Morphokinetics analysis of embryos derived from vitrified/warmed oocytes

D. Montjean¹ · C. Geoffroy-Siraudin¹ · M. Gervoise-Boyer¹ · P. Tourame¹ · P. Boyer¹

Received: 26 May 2015 / Accepted: 7 September 2015 / Published online: 26 September 2015
© Springer Science+Business Media New York 2015

Abstract

Purpose Oocyte vitrification is a worldwide used technique that has proved its worth. Although it was shown not to alter oocyte integrity, a recent study concluded that it may affect oocyte embryo development. As the morphology and kinetics of embryos derived from sibling fresh and vitrified oocytes have not been described previously, the present study evaluates cleavage rate, blastomeres size, fragmentation rate, and blastocyst formation in vitrified/warmed oocyte derived embryos (VODE) as compared with sibling fresh oocytes derived embryos (FODE).

Methods This investigation included 90 infertility cases displaying large cohort of mature oocytes at pick up, divided into 2 groups after denudation. A part of oocytes underwent ICSI while others were vitrified. Oocyte warming cycles were performed when no pregnancy was achieved using fresh eggs. Zygote to blastocyst development was recorded prospectively in an image database up to day 5.

Results VODE did not show major difference as compared with FODE in terms of cleavage rate, number of blastomeres,

fragmentation rate, and blastomeres size. Furthermore, percentage of morulae at day 4 and blastocysts at day 5 are not affected by oocyte vitrification.

Conclusion Although our results show that embryo development is not altered by oocyte vitrification, offspring follow-up is essential to exclude any adverse developmental effect of the technique.

Keywords Oocyte vitrification · Embryo development · Blastocyst · Fragmentation · Blastomeres

Introduction

Oocyte vitrification is widely used in assisted reproductive technology (ART) laboratories for several indications like diminution of frozen embryo number and egg banking for donation to fertility preservation [1–8]. To date vitrification is the most attractive and reliable option to store oocytes, bringing about a dramatic change in the field of ART [9, 10]. Although this technique was shown to have no major adverse effect on oocyte ultrastructure and oocyte in vitro performance [6, 11], it was suggested to indirectly impact on derived embryo development [12]. To date, vitrified/warmed oocyte-derived embryo kinetics and morphology have not yet been entirely documented. After an internal validation of the technique [13], followed by the use of the technique in routine for infertile couples management, we chose to investigate vitrified/warmed oocyte derived embryos (VODE) kinetics, development and morphology as compared with fresh oocyte derived embryos (FODE). The present study investigates cleavage rate, blastomeres size, embryo fragmentation degree and blastocyst formation at embryo culture day 5 in VODE as compared with sibling FODE.

Capsule This is the first report on morphologic and morphokinetic parameters in embryos derived from sibling oocytes that were either vitrified/warmed prior to fertilization or fertilized shortly after pick up. The present data show that the development of embryos obtained from vitrified/warmed oocytes is not significantly different from embryos formed from sibling fresh non-vitrified oocytes.

✉ D. Montjean
debbie_montjean@hotmail.com;
dmontjean@hopital-saint-joseph.fr

¹ Service de Médecine et Biologie de la Reproduction, Hôpital Saint-Joseph, 26 boulevard de Louvain, 13008 Marseille, France

Materials and methods

Patients

The 90 couples included in this study had oocytes that were subjected to ICSI procedure for medical reasons (between 2012 and 2013) and showed no parental constitutional karyotype anomalies. French legal conditions were respected for ART treatment especially for viral risk. The study was approved by the local ethical committee on 3 April 2008 (reference number 208 R06).

Study design

Couples included in this study were enrolled in an In Vitro Fertilization (IVF) program for medical reasons requiring the use of ICSI. The number of metaphase II oocytes recovered was over 6 after eggs denudation, and informed consent for oocyte vitrification was signed by women after egg maturation assessment.

