Chromosomal meiotic segregation, embryonic developmental kinetics and DNA (hydroxy)methylation analysis consolidate the safety of human oocyte vitrification

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ABSTRACT: Oocyte vitrification has been introduced into clinical settings without extensive pre-clinical safety testing. In this study, we analysed major safety aspects of human oocyte vitrification in a high security closed system: (i) chromosomal meiotic segregation, (ii) embryonic developmental kinetics and (iii) DNA (hydroxy)methylation status. Fresh and vitrified sibling oocytes from young donors after intracytoplasmic sperm injection (ICSI) were compared in three different assays. Firstly, the chromosomal constitution of the fertilized zygotes was deduced from array comparative genomic hybridization results obtained from both polar bodies biopsied at Day 1. Secondly, embryo development up to Day 3 was analysed by time-lapse imaging. Ten specific time points, six morphokinetic time intervals and the average cell number on Day 3 were recorded. Thirdly, global DNA methylation and hydroxymethylation patterns were analysed by immunostaining on Day 3 embryos. The nuclear fluorescence intensity was measured by Volocity imaging software. Comprehensive chromosomal screening of the polar bodies demonstrated that at least half of the zygotes obtained after ICSI of fresh and vitrified oocytes were euploid. Time-lapse analysis showed that there was no significant difference in cleavage timings, the predictive morphokinetic time intervals nor the average cell number between embryos developed from fresh and vitrified oocytes. Finally, global DNA (hydroxy)methylation patterns were not significantly different between Day 3 embryos obtained from fresh and from vitrified oocytes. Our data further consolidate the safety of the oocyte vitrification technique. Nevertheless, additional testing in young and older sub-fertile/infertile patients and sound follow-up studies of children born after oocyte cryopreservation remain mandatory.

Key words: oocyte / vitrification / DNA methylation / PB biopsy / morphokinetics

Introduction

Oocyte cryopreservation offers a range of perspectives as it: (i) permits women to cryopreserve oocytes prior to gonadotoxic radio- or chemotherapy and/or ovariectomy (Tao and Del Valle, 2008; Ata et al., 2010); (ii) allows women to delay childbearing (Stoop et al., 2011, 2014); (iii) eliminates donor-recipient endometrium synchronization problems and (iv) avoids ethical and legal concerns regarding supernumerary cryopreserved embryos and embryo ownership (Schoolcraft et al., 2009). Slow freezing has been the traditional cryopreservation method until the introduction of the vitrification protocol by Kuwayama et al. (2005). The efficiency of oocyte cryopreservation has substantially increased with this protocol as it allows better oocyte survival and fertilization and higher implantation and pregnancy rates.

Today, there is an increasing awareness of the potential impact of assisted reproductive technologies (ART) on embryo development, gene expression, epigenetic modifications (in particular genomic imprinting) and the developmental origins of health and disease (Brison et al., 2013; Harper et al., 2012). The safety of cryopreservation procedures has particularly been questioned after reports showing that singleton babies born after frozen embryo transfer have an increased risk of being born large for gestational age (Pinborg et al., 2010, 2014).
Ideally, new ART procedures should be introduced into clinical practice only following well-planned safety studies. The information available on the neonatal outcome and the long-term follow-up of children conceived after oocyte cryopreservation is limited (Chian et al., 2008, 2014; Noyes et al., 2009; Wennerholm et al., 2009; Cobo et al., 2014) and only small studies have specifically addressed the safety of oocyte vitrification (Costicchio et al., 2009; Bonetti et al., 2011; Chen et al., 2012; Forman et al., 2012; Khalili et al., 2012; Monzo et al., 2012; Dominguez et al., 2013; Gugliemo et al., 2014; Konc et al., 2014; Nohales Córcoles et al., 2014; Palmerini et al., 2014).

