, in the case of normal distribution; (ii) misaligned, if the plate showed some disorganization, with one or two chromosomes separated from the plate; (iii) displaced, in the case of scattered and decondensed chromosomes or disorganized chromosomal plate; and (iv) activated, if sister chromatid separation was observed.

### Statistical analysis

Calculation of sample size was based on this group's previous study performed on 437 slow-frozen oocytes, with a survival rate after thawing of 75% (Parmegiani et al., 2008a). This analysis revealed that at least 174 oocytes would be necessary to obtain a confidence level of 95% and a margin of error (confidence interval) of 5%. For this reason, the study was performed on more than 200 oocytes. Continuous variables are presented as mean  $\pm$  standard error. Categorical variables are presented as percentage. Normality of distribution of continuous variables was assessed with a Kolmogorov–Smirnov test (with Lillefor correction). Between-group differences of normally distributed continuous variables were assessed with parametric statistic (Student's *t*-test), whereas non parametric statistics (Mann–Whitney rank sum test) were employed when the normality test was not passed. Between-group differences in noncontinuous variables were assessed using chi-squared test with Yates correction if needed or with Fisher's exact test. Difference was considered significant when a *P*-value was  $<0.05$ .

## Results

### Survival

The mean number of oocytes warmed/thawed per group was comparable:  $2.55 \pm 0.29$  in the rapid warming group versus  $2.85 \pm 0.30$  in the rapid thawing group. Survival rate was 90.2% (92/102) in the rapid warming group versus 74.6% (85/114) in the rapid thawing group ( $P = 0.005$ ).

An interesting observation was that, during warming, slow-frozen oocytes in the rapid warming group displayed the behaviour typical of vitrified oocytes: as soon as the oocytes were expelled from the straw, they tended to float and appear vitreous in 1 mol/l sucrose solution; they subsequently shrank in 0.5 mol/l sucrose, and then in 0.25 mol/l sucrose and the washing solution they progressively recovered their original shape. (Parmegiani in [The Alpha Consensus Meeting, 2012](#)). This similarity is due to the ideal sucrose concentration (1 mol/l) and temperature (37°C) in the first warming solution, which both determine better osmolarity conditions for propanediol dilution compared with the conventional slow-freezing thawing solution used in the rapid thawing group (0.3 mol/l sucrose, 1 mol/l propanediol;

room temperature). For this reason it was critical to expel the oocytes into the first warming solution at 37°C as soon as the external ice around the straw had melted to avoid or minimize fluid exchanges between oocytes and melted holding medium inside the straw. Rapidly warmed oocyte were expelled in 1 mol/l sucrose at 37°C still shrunken and rehydration occurred within 1 min ([Figure 1A, B](#)); subsequently, the oocytes went through the same recovery process as vitrified oocytes ([Figure 1C–E](#)). Full recovery of the original cell shape is obtained after 10 min from expulsion from the straw, compared with 30 min for rapidly thawed oocytes ([Figure 1F–J](#)). The rapid warming in 1 mol/l sucrose at 37°C (which may better protect against the risk of water recrystallization) and the faster dilution of intracellular cryoprotectants may both contribute to the significantly better performance of rapidly warmed oocytes compared with rapidly thawed oocytes.

### Parthenogenetic development

A total of 96 sibling oocytes from 26 patients were activated for parthenogenesis. The patient age was  $33.69 \pm 0.94$  years. In the rapid warming and the rapid thawing groups, the parthenogenetic activation rate was comparable (90.4% versus 81.8%, respectively). Parthenogenetic development was comparable at day 2 ( $2.67 \pm 0.42$  blastomeres versus  $2.00 \pm 0.00$  blastomeres, respectively); however, at day 3, the rapidly warmed parthenotes had  $5.00 \pm 0.47$  blastomeres versus  $3.56 \pm 0.26$  blastomeres for the rapidly thawed parthenotes ( $P = 0.042$ ). Blastocyst development was comparable (10.6% versus 5.6%, respectively). The results for parthenogenetic development are summarized in [Table 1](#).