For every patient where at least 6 eggs in metaphase II were recovered, sibling oocytes were arbitrarily divided into 2 groups: A part of oocytes underwent ICSI while others were vitrified. In general, 4–5 oocytes were fertilized to obtain at least one blastocyst stage embryo at day 5, and remaining oocytes were vitrified. In cases where patients refused embryo freezing, only 3 oocytes were microinjected at day 0 while others were cryopreserved. Oocyte warming cycles were proposed when clinical pregnancy failed to happen using fresh eggs. Sperm used for injection was obtained from the same partner for the microinjection of both fresh and vitrified oocytes. Zygote to blastocyst embryo development was recorded daily in a photo database (Pegase software, 2iSYSTEM, France). The development of 537 and 442 microinjected fresh oocytes and vitrified/warmed oocytes, respectively, was evaluated and recorded daily up to embryo culture day 5. A total of 3893 photos were analysed for the purpose of the present study (2288 images for the investigation of FODE development and 1605 for VODE).

One-hundred-seventeen oocyte warming cycles were performed after failure of the fresh cycle. Failed cycles included those with no pregnancy, biochemical pregnancy (β hCG blood measurement > 100 UI/l but no foetal heartbeat detected) and miscarriage (pregnancy loss at any time after the detection of foetal heartbeat by ultrasound). This permitted comparison of VODE development to that of sibling FODE based on the image database. The number of oocytes warmed was decided based on several criteria: fertilization rate of fresh sibling oocytes, oocyte survival rate after warming, number of embryo to be transferred depending on the age of the woman and rank of attempt. Oocyte warming cycles were organized along with a natural ovarian cycle where oocyte warming was performed the day following LH surge or using hormone replacement treatment (a combination of oestrogen and progesterone). No

ovarian hyperstimulation using gonadotropin treatment was used for oocyte warming cycles.

Ovarian stimulation and oocyte pick up

Ovarian stimulation was conducted using agonist or antagonist pituitary control. Administration of urinary FSH or recombinant FSH was used to induce ovulation. Oocyte recovery was performed approximately 36 h after triggering ovulation by hCG administration, under transvaginal ultrasound-guided aspiration. Depending on the number of cumuli found in follicular fluid, oocyte denudation was performed 15–35 min after pick up. Subsequently to denudation, oocytes were cultured 30–120 min or 30–60 min in BM1 medium (Eurobio, France) prior to ICSI or vitrification, respectively.

Oocyte vitrification and warming

Oocytes were vitrified on Cryotop[®] using a Kitazato vitrification kit with the following protocol. Briefly, equilibration solution (ES) and vitrification solution (VS) were allowed to warm up to room temperature (20–27 °C). Oocytes were subjected to a gradient of ES solution during 15 min, then vitrified in VS solution and immersed into liquid nitrogen within 1 min.

Oocyte warming was performed using a Kitazato warming kit following the manufacturer's recommendations: The thawing solution (TS) was let overnight to warm up at 37 °C in a dish. The sheet of the Cryotop[®] was quickly immersed into the TS. Oocytes were aspirated within the end of the capillary 1 min after immersion into TS. Oocytes were transferred into the diluent solution (DS) for 3 min and washed twice in droplets of wash solution (WS) (5 and 1 min). Oocytes were incubated at 37 °C at least for 2 h in BM1 medium (Eurobio, France) before microinjection.

Embryo culture

Fertilization rate was calculated by dividing the number of oocytes showing 2 pronuclei (16–18 h after ICSI) by the number of microinjected oocytes. ICSI program fertilization rate was stable (mean fertilization rate per patient: 65.4 ± 25.0 %) over the span of the study. Embryos were cultured in Global[®] medium (Life global) micro drops covered with mineral oil at 37.2 °C under 6%CO₂. Embryo transfer was performed at embryo culture day 2, day 3 and day 5 depending on embryo development and the number of embryos to be transferred. Non-cleaving and poor morphology embryos were discarded. No laboratory protocol changes were made over the duration of the study.

Embryo morphology from culture day 2 to day 5 was assessed using the BLEFCO (Biologistes des Laboratoires d'Etudes de la Fécondation et de la Conservation de l'Oeuf) classification that is widely used in France [14].