In this study, we addressed some safety aspects after closed vitrification by comparing human fresh and vitrified sibling oocytes and derived embryos while focusing on three major safety aspects: (i) chromosomal meiotic segregation, (ii) embryonic developmental kinetics and (iii) DNA (hydroxy)methylation status. Firstly, it is known that the meiotic spindle is very sensitive to cryodamage (Ghetler et al., 2005) and, thus, the second meiotic division may be particularly vulnerable to chromosomal segregation errors. In order to detect aneuploidies of female meiotic origin, we used the array-based comparative genomic hybridization (aCGH) technique (Handyside, 2013) on polar bodies (PBs) to distinguish errors occurring before oocyte vitrification in meiosis I (PB1) from errors occurring after oocyte warming in meiosis II (PB2). Secondly, time-lapse imaging is useful to study the developmental kinetics of preimplantation embryos. However, it has not yet been proven that time-lapse monitoring yields higher pregnancy rates in randomized clinical trials (Albertini, 2014; Kaiser and Racowsky, 2014). Finally, DNA methylation is a key epigenetic modification, and timely patterns of genome-wide DNA demethylation and subsequent remethylation are important for correct preimplantation development. One of the pathways of DNA demethylation is the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) methyltransferase enzymes (Ito et al., 2011; Pastor et al., 2013). Previous experiments showed that the spatial and temporal expression patterns of DNA methyltransferase enzymes are disturbed after embryo slow freezing procedures (Petrussa et al., 2014).

**Materials and Methods**

**Oocyte source**

For this study, approval was obtained by the Local Ethical Committee of UZ Brussel and by the Federal Ethical Committee for medical and scientific research on human embryos in vitro. Fresh oocytes were obtained from 18 young donors (30.5 ± 3.8 years) between December 2011 and March 2013. They donated a few of their retrieved oocytes for research after informed consent, allowing us to create embryos for this study. Oocytes were denuded with Cumulase® (80 USP Units/ml, Holzyme Therapeutics, Inc., San Diego, CA, USA) from the surrounding cumulus and corona cells (De Vos et al., 2008).

We studied two groups of sibling oocytes: fresh versus vitrified. The oocytes were collected over a time period of 16 months and, therefore, the comparisons of fresh and vitrified oocytes were carried out in two consecutive experiments. We used three assays to analyse the oocytes and the derived embryos: chromosomal meiotic segregation, embryonic developmental kinetics and DNA (hydroxy)methylation. Supplementary data, Table S1, shows the allocation of the oocytes to each experiment and assay.

**Oocyte vitrification and warming**

Vitrification and warming was carried out as previously described (De Munck et al., 2013) with minor adaptations.

For vitrification, we used the Irvine Scientific® Vitrification Freeze Kit (Irvine Scientific, USA), containing 7.5% (v/v) ethylene glycol (EG) + 7.5% (v/v) dimethylsulphoxide (DMSO) in an M-199 HEPES-buffered medium supplemented with 20% dextran serum supplement (DSS), referred to as equilibration solution (ES) and vitrification solution (VS) containing 15% (v/v) EG + 15% (v/v) DMSO + 0.5 M sucrose. Oocytes were placed in 25 µl HTF-hepese supplemented with HSA and immediately merged with 25 µl ES for 2 min at room temperature followed by a second merging with 25 µl ES for 2 min. Then, oocytes were transferred into a new 25 µl ES droplet for 10 min, followed by two consecutive 50 µl VS droplets, and they were loaded on the CBS vit straw (CryoBiosystems, France), and the straws were thermosealed and plunged into liquid nitrogen (LN2) within 60 s. The high security closed system prevents any contact with LN2. The straws are thermosealed before they are plunged into the LN2 so during vitrification, storage and warming, the oocytes are never in direct contact with the LN2. Depending on the number of oocytes available, vitrified oocytes were vitrified individually or in pairs.

For warming, we used the Irvine Scientific® Vitrification Thaw Kit (Irvine Scientific, USA), containing a thawing solution (TS) with 1 M sucrose in an HEPES-buffered medium supplemented with 20% DSS, a dilution solution (DS) containing 0.5 M sucrose in an HEPES-buffered medium supplemented with 20% DSS and a washing solution (WS) containing HEPES-buffered medium supplemented with 20% DSS. Oocytes were immediately placed in 200 µl preheated TS at 37°C for 1 min, followed by 3 min in 50 µl DS and two washes for 5 min each in 50 µl WS, both at room temperature.

After washing, oocytes were transferred into individual 25 µl droplets of Quinn’s Advantage Fertilization medium (Sage, Cooper Surgical, USA) under oil and scored for survival. Subsequently, they were cultured for 2 h in an incubator with 5% O2 and 6% CO2.