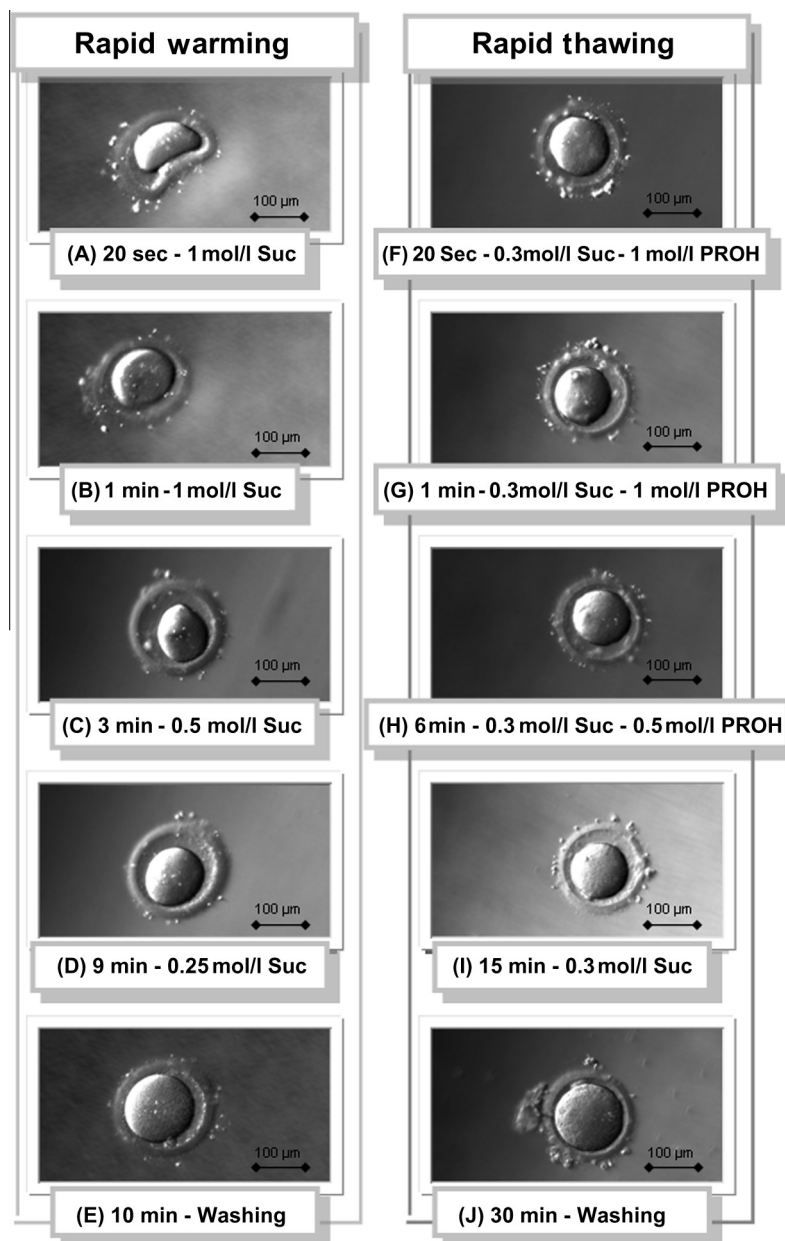
### Spindle/metaphase configuration

A total of 48 sibling oocytes taken from seven patients were fixed and observed for meiotic spindle/metaphase configuration. More specifically, in 35 sibling oocytes taken from six patients, the meiotic spindle together with the metaphase asset was studied, and in 13 sibling oocytes taken from one patient, only the metaphase asset was studied. The mean patient age was  $34.17 \pm 2.50$  years.

In the rapid warming and the rapid thawing groups, the meiotic spindle was present in the majority of the oocytes observed (94.4% and 88.2%, respectively; not significant). No significant differences were observed between the two groups in normal and abnormal spindle and metaphase plate configurations. Nevertheless, a better trend in spindle restoration and normal spindle/chromosomal pattern was observed in the rapid warming group. The results are summarized in [Table 2](#).

## Discussion

Human reproductive cells can be cryopreserved either via vitrification or via slow freezing. Especially for embryos and oocytes, vitrification is increasingly used due to the high survival rate obtainable, and this procedure is overtaking slow freezing in human assisted reproduction treatment ([Edgar and Gook, 2012](#)).



**Figure 1** Rapid warming and rapid thawing of slow-frozen sibling oocytes. Slow-frozen sibling oocytes from the same patient were warmed via rapid warming or via a conventional rapid thawing procedure. (A–E) A rapidly warmed oocyte was expelled into 1 mol/l sucrose at 37°C still shrunken (A) and its rehydration occurred within 1 min (B); then, it was moved to 0.5 mol/l sucrose, where it shrank again (C); subsequently, in 0.25 mol/l sucrose (D) and washing solution (E), the oocyte completely recovered its original cell shape. (F–J) A rapidly thawed oocyte was expelled in 0.3 mol/l sucrose at room temperature when intra/extracellular fluids (water and cryoprotectants) had already been exchanged inside the cryostraw; for this reason, its shape was already partially recovered (F, G); the oocyte remained partially rehydrated throughout the third step of thawing, in 0.3 mol/l sucrose solution (H–I) and fully recovered its original shape only in the washing solution, at 30 min after its release from the straw (J).