Day 2/day 3 embryos were characterized using 3 values. The first value represents the number of blastomeres, the second one (1 or 2) characterizes the size and regularity of blastomeres (1: typical, 2: atypical) and the last value corresponds to the fragmentation rate (1: <10 % of fragmentation, 2: 10–30 % of fragmentation and 3: >30 % of fragmentation). An embryo of optimal quality at embryo culture day 2 and day 3 would be 411 and 811 (Fig. 1a), respectively. This classification does not consider blastomere multinucleation or cytoplasmic abnormalities. An overview of possible embryos and corresponding grades at embryo culture day 2 and day 3 is depicted in Fig. 1a. Embryos that did not correspond to one of the described situations were classified as “non-evaluable” and discarded.

In order to strengthen the results, the overall grade of embryos was included to the analysis using a 4-point embryo score [15]. Cleaved embryos received 1 point, and additional points were added when embryos (i) present no fragmentation, (ii) display no irregularities, and (iii) have four cells at day 2 or

8 cells at day 3. Based on this scoring system, top quality embryos score 4. The lower is the score; the poorer is the embryo morphology.

Blastocyst stage embryos were graded from B0 to B6 (Fig. 1b) using an adaptation of Gardner day 5 embryo classification [16, 17] B0 are blastocysts with an early forming blastocoele; B1: early blastocyst with a clear but limited to less than half of the embryonic volume blastocoele; B2 displays blastocoele occupying half the volume of the embryo; B3 have a blastocoele occupying more than half of the embryo volume and have an augmented total volume; B4 are blastocysts whose blastocoele occupies the entire embryo; B5 are blastocysts of increased volume with a thinned zona pellucida and a hernia of trophectoderm through the zona pellucida; and B6 are hatching blastocyst [14]. From B3 stage to further stages, inner cell mass was considered and only blastocysts displaying inner cell mass composed of many/several cells tightly/loosely packed and a trophectoderm showing many to few cells forming a cohesive or loose layer (A or B based

Fig. 1 Embryo development grading using BLEFCO classification. Adapted from Boyer & Boyer 2009 [14]. **a** Overview of possible embryo grades at day 2 and day 3 using BLEFCO system. **b** Day 5 embryos grading using BLEFCO classification from B0 to B6. Embryos that did not reach one of these morphological criteria were classified as “non-evaluable”. ICM inner cell mass. B Blastocoele. TE trophectoderm

on Gardner classification system) [16, 17] were further characterized using the simplified BLEFCO grading system while the others were classified as “non-evaluable” and discarded.

Statistical analysis

Chi-squared and Student's *t* test were used for observed differences analysis, and *p* value <0.05 was considered statistically significant.

Results

A summary of clinical and biological data concerning the 90 women included in the present study is presented in Table 1. Significantly fewer oocytes per patient were microinjected during warming cycles than during fresh cycles (4.9 ± 2.1 vs. 6.0 ± 1.4 , $p < 0.001$) as a result of the will to reduce the number of fertilized eggs in order to limit embryo cryopreservation. Our data show mean oocyte survival rate per patient after warming of 86.2 % and similar mean fertilization rate (64.9 ± 29.3 % vs 58.0 ± 27.4 %, $p > 0.05$) and cleavage rate (64.2 vs 70.1 %, $p > 0.05$) per patient after vitrification/warming cycle when comparing with fresh eggs.

Overall, no statistically significant differences were found in the number of blastomeres, blastomere fragmentation rate, blastomere morphology and percentage of morula and blastocysts stage embryos.

However, at embryo culture day 2 (43–45 h post-ICSI), VODE showed lower fragmentation rate than FODE. In fact, 1.0 % of VODE displayed more than 30 % fragmentation whereas 3.2 % of FODE showed more than 30 % fragmentation ($p < 0.05$). Besides, a higher proportion of VODE showed mild fragmentation (10–30 %) than FODE (45.2 vs 39.4 %, $p < 0.05$).