**Evaluation of survival**

Warmed oocytes were considered to have ‘morphologically survived’ if they had no dark/degenerated or contracted ooplasm and no cracked zona pellucida.

**Fertilization and embryo quality**

After 2 h incubation in fertilization medium, intracytoplasmic sperm injection (ICSI) was performed on all surviving oocytes (Palermo et al., 1992) with cryopreserved sperm of one consenting donor. The injected oocytes were cultured individually in 25 µl droplets of Quinn’s Advantage Cleavage medium (Sage, Cooper Surgical) under oil (Ovol, Vitrolife, Sweden) until Day 3.

Fertilization was checked 16–18 h after injection. Preimplantation embryo development was evaluated on Day 3 and only good-quality Day 3 embryos (defined as having at least six blastomeres and <20% fragmentation) were fixed. For eight donors, injected sibling oocytes were cultured in the Embryoscope™ (Unisense FertilTech, Aarhus, Denmark); fertilization and development were analysed through time-lapse imaging. Good-quality embryos were fixed on Day 3.

**PB biopsy**

Oocytes were immediately subjected to PB biopsy after the fertilization check. Biopsy was performed on a heated stage of an inverted microscope (Nikon) using laser-assisted biopsy (Fertilase, Octax, Herbron, Germany). The biopsy was performed in dishes prepared per oocyte, each containing three droplets (5–10–10 µl) HTF-Hepes medium under oil (Vitrolife) and pre-equilibrated at 37°C. The smallest droplet was used for the oocyte and the other two droplets were used for sampling of PB1 and PB2 of the
corresponding oocyte after biopsy. The bigger PB was defined as the first PB (two chromatids) and the smaller one as the second PB (one chromatid). Further technical aspects of the biopsy and transfer of the PBs into the reaction tube are described elsewhere (Magli et al., 2011).

Chromosomal meiotic constitution
Biopsied PBs were collected into sterile PCR tubes. The samples and positive/negative control samples were lysed, fragmented and amplified using the SurePlex whole genome amplification (WGA) kit (BlueGnome, Cambridge, UK) according to the manufacturer’s instructions (www.illumina.com). The occurrence of amplification was ensured by gel electrophoresis. Sample and reference DNA (male and female) were labelled with Cy3 or Cy5 fluorophores following the manufacturer’s instructions. Labelling mixes were combined and hybridized overnight on 24sure Cytochip (V3, BlueGnome, Cambridge, UK). Fluorescence intensity was detected using a laser scanner (Agilent scanner G2565BA), and BlueFuse Multi software was used for data processing (BlueGnome, Cambridge, UK). Each sample is compared with both a male and a female reference. Once a specific amplification was observed, autosomal profiles were analysed for gain or loss of whole or partial chromosomal ratios using a 3SD assessment, >0.3 log2 ratio call or both. To pass hybridization quality controls, sex-mismatched female samples had to show a consistent gain on chromosome X and a consistent loss of chromosome Y. Sex-matched female samples had to consistently show no change on either chromosome X or Y. PBs were classified as: ‘normal or euploid’ if the generated array CGH plot showed no gains or losses in any of the autosomes, ‘abnormal or aneuploid’ if total or segmental chromosome gains or losses were observed or, ‘inconclusive’ due to increased levels of random noise. The ‘24sure microarray product description’ describes 20 Mb effective resolution for 24sure using BlueFuse software, being this the minimum size specified by BlueGnome for segmental aneuploidies.

All array data are available at the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/gds) with accession number GSE63866.

Embryonic developmental kinetics
Each well of the EmbryoSlide® was filled with a 25 µl droplet of cleavage medium and covered with 1.2 ml preheated oil to form a confluent oil layer preventing evaporation. The slides were equilibrated during at least 4 h in an incubator with 5% O2 and 6% CO2 at 37°C. After pre-equilibration, air bubbles were removed and the injected sibling oocytes were placed individually in the Embryoscope™ microwells and incubated in the time-lapse monitoring system under 5% O2 and 6% CO2 at 37°C. When all six slides of the embryooscope are filled with 12 embryos, it is impossible to make images at many planes and in a very short time interval. Therefore, pictures were taken every 20 min at 5 focus planes through every oocyte/zygote/embryo.