It is important to note that, during both these procedures, despite the different behaviour of the cryopreservation medium when cooled below the solidification point (vitrification medium appears 'glassy' whereas slow freezing medium is 'iced' due to the presence of ice crystals), the cell cytoplasm itself is converted from liquid to solid state without formation of ice crystals. In other words, slow freezing is just a method of intracellular vitrification with ice being present in the extracellular compartment (Katkov

et al., 2012). In fact, intracellular formation of ice crystals has a detrimental effect on cellular organelles and membranes and must therefore be avoided during both cooling and thawing phases (Leibo et al., 1978; Luyet, 1937; Mazur et al., 2005). This is ensured in different ways during vitrification or slow freezing, but at the end of both these procedures the cell is vitrified. 'Vitrification' is defined as the conversion of a superviscous liquid into a glassy state when it is cooled below its glass transition temperature

**Table 1** Parthenogenetic development.

Parameter	Rapid warming (n = 58)	Rapid thawing (n = 60)	P value
Survival rate	52 (89.7)	44 (73.3)	0.041
Activated oocytes	52 (2.00 ± 0.16)	44 (1.69 ± 0.22)	NS
Parthenogenetic activation rate	47 (90.4)	36 (81.8)	NS
Blastomeres on day 2 (42–44 h)	2.67 ± 0.42	2.00 ± 0.00	NS
Blastomeres one day 3 (66–68 h)	5.00 ± 0.47	3.56 ± 0.26	0.042
Blastocyst rate	5 (10.6)	2 (5.6)	NS

Values are n (%), n (mean ± standard error) or mean ± standard error.  
NS = not statistically significant.

**Table 2** Spindle/metaphase configuration.

Parameter	Rapid warming	Rapid thawing
Meiotic spindle		
No. of oocytes observed	18	17
Oocytes displaying meiotic spindle at 2 h from thawing/warming	17 (94.4)	15 (88.2)
Type of meiotic spindle		
Normal	8 (44.4)	4 (23.5)
Slightly aberrant	4 (22.2)	6 (35.3)
Aberrant	5 (27.8)	5 (29.4)
Absent	1 (5.6)	2 (11.8)
Chromosomal distribution		
No. of oocytes observed	23	25
Normal	10 (43.5)	9 (36.0)
Misaligned	5 (21.7)	6 (24.0)
Displaced	8 (34.8)	8 (32.0)
Activated	0 (0)	2 (8.0)

Values are n (%) unless otherwise stated. There were no statistically significant differences between the groups.

(Fahy et al., 1987). Any material can vitrify as long as the cooling rate is fast enough to prevent the formation of organized crystals; the probability of obtaining a glassy state depends on the relationship between viscosity, cooling rate and sample volume (Vajta and Kuwayama, 2006; Yavin and Arav, 2007).

During vitrification for cryopreservation of reproductive cells, cells are converted to the glassy state by using a viscous medium with very high concentration of cryoprotectants (Rall and Fahy, 1985). Two types of cryoprotectants are involved in cell cryopreservation: intracellular, which can permeate the cell membrane (e.g. ethylene glycol, propanediol, dimethylsulphoxide) and extracellular, which cannot permeate inside the cell (e.g. sucrose). During vitrification, high concentrations of cryoprotectants in the holding medium (about 4.5–6.5 mol/l for intracellular and about 0.5–1 mol/l for extracellular cryoprotectants) encapsulate the cell in a vitrifying sheath and, at the same time, determine a viscous environment inside the cell (Vanderzwalmen et al., 2013). The intracellular cryoprotectants permeate the cell membrane and replace the inner water which has drained out from the cell due to the very high

osmotic gradient generated during the consecutive steps of vitrification (in equilibration and vitrification solutions). Subsequently the cells (loaded in specifically designed carriers which allow fast cooling rates) vitrify by direct contact with liquid nitrogen or exposure to nitrogen vapours (Kuwayama et al., 2005; Parmegiani, 2011; Vajta and Kuwayama, 2006; Yavin and Arav, 2007).