At embryo culture day 3 (67–69 h post-ICSI), VODE presented significantly smaller number of blastomeres than FODE (6.3 ± 1.9 in VODE vs 6.8 ± 2.1 in FODE, $p < 0.05$).

No difference was found in the percentage of high score embryo in VODE as compared to FODE (Fig. 2).

Although the proportion of blastocysts available at embryo culture day 5 was the same, a statistically significantly lower percentage of B3 blastocysts was observed in VODE when comparing with FODE (15.2 % in FODE vs 3.8 % in VODE, $p < 0.05$) (Fig. 3b). However, this difference is likely to be a consequence of small number of embryos rather than a meaningful dissimilarity.

Since warming cycles were proposed only when implantation failure and miscarriage occurred after the transfer of FODE, VODE embryos showed twice the implantation rate (9.1 % in FODE and 19.8 % VODE) and clinical pregnancy rate (12.6 % in FODE and 28.9 % in VODE). These differences are not assessable.

Discussion

The aim of the present study was to compare in vitro development of vitrified/warmed oocyte derived embryos (VODE) with fresh oocyte derived embryos (FODE) in order to evaluate the impact, if any, of oocyte vitrification on embryo development. For this purpose, 3893 photos of embryos taken daily with Pegase software (2iSYSTEM, France) software were retrospectively analysed as previously reported [14]. Our investigation did not show any detrimental effect of vitrification on embryo development. Indeed, at embryo culture day 2, VODE exhibited lower fragmentation degree than FODE. However, a higher number of VODE was found with mild fragmentation and although not significant, our data show more low-fragmented embryos among FODEs. Although statistically significant differences were described, they may not be informative as long as they were not detectable at further stages of development. Afterwards, at day 3, smaller number of blastomeres was observed in VODE as compared with FODE, but at culture day 4, no difference was observed between the two groups. We assume that the slight lag described for VODE at day 3 might be due to delayed intra-cytoplasmic ultrastructure reorganization of the egg after warming. If so, selecting only optimal quality of eggs before vitrification can be discussed or more than a 2-h post-warming incubation may be applied before egg microinjection [18]. Although VODE displayed lower percentage of B3 blastocysts at embryo culture day 5, percentage of blastocysts available at embryo culture day 5 is equal in VODE and FODE. Overall, present data assessing morphology and development of VODE and FODE do not show any major difference between the two groups. Our results are reassuring, and they are in line with published data showing that vitrification/warming cycles do not alter oocyte quality or resulting embryo development [6, 11].

Although molecular data concerning oocyte vitrification are very limited, they appear to be necessary for

Table 1 Baseline patient characteristics and laboratory outcome of the 90 women included in the study

	<i>n</i> =90 women
Mean age (SD)	33.0 (4.3)
Mean attempt number (SD)	2.0 (1.2)
Mean number of mature oocytes available at pick up (SD)	13.2 (4.1)
Mean number vitrified oocytes (SD)	7.2 (3.3)
Mean number of warmed oocytes (SD)	5.8 (2.5)
Mean oocyte survival rate (SD)	86.2 % (22.4)

NS not significant

Fig. 2 Distribution of day 2 and day 3 embryo score. **a** Embryo scores at day 2. **b** Embryo scores at day 3. No statistically significant difference was found

comprehensive knowledge of the impact of vitrification on oocyte and derived embryo. Interestingly, a recent study suggested an adverse effect of vitrification on subsequent embryo development via an alteration of calcium signalling in fertilized vitrified/warmed oocyte [12]. The cell division delay noticed at day 3 in VODE might be a consequence of calcium signalling alteration caused by oocyte vitrification, but no molecular data are available to challenge this issue. Further, similar investigations are required, although our observational data do not support this hypothesis since VODE and FODE show similar development and quality.

Large oocyte cohort associated with ovarian hyper stimulation may account for reduced oocyte quality [19] and thus may explain inferior survival rate after warming, reduced cleavage rate and lower percentage of blastocysts available at embryo culture day 5 when comparing with the published data of other clinics using identical embryo culture system [3, 20, 21].