We intended to analyse the following 10 time points: (i) appearance of the second PB, (ii) pronuclear abuttal, (iii) disappearance of the pronuclei (PN), (iv) appearance of the first cleavage furrow or cell elongation, (v) time to reach the two-cell stage, (vi) time of appearance of the first nucleus at the two-cell stage, (vii) time of appearance of the second nucleus at the two-cell stage, (viii) time to reach the three-cell stage, (ix) time to reach the four-cell stage and (x) time to reach the five-cell stage. Based on these time points, we aimed to calculate the following six time intervals as described by Wong et al. (2013): (i) time from injection to pronuclear abuttal, (ii) duration of the first cytokinesis: time between cell elongation and the time the two-cell stage is reached, (iii) time between the appearance of both nuclei at the two-cell stage, (iv) duration of the first mitosis: the time an embryo needs to go from two cells to three cells, (v) duration of the second mitosis: the time an embryo needs to go from three cells to four cells and (vi) time from injection to reach the five-cell stage.

DNA (hydroxy)methylation analysis
Embryos were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, Bornem, Belgium) supplemented with 2% bovine serum albumin (BSA; Sigma-Aldrich), fixed with 3.7% formaldehyde (Merck; VWR International, Leuven, Belgium) in PBS for 10 min at room temperature. They were stored in PBS with BSA at 4°C.

Embryos were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 30 min at room temperature. They were treated with 6 M HCl for 10 min at room temperature to obtain DNA denaturation, and washed for 20 min in PBS with 2% BSA.

The optimal incubation time, temperature and antibody concentration required for saturable staining were determined in preliminary experiments. Embryos were incubated with a 1:30 dilution mouse anti-5-methylcytosine (Bi-ME CY Eurogentec, Belgium) and a 1:500 dilution of rabbit anti-5-hydroxymethylcytosine (39 769, Active Motif, Belgium) in PBS with 2% BSA at 4°C overnight. Negative controls were incubated with normal mouse IgG (SC-2025, Santa Cruz Biotechnology) or normal rabbit IgG (SC-2027, Santa Cruz Biotechnology, Tebu-bio, Boechout, Belgium) depending on the host species of the primary antibody. Next, samples were incubated simultaneously with 10 µg/ml of both goat anti-rabbit IgG F(AB’)2 conjugated to Alexa Fluor 488 (A-11070, Life Technologies, Belgium) and goat anti-mouse IgG F(AB’)2 conjugated to Alexa Fluor 647 (A-21237, Life Technologies, Belgium) in PBS with 2% BSA for 2 h at room temperature. Between every step, samples were washed three times for 5 min in PBS with 2% BSA at room temperature. Embryos of similar developmental stages were stained with normal serum of the same host species to serve as an internal negative control for the primary antibody. Control reactions for the secondary antibody were carried out by omitting the primary antibody. After staining, embryos were put between two glass cover slips in SlowFade Gold Antifade reagent (Life Technologies, Belgium). To prevent squeezing of the embryos, round glass cover slips were used as spacers. Each staining protocol took 2 days and imaging was always done on the second day of the protocol, so the signal would not quench.

Embryos were visualized by confocal scanning microscopy with an Ar-HeNe laser (488/633 nm) (IX71 Fluoview 300; Olympus, Aartselaar, Belgium) and a relative quantification of the signals was done with Velocity imaging software (Perkin Elmer, Inc.): all embryos were visualized in 3D and the individual nuclei were delineated by the software. The nuclear fluorescence intensity of 5mC and 5hmC staining was measured in all individual non-dividing nuclei. This measurement provides the total fluorescence intensity of all the voxels in the nucleus. Per embryo, the total fluorescence intensities were summed and divided by the number of nuclei to calculate the mean value. Ratios of 5mC/5hmC were calculated with the mean fluorescence intensity value of the whole embryo.

The embryos were stained in two independent immunocytochemistry experiments. The mean fluorescence intensity of the fresh embryos was arbitrarily set as 1 for 5mC and 5hmC and the ratio of 5mC/5hmC was set as 1 to combine the measurements of the two different experiments.