On the other hand, during slow freezing, the cryoprotectant concentration in the cryopreservation medium is lower than in the vitrification medium (about 1–2 mol/l for intracellular and about 0.1–0.3 mol/l for extracellular cryoprotectants). During slow freezing, the cells are exposed to this moderate concentration of extracellular cryoprotectants which establish an osmotic gradient to facilitate a progressive movement of water out of the cell (cell dehydration), while intracellular cryoprotectants enter the cell, replacing the intracellular water drawn out. At the end of this dehydration process, intracellular cryoprotectants are estimated to be approximately 1.5–1.7 mol/l (McGrath, 2009; Son and Tan, 2009). However, it must be pointed out that, at the moment of cytoplasm solidification, intracellular cryoprotectant concentration is postulated to be

higher than this. This is explained by the 'solution effect': as the solution is cooled to lower and lower subzero temperatures, more of the water freezes and the solution becomes increasingly concentrated (Leibo, 2008). Thus, as ice crystals form and the osmolarity rises in the cryopreservation solution, intracellular cryoprotectants continue to progressively permeate the cell and further dehydration, shrinkage and increase in intracellular solute concentration occur (Leibo and Pool, 2011; Son and Tan, 2009). This happens in the programmable freezer chamber during the seeding of ice nucleation along the length of the straw/vial containing the cell; after this, the viscous cell cytoplasm is supercooled and converted to a glassy state (McGrath, 2009). By using computerized predictive models, McGrath (2009) calculated an intracellular cryoprotectant concentration (1,2-propanediol) of 2.7 mol/l for human oocytes slow cooled via a 0.3 mol/l sucrose protocol (Fabbri et al., 2001) after 10 min holding time after ice nucleation seeding. At this point the oocyte is postulated to be close to equilibrium, the freezer chamber temperature slightly decreases at 0.3°C/min from -7°C to -20°C and cell vitrification occurs. In a recent paper, Vanderzwalmen et al. (2013) demonstrated that cryoprotectant concentrations at the moment of solidification inside slow-frozen mouse oocytes are actually higher than inside vitrified oocytes (approximately 2.14 mol/l). This may be one of the reasons why slow-frozen oocytes/embryos can survive the low thawing rates (about 600°C/min when straws/vials are air warmed at 22–25°C) during conventional slow freeze-thaw protocols. In fact, warming speeds play an even more important role for cell survival than cooling rates (Seki and Mazur, 2008, 2009). This is well accepted in the case of vitrification, where a high warming speed (about 20,000°C/min) is critical to prevent cell lysis; if the warming speed is not fast enough, the supercooled liquid is rapidly transformed into ice crystals because intracellular cryoprotectant concentration is too low to prevent recrystallization (Vanderzwalmen et al., 2012, 2013).

During the thawing/warming process, extracellular cryoprotectants are used to generate an osmotic gradient which favours cell rehydration and drains intracellular cryoprotectants. Since for any given concentration of cryoprotectants, the warming rates are much faster than the critical cooling rates (Fahy et al., 1987), the minimum concentration of cryoprotectant to prevent crystallization during warming must be higher than during cooling (Vanderzwalmen et al., 2012). This means that, when thawing slow-frozen oocytes/embryos, vitrification warming solution could be used to optimize cell survival because extracellular cryoprotectant concentration in the first warming solution in any vitrification protocol is usually 1 mol/l, which is higher than in slow-freezing thawing solution (e.g. 0.3–0.5 mol/l for human oocytes). For this reason, this high extracellular cryoprotectant concentration in the medium should better protect against the risk of the recrystallization of the cellular water during warming. This was the rationale of the present study, which tested the vitrification warming protocol on slow-frozen human oocytes, which are well known to be the most sensitive reproductive cells during cryopreservation/thawing process. Furthermore, predictive models have suggested that there may be opportunities for optimizing the thawing protocol for oocytes slow frozen with

0.3 mol/l sucrose (McGrath, 2009). As far as is known, this study is the first to investigate the possibility of using vitrification warming solution for thawing human oocytes.

Interestingly, the survival rate was significantly improved when the slow-frozen oocytes were rapidly warmed (90.6%) rather than thawed with the conventional procedure (75.0%). In the rapid thawing group, the survival rate was comparable to the survival previously reported in clinical studies on oocytes slow frozen with 0.3 or 0.2 mol/l sucrose (Bianchi et al., 2012; Parmegiani et al., 2008a,b, 2009a,b); in fact, evidence from widespread practice suggests that a plateau has been reached at 70–80% with currently available slow freezing/rapid thawing methodology (Edgar and Gook, 2012; Gook and Edgar, 2007). On the other hand, the rapidly warmed oocyte survival rate in the current study (about 90%) is comparable with survival obtained in the majority of vitrification studies (Edgar and Gook, 2012; Kuwayama et al., 2005), particularly in those performed on infertile patients (Parmegiani et al., 2011; Practice Committees of American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2013; Rienzi et al., 2010; The Alpha Consensus Meeting, 2012).