Besides, we were not able to investigate blastocyst development rate per se (number of blastocyst at day 5/number of zygotes at day 1) in both groups because day 3 embryo transfer was frequently performed for VODE as a result of fewer embryos available for transfer than during ICSI cycles with fresh oocyte. In fact, warming cycles involved smaller number of oocyte as compared with fresh ICSI cycles in order to limit embryo cryopreservation as recommended by French authorities (Table 1).

This study demonstrates no negative effect of oocyte vitrification on embryo development. The objective was to evaluate and compare the morphology of embryos deriving from fresh and vitrified oocytes. As a consequence, the design of present study did not allow comparing clinical pregnancy rate achieved after the transfer of VODE (28.9 %) vs to FODE (12.6 %) since oocyte warming cycles were proposed subsequently to implantation failure or miscarriage with FODE. The implantation and pregnancy rates were calculated in the

Fig. 3 Distribution of day 5 embryo subclasses. **a** Proportion of morulae subclasses, as previously described [14], at embryo culture day 4. **b** Percentage of the different blastocyst subclasses at day 5. *VODE* vitrified/warmed oocyte derived embryos, *FODE* fresh oocyte derived embryos. * $p < 0.05$ FODE vs VODE. Numbers inside the bars correspond to the number of embryos in each category

reported population that is a sample and does not represent the total population managed in our centre. The reported group was also selected based on ovarian hyper stimulation. Oocyte warming cycle was proposed to these couples subsequently to pregnancy failure after fresh embryo transfer, probably due to poor endometrial quality induced by supra physiological environment. This situation may be avoided by delaying embryo transfer as proposed in “freeze all” strategy [22, 23]. We assume that the difference in clinical outcome of FODE vs VODE does not rely on embryo intrinsic quality but likely on clinical context. Indeed, VODE are replaced in the context of spontaneous or using hormonal replacement therapy allowing better endometrial receptivity. This difference in clinical outcome seems to illustrate a selection bias. Nevertheless, internal evaluation [13], along with other published data, demonstrate that oocyte vitrification does not impact subsequent biological and clinical outcomes in the context of infertility management [7, 8, 24].

We need to bear in mind that embryo grading is quite subjective and likely to vary between operators and microscope focus. In case of difficulty to evaluate embryo morphology, embryo grade was assessed based on the evaluation of another embryologist. Our image database has proved its worth the last 16 years since it provides a retrospective overview of embryo development for each attempt while also permitting to compare embryo morphology in each couple from one IVF cycle to another. Despite the fact that daily photos of embryos are appreciable records of embryo morphology, they are not the ideal tool for the investigation of embryo development per se.

Overall, results obtained in the present investigation confirmed previous studies demonstrating the safety and the efficiency of opened system based vitrification techniques (oocytes are directly in contact with liquid nitrogen during vitrification) [1–8, 13]. Since closed system based vitrification techniques are mostly used in France, it is worth testing these

vitrification systems using comparable criteria to better understand efficiency variation observed from one vitrification system to another.

Although we show that embryo development is not altered by oocyte vitrification, offspring follow-up remains essential to assess the safety of the technique and to exclude any adverse developmental effect. Therefore, babies born after oocyte vitrification benefit from the same long-term follow-up as other children born in our centre.

Acknowledgments We acknowledge François Lecluyse and Anne-Laure Benedetti for technical support. We would also like to thank clinical staff for addressing the couples to us and for allowing their participation in the present study.

Conflict of interest The authors declare that they have no competing interests.