Some embryos were counterstained with propidium iodide (PI) (Sigma-Aldrich, Belgium) after the 5mC/5hmC double immunocytochemistry. Double-stained embryos were washed three times for 5 min in PBS with 2% BSA at room temperature, incubated in 0.01 mg/ml PI for 10 min at 37°C, washed three times and put in SlowFade Gold Antifade reagent (Life technologies). Embryos were visualized on an inverted fluorescence microscope (IX81 Fluoview 300; Olympus).

Statistical analysis
Fisher’s exact test was used to assess differences between fresh and vitrified oocytes for the array results. Differences between embryos obtained from fresh and vitrified oocytes were assessed with the Student’s t-test. We were unable to use the paired t-test since the oocytes of three donors
were not used as sibling oocytes. A difference was considered statistically significant when the P-value was <0.05.

Results

Chromosomal meiotic constitution

A total of 27 zygotes were biopsied on Day 1. WGA was successful on both PBs of 23 zygotes: 12 from fresh and 11 from vitrified oocytes. For four zygotes, one from a fresh oocyte and three from vitrified oocytes, only one PB was obtained. Supplementary data, Table S2 shows the array results for fresh and vitrified oocytes with two PBs analysed. For the fresh oocytes, aCGH results showed six (50.0%) euploid zygotes, two zygotes were inconclusive, one was without result and three were aneuploid. For the vitrified oocytes, aCGH results showed that eight (72.7%) zygotes were euploid, one zygote was inconclusive and two zygotes were aneuploid. There was no significant difference between the two groups (P = 0.400). The aCGH outcome for the four zygotes of which only one PB was analysed is also given in Supplementary data, Table S2. Those PBs were all euploid after aCGH. Array plots representing a fresh and a vitrified oocyte are shown in Fig. 1.

Embryonic developmental kinetics

From both experiments, a total of 24 good quality embryos were obtained on Day 3 from the fresh oocytes. The average cell number on Day 3 was 8.5 ± 1.4. For the vitrified oocytes, 19 good quality embryos were obtained on Day 3 and the average cell number on Day 3 after oocyte vitrification was 8.1 ± 1.9. In each group, one compacting embryo was excluded from the cell number calculation. The number of blastomeres on Day 3 was not significantly different between both groups (P = 0.371). A total of 32 sibling oocytes from eight oocyte donors were evaluated by time-lapse analysis after injection: 16 fresh and 16 vitrified oocytes. From the fresh oocytes, 12 were fertilized after ICSI (75%) and 11 good-quality embryos were obtained on Day 3 (92%). After oocyte vitrification, 13 oocytes were fertilized after ICSI (81%) and 10 developed into good-quality embryos (77%). For one embryo, we were unable to analyse the images due to an unexplained shift in the time-lapse analysis. For the second time point (or first time interval), pronuclear abuttal, we were unable to determine the exact timing because the quality of the images was low and because pictures were taken every 20 min. This made it impossible to determine whether the pronuclear abuttal occurred immediately at the time the second PN appeared or happened within the 20 min interval. For the nine remaining time points tested, there was no significant difference between embryos obtained from fresh or vitrified oocytes. Results for the two groups of oocytes are shown in Fig. 2. When analysing the five remaining time intervals, no significant differences were observed between fresh and vitrified oocytes. Supplementary data, Table S3 shows the time points and time intervals for all individual embryos analysed.

DNA (hydroxy)methylation analysis

From both experiments, 31 Day 3 embryos, 17 from fresh and 14 from vitrified oocytes, were analysed for their global DNA (hydroxy)methylation pattern. All clearly delineated nuclei were analysed. The number of blastomeres with one nucleus, two nuclei or fragmented nuclei and the