Furthermore, rapidly warmed oocytes showed a better development after parthenogenetic activation. Parthenogenesis is a tool for the preclinical evaluation of experimental procedures, because the developmental competence of activated oocytes is similar to fertilized oocytes (Paffoni et al., 2007). The mean number of blastomeres in day 3 was significantly higher in rapid warming than in rapidly thawed parthenotes, and this was comparable with that of parthenotes derived from fresh oocytes in the study by Paffoni et al. (2007), as was the blastulation rate (about 10%). These findings seem to demonstrate that it is possible to increase the efficiency of oocyte slow freezing simply by improving the thawing procedure.

This study also analysed the oocytes by confocal microscopy in order to observe the meiotic spindle rearrangement dynamics (Bromfield et al., 2009). The human metaphase-II oocyte has its chromosomes equatorially located on a spindle, a temporary and dynamic structure of microtubules which is very sensitive to cooling (Pickering et al., 1990). It is already known that prolonged chilling at temperatures about 0°C is highly detrimental and causes oocyte meiotic spindle disassembly (Zenzes et al., 2001). However, in oocytes cryopreserved via slow freezing, the spindle should be protected by intracellular cryoprotectants (George et al., 1996; Gook et al., 1993; Van der Elst et al., 1988; Yang et al., 2010). Nevertheless, spindle alterations and disassembly have been displayed during the rapid thawing procedure (Coticchio et al., 2006; Rojas et al., 2004; Wu et al., 2006). In studies with polarization microscopy, oocyte spindles were identified immediately after thawing, disappeared during the subsequent removal of cryoprotectants and finally reappeared after 3–5 h of culture (Bianchi et al., 2005; Rienzi et al., 2004). On the contrary, during vitrification protocols, the meiotic spindle and chromosome alignment are both maintained throughout the processes of vitrification, warming and post warming (Chang et al., 2011; Larman et al., 2007). Through the use of a vitrification warming protocol on slow-frozen oocytes, the present study might give interesting information on the mechanism of spindle disruption/recovery during slow freezing. Thus,

spindle and chromosome dynamics were studied at 2 h from thawing/warming in order to discover if different protocols might influence spindle recovery. The presence of spindles were observed in the majority of the oocytes; this is in agreement with previous studies showing time-dependant spindle repolymerization (Bianchi et al., 2005; Mandelbaum et al., 2004; Pickering et al., 1990; Rienzi et al., 2004). Furthermore, both normal spindle and chromosomal configuration were comparable between the two study groups (Table 2). Although slightly better figures for spindle presence and normal spindle/chromosome pattern may suggest a better dynamic in the rapid warming group, the spindle repolymerization does not seem to be influenced by the choice of thawing/warming protocol, and it is at least clear that the quick removal of intracellular cryoprotectants during rapid warming does not negatively affect meiotic spindle reformation.

Nowadays, human oocyte cryopreservation has moved from bench-to bedside and it is no longer considered an experimental technique but a valuable clinical tool (ASRM Practice Committee, 2012); this is due particularly to studies on vitrification demonstrating that vitrified oocytes perform as well as their fresh counterparts (Cobo et al., 2008b; Parmegiani et al., 2011; Rienzi et al., 2010). The present study demonstrates that it is possible to increase the survival of slow-frozen oocytes by changing the thawing protocol and to potentially obtain results comparable to vitrification; furthermore, this study demonstrates that the fast removal of intracellular cryoprotectants during rapid warming does not negatively affect meiotic spindle reformation. Even though oocyte slow freezing has been optimized in the last 5 years (Bianchi et al., 2007, 2012; Parmegiani et al., 2008a, 2009a), the oocyte still remains the most fragile human cell with the highest risk of ice crystal formation and cryoinjury during freezing/thawing, due to an unfavourable nucleus/cytoplasm ratio and elevated intracellular water concentration. Since the rapid warming procedure proposed in this study is viable for oocytes, it is also potentially applicable to other less fragile reproductive cells frozen via slow freezing, such as zygotes or embryos at any stage of cleavage (from 2 cells to blastocyst). A recent prospective study performed on 359 slow-frozen pronuclear embryos in Japan confirmed the positive effect on embryo development of the rapid warming protocol: the blastocyst formation rate was significantly increased when the embryos were warmed with vitrification warming media rather than with conventional rapid thawing (Kojima et al., 2012). Furthermore, since in any protocol or commercial kit, vitrification warming solutions have a concentration of 1 mol/l (first warming step) and 0.5 mol/l (second warming step) of extracellular cryoprotectants, these solutions can be used for the warming of any reproductive cell irrespective of the freezing protocols, thus streamlining laboratory activity and potentially reducing costs.