Funding This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

- Anderson RA, Wallace WH. Fertility preservation in girls and young women. *Clin Endocrinol*. 2011;75:409–19.
- 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.
- Cobo A, Remohi J, Chang CC, Nagy ZP. Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online*. 2011;23:341–6.
- Cobo A, de Los Santos MJ, Castellò D, Gámiz P, Campos P, Remohi J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: evaluation of 3,150 warming cycles. *Fertil Steril*. 2012;98:1138–46.
- Cobo A, Garrido N, Crespo J, José R, Pellicer A. Accumulation of oocytes: a new strategy for managing low-responder patients. *Reprod Biomed Online*. 2012;24:424–32.
- Rienzi L, Romano S, Albricci L, Maggiulli R, Capalbo A, Baroni E, et al. Embryo development of fresh ‘versus’ vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. *Hum Reprod*. 2010;25:66–73.
- Rienzi L, Cobo A, Paffoni A, Scarduelli C, Capalbo A, Vajta G, et al. Consistent and predictable delivery rates after oocyte vitrification: an observational longitudinal cohort multicentric study. *Hum Reprod*. 2012;27:1606–12.
- Ubaldi F, Anniballo R, Romano S, Baroni E, Albricci L, Colamaria S, et al. Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. *Hum Reprod*. 2010;25:1199–205.
- Boyer P, Tourame P, Le Coz P. New assisted reproduction techniques: France, an absent subscriber. *Gynecol Obstet Fertil*. 2010;38:561–2.
- Cobo A. Oocyte vitrification: a watershed in ART. *Fertil Steril*. 2012;98:600–1.
- Khalili MA, Maione M, Palmerini MG, Bianchi S, Macchiarelli G, Nottola SA. Ultrastructure of human mature oocytes after vitrification. *Eur J Histochem*. 2012;56, e38.
- Nikiforaki D, Vanden Meerschaut F, Qian C, De Croo I, Lu Y, Deroo T, et al. Oocyte cryopreservation and in vitro culture affect calcium signalling during human fertilization. *Hum Reprod*. 2014;29:29–40.
- Boyer P, Montjean D, Tourame P, Gervoise-Boyer M. Oocyte vitrification in an ART laboratory. *Gynecol Obstet Fertil*. 2013;41: 551–3.
- Boyer P, Boyer M. Non invasive evaluation of the embryo: morphology of preimplantation embryos. *Gynecol Obstet Fertil*. 2009;37:908–16.
- Giorgetti C, Terriou P, Auquier P, Hans E, Spach JL, Salzmann J, et al. Embryo score to predict implantation after in-vitro fertilization: based on 957 single embryo transfers. *Hum Reprod*. 1995;10: 2427–31.
- Gardner DK, Schoolcraft WB. In vitro culture of human blastocysts. In: Jansen R, Mortimer D, editors. *Towards reproductive certainty: fertility and genetics beyond*. London: Parthenon Publishing; 1999.
- Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. *Curr Opin Obstet Gynecol*. 1999;11:307–11.
- Cobo A, Pérez S, De los Santos MJ, Zulategui J, Domingo J, Remohi J. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. *Reprod Biomed Online*. 2008;17:350–9.
- Sunkara SK, Rittenberg V, Raine-Fenning N, Bhattacharya S, Zamora J, Coomarasamy A. Association between the number of eggs and live birth in IVF treatment: an analysis of 400 135 treatment cycles. *Hum Reprod*. 2011;26:1768–74.
- Zech N, Stecher A, Zech H, Uher P, Vanderzwalmen P. Prospective analysis of embryo development to day 5 and transfer outcomes in sequential medium (G1.3-G2.3) vs a one step protocol (Global medium). *Hum Reprod*. 2006;21:i162.
- Vansteenberghe A, Vastersaegher C, Fontaine P, Klay C, Rodez D, Pauwels P. Comparison of a single medium with sequential media for the development of human zygote to the blastocyst stage. *Fertil Steril*. 2006;88:S319.
- Roque M. Freeze-all policy: is it time for that? *J Assist Reprod Genet*. 2015;32:171–6.
- Shapiro BS, Daneshmand ST, Gamer FC, Aguirre M, Hudson C. Freeze-all can be a superior therapy to another fresh cycle in patients with prior fresh blastocyst implantation failure. *Reprod Biomed Online*. 2014;29:286–90.
- Ubaldi F, Rienzi L, Baroni E, Ferrero S, Iacobelli M, Minasi MG, et al. Cumulative pregnancy rates after transfer of fresh and thawed embryos. *Eur J Obstet Gynecol Reprod Biol*. 2004;115:106–9.