Figure 1  Polar body aCGH. Array plot for the PBs of a fresh (left) and a vitrified (right) oocyte. For the fresh oocyte, both PB1 (top left) and PB2 (bottom left) were euploid indicating a euploid zygote. The vitrified oocyte showed a meiosis I error for Chromosome 16, where the loss in PB1 (top) was compensated by a gain in PB2 (bottom), and a meiosis II error for Chromosome 11, indicating a zygote with Monosomy 11.
number of anucleated blastomeres are given in Supplementary data, Table S4. The fluorescence intensity of methylation and hydroxymethylation was quantified. Both 5mC and 5hmC signals were present on Day 3. Figure 3 shows the intra-embryonic variability for an embryo obtained from a fresh and a vitrified oocyte. There was no statistically significant difference between Day 3 embryos obtained from fresh or vitrified oocytes when we compared the mean fluorescence intensities for 5mC (1.0 ± 0.49 versus 0.83 ± 0.41), 5hmC (1.0 ± 0.40 versus 0.81 ± 0.36) and 5mC/5hmC ratios (1.0 ± 0.18 versus 1.05 ± 0.28) (Table I). Figure 4 shows the inter-embryonic variability for both stainings in the two study groups.

Discussion

The aim of this study was to analyse three safety aspects of oocyte vitrification in a high security closed system by comparing fresh and vitrified sibling oocytes from young donors. Comprehensive chromosomal screening of the PBs demonstrated that at least half of the zygotes were euploid in both groups and this was not significantly different. Time-lapse analysis showed that there was no significant difference in the time points and time intervals analysed between embryos developed from fresh or vitrified oocytes. Also, the number of blastomeres on Day 3 did not differ between the two groups of embryos. Finally, the global DNA methylation pattern, analysed by 5mC and 5hmC immunocytochemistry stainings, was not significantly different between the Day 3 embryos obtained from fresh or vitrified oocytes.

The use of vitrification to cryopreserve human oocytes is a highly efficient technique. Nowadays, survival rates over 90% and fertilization rates ranging from 70 to 95% are obtained, which has led to a tremendous increase in clinical outcomes and live birth rates after oocyte cryopreservation (Cil and Seli, 2013). Although over 1500 babies are born after oocyte vitrification, it is too early for information on the long-term developmental influence of oocyte vitrification. In order to address some of the possible long-term effects of oocyte vitrification, the follow-up of a sufficient number of pregnancies and infants is needed.

The oocyte vitrification technique has been introduced into clinical practice without proper pre-clinical testing in animal models and safety testing on human research oocytes as recommended by Harper et al. (2012) and Brison et al. (2013). Pre-clinical testing is crucial since the oocytes are exposed to high concentrations of cryoprotectants during the vitrification procedure which may damage the oocyte through cytotoxic or osmotic effects. The osmotic imbalance created by addition and removal of cryoprotectants results in large volumetric changes and may cause damage to the oocyte’s morphology, cytoskeletal structures and function (Paynter, 2005; Smith et al., 2011). This study is the first one addressing some important safety issues on oocyte vitrification. Although this study was conducted after introduction of the vitrification technique into the clinical setting, we believe that it is important to continue this type of pre-clinical/fundamental research in order to consolidate the safety of a new technique.

Chromosomal meiotic constitution

The molecular analysis of the first and the second PB provides an indirect assessment of the chromosomal constitution of the oocyte because it examines the segregation during meiosis I and II, respectively. In this way, aneuploidies of maternal meiotic origin will be detected (Fragouli et al., 2011). In the present study, the percentage of aneuploid zygotes from fresh and vitrified oocytes was 50.0 and 27.3%, respectively, indicating that the post-warming meiotic spindle is perfectly able to complete the second meiotic division and the cytoskeletal structure and function are completely restored.

It is known that oocytes from women of advanced maternal age (>40 years) have a high rate of chromosomal abnormalities (>50%) after FISH analysis (usually testing five chromosomes: 13, 16, 18, 21 and 22) (Haaf et al., 2009). However, Haaf et al. (2009) also found chromosomal errors in fresh oocytes from young infertile patients (≤35 years) at a rate of
34.4% in their first cycle or an increased rate of 43.8% in subsequent cycles. The oocytes of young donors (26.1 years) with no known fertility problems revealed an aneuploidy rate of only 19.1% (Velilla et al., 2013). In the present study, when we only look at the five chromosomes usually tested by FISH and exclude all aberrations detected on other chromosomes, the aneuploidy rate would be 33.3% (4/12) for fresh oocytes and 18.2% (2/11) for vitrified oocytes from young donors, which is in line with the results of Velilla