In conclusion, this is a pilot study that need confirmation in large-scale clinical studies but, if these preliminary data are confirmed, this finding will redefine the scenario of slow freezing and vitrification, as it emphasizes the pivotal role of warming and indicates that the results obtained until now with slow freezing/rapid thawing can be improved. The application of this warming protocol increased the

efficiency of the oocyte/embryo slow freezing procedure, enabling survival rates comparable with those reported in vitrification studies. This could be the biggest breakthrough in human oocyte cryopreservation since the introduction of vitrification. Furthermore, this finding demonstrates that it is possible to optimize the management of cryopreservation cycles in the IVF laboratory by using a single warming protocol for both slow-frozen and vitrified oocytes and potentially other reproductive cells.

## Acknowledgements

Thawing and warming solutions were kindly supplied by Origio. The authors wish to thank Ms Maggie Baigent for revising the manuscript and Dr Azzurra Rastellini and Dr Sara Lanzilotti (GynePro Medical Centres) for their valuable contribution in thawing/warming and parthenogenesis experiments.

## References

- Alpha Scientists in Reproductive Medicine, 2012. The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting. *Reprod. Biomed. Online* 25, 146–167.
- Antczak, M., Van Blerkom, J., 1997. Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol. Hum. Reprod.* 3, 1067–1086.
- Bianchi, V., Coticchio, G., Fava, L., Flamigni, C., Borini, A., 2005. Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. *Hum. Reprod.* 20, 1078–1083.
- Bianchi, V., Coticchio, G., Distratis, V., Di, G.N., Flamigni, C., Borini, A., 2007. Differential sucrose concentration during dehydration (0.2 mol/l) and rehydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. *Reprod. Biomed. Online* 14, 64–71.
- Bianchi, V., Lappi, M., Bonu, M.A., Borini, A., 2012. Oocyte slow freezing using a 0.2–0.3 M sucrose concentration protocol: is it really the time to trash the cryopreservation machine? *Fertil. Steril.* 97, 1101–1107.
- Boldt, J., Tidswell, N., Sayers, A., Kilani, R., Cline, D., 2006. Human oocyte cryopreservation: 5-year experience with a sodium-depleted slow freezing method. *Reprod. Biomed. Online* 13, 96–100.
- Bromfield, J.J., Coticchio, G., Hutt, K., Sciajno, R., Borini, A., Albertini, D.F., 2009. Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. *Hum. Reprod.* 24, 2114–2123.
- Chang, C.C., Lin, C.J., Sung, L.Y., Kort, H.I., Tian, X.C., Nagy, Z.P., 2011. Impact of phase transition on the mouse oocyte spindle during vitrification. *Reprod. Biomed. Online* 22, 184–191.
- Cobo, A., Perez, S., de los Santos, M.J., Zulategui, J., Domingo, J., Remohi, J., 2008a. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. *Reprod. Biomed. Online* 17, 350–359.
- Cobo, A., Kuwayama, M., Perez, S., Ruiz, A., Pellicer, A., Remohi, J., 2008b. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertil. Steril.* 89, 1657–1664.
- Coticchio, G., De, S.L., Rossi, G., Borini, A., Albertini, D., Scaravelli, G., Alecci, C., Bianchi, V., Nottola, S., Cecconi, S., 2006. Sucrose concentration influences the rate of human



- oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Hum. Reprod.* 21, 1771–1776.
- Edgar, D.H., Gook, D.A., 2012. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum. Reprod. Update* 18, 536–554.
- Fabbri, R., Porcu, E., Marsella, T., Rocchetta, G., Venturoli, S., Flamigni, C., 2001. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum. Reprod.* 16, 411–416.
- Fahy, G.M., Levy, D.I., Ali, S.E., 1987. Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. *Cryobiology* 24, 196–213.
- George, M.A., Pickering, S.J., Braude, P.R., Johnson, M.H., 1996. The distribution of alpha- and gamma-tubulin in fresh and aged human and mouse oocytes exposed to cryoprotectant. *Mol. Hum. Reprod.* 2, 445–456.
- Gook, D.A., Edgar, D.H., 2007. Human oocyte cryopreservation. *Hum. Reprod. Update* 13, 591–605.
- Gook, D.A., Osborn, S.M., Johnston, W.I., 1993. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. *Hum. Reprod.* 8, 1101–1109.
- Katkov, I.I., Bolyuk, V.F., Chernetsov, O.A., Dudin, P.I., Grigoriev, A.Y., Isachenko, V., Isachenko, E., Lulat, A.G.M., Moskovtsev, S.I., Petrushko, M.P., Pinyaev, V.I., Sokol, K.M., Sokol, Y.I., Sushko, A.B., Yakhnenko, I., 2012. Kinetic vitrification of spermatozoa of vertebrates: what can we learn from nature? In: Katkov, I.I. (Ed.), *Current Frontiers in Cryobiology*. InTech, New York, pp. 3–40.
- Kojima, E., Fukunaga, N., Nagai, R., Kitasaka, H., Ohno, H., Asada, Y., 2012. The vitrification method is significantly better for thawing of slow-freezing embryos. *Fertil. Steril.* 98, S124.
- Kuwayama, M., Vajta, G., Kato, O., Leibo, S.P., 2005. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod. Biomed. Online* 11, 300–308.
- Larman, M.G., Minasi, M.G., Rienzi, L., Gardner, D.K., 2007. Maintenance of the meiotic spindle during vitrification in human and mouse oocytes. *Reprod. Biomed. Online* 15, 692–700.
- Leibo, S.P., 2008. Cryopreservation of oocytes and embryos: optimization by theoretical versus empirical analysis. *Theriogenology* 69, 37–47.
- Leibo, S.P., Pool, T.B., 2011. The principal variables of cryopreservation: solutions, temperatures, and rate changes. *Fertil. Steril.* 96, 269–276.
- Leibo, S.P., McGrath, J.J., Cravalho, E.G., 1978. Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. *Cryobiology* 15, 257–271.
- Loi, P., Ledda, S., Fulka Jr., J., Cappai, P., Moor, R.M., 1998. Development of parthenogenetic and cloned ovine embryos: effect of activation protocols. *Biol. Reprod.* 58, 1177–1187.
- Luyet, B.J., 1937. The vitrification of organic colloids and of protoplasm. *Biodynamica* 1, 1–14.
- Mandelbaum, J., Anastasiou, O., Levy, R., Guerin, J.F., de Larouzière, V., Antoine, J.M., 2004. Effects of cryopreservation on the meiotic spindle of human oocytes. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 113, S17–S23.
- Mazur, P., Seki, S., Pinn, I.L., Kleinhans, F.W., Edashige, K., 2005. Extra- and intracellular ice formation in mouse oocytes. *Cryobiology* 51, 29–53.
- McGrath, J.J., 2009. Predictive models for the development of improved cryopreservation protocols for human oocytes. In: Borini, A., Coticchio, G. (Eds.), *Cryopreservation of Human Oocytes*. Informa, London, pp. 62–82.
- Messinger, S.M., Albertini, D.F., 1991. Centrosome and microtubule dynamics during meiotic progression in the mouse oocyte. *J. Cell Sci.* 100, 289–298.
- Paffoni, A., Brevini, T.A., Somigliana, E., Restelli, L., Gandolfi, F., Ragni, G., 2007. In vitro development of human oocytes after parthenogenetic activation or intracytoplasmic sperm injection. *Fertil. Steril.* 87, 77–82.
- Parmegiani, L., 2011. Vitrification with UV-sterilized supercooled air. *Fertil. Steril.* 95, e43.
- Parmegiani, L., Cognigni, G.E., Bernardi, S., Ciampaglia, W., Infante, F., Pocognoli, P., de Fatis, C.T., Troilo, E., Filicori, M., 2008a. Freezing within 2 h from oocyte retrieval increases the efficiency of human oocyte cryopreservation when using a slow freezing/rapid thawing protocol with high sucrose concentration. *Hum. Reprod.* 23, 1771–1777.
- Parmegiani, L., Fabbri, R., Cognigni, G.E., Bernardi, S., Pocognoli, P., Filicori, M., 2008b. Blastocyst formation, pregnancy, and birth derived from human oocytes cryopreserved for 5 years. *Fertil. Steril.* 90, 2014.e7–2014.e10.
- Parmegiani, L., Garello, C., Granella, F., Guidetti, D., Bernardi, S., Cognigni, G.E., Revelli, A., Filicori, M., 2009a. Long-term cryostorage does not adversely affect the outcome of oocyte thawing cycles. *Reprod. Biomed. Online* 19, 374–379.
- Parmegiani, L., Bertocci, F., Garello, C., Salvarani, M.C., Tambuscio, G., Fabbri, R., 2009b. Efficiency of human oocyte slow freezing: results from five assisted reproduction centres. *Reprod. Biomed. Online* 18, 352–359.
- Parmegiani, L., Cognigni, G.E., Bernardi, S., Cuomo, S., Ciampaglia, W., Infante, F.E., Tabarelli de, F.C., Arnone, A., Maccarini, A.M., Filicori, M., 2011. Efficiency of aseptic open vitrification and hermetical cryostorage of human oocytes. *Reprod. Biomed. Online* 23, 505–512.
- Pickering, S.J., Braude, P.R., Johnson, M.H., Cant, A., Currie, J., 1990. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil. Steril.* 54, 102–108.
- Practice Committees of American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2013. Mature oocyte cryopreservation: a guideline. *Fertil. Steril.* 99, 37–43.
- Rall, W.F., Fahy, G.M., 1985. Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. *Nature* 313, 573–575.
- Rienzi, L., Martinez, F., Ubaldi, F., Minasi, M.G., Iacobelli, M., Tesarik, J., Greco, E., 2004. Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. *Hum. Reprod.* 19, 655–659.
- Rienzi, L., Romano, S., Albricci, L., Maggilli, R., Capalbo, A., Baroni, E., Colamaria, S., Sapienza, F., Ubaldi, F., 2010. Embryo development of fresh ‘versus’ vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. *Hum. Reprod.* 25, 66–73.
- Rojas, C., Palomo, M.J., Albarracin, J.L., Mogas, T., 2004. Vitrification of immature and in vitro matured pig oocytes: study of distribution of chromosomes, microtubules, and actin microfilaments. *Cryobiology* 49, 211–220.
- Seki, S., Mazur, P., 2008. Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice. *Biol. Reprod.* 79, 727–737.
- Seki, S., Mazur, P., 2009. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology* 59, 75–82.
- Son, W.Y., Tan, S.L., 2009. Comparison between slow freezing and vitrification for human embryos. *Expert. Rev. Med. Devices* 6, 1–7.
- Vajta, G., Kuwayama, M., 2006. Improving cryopreservation systems. *Theriogenology* 65, 236–244.
- Van der Elst, J., Van den Abbeel, E., Jacobs, R., Wisse, E., Van, S.A., 1988. Effect of 1,2-propanediol and dimethylsulphoxide on the meiotic spindle of the mouse oocyte. *Hum. Reprod.* 3, 960–967.

- Vanderzwalmen, P., Zech, N.H., Ectors, F., Stecher, A., Lejeune, B., Vanderzwalmen, S., Wirleitner, B., 2012. Blastocyst transfer after aseptic vitrification of zygotes: an approach to overcome an impaired uterine environment. *Reprod. Biomed. Online* 25, 591–599.
- Vanderzwalmen, P., Connan, D., Grobet, L., Wirleitner, B., Remy, B., Vanderzwalmen, S., Zech, N., Ectors, F.J., 2013. Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions. *Hum. Reprod.* 28, 2101–2110.
- Wu, C., Rui, R., Dai, J., Zhang, C., Ju, S., Xie, B., Lu, X., Zheng, X., 2006. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. *Mol. Reprod. Dev.* 73, 1454–1462.
- Yang, D., Winslow, K.L., Nguyen, K., Duffy, D., Freeman, M., Al-Shawaf, T., 2010. Comparison of selected cryoprotective agents to stabilize meiotic spindles of human oocytes during cooling. *J. Exp. Clin. Assist. Reprod.* 7, ii.
- Yavin, S., Arav, A., 2007. Measurement of essential physical properties of vitrification solutions. *Theriogenology* 67, 81–89.
- Zenzes, M.T., Bielecki, R., Casper, R.F., Leibo, S.P., 2001. Effects of chilling to 0 degrees C on the morphology of meiotic spindles in human metaphase II oocytes. *Fertil. Steril.* 75, 769–777.

*Declaration: LP holds an international patent: 'Device and method for sterilizing liquid nitrogen by ultraviolet radiation'. The other authors report no financial or commercial conflicts of interest.*

Received 22 August 2013; refereed 22 January 2014; accepted 22 January 2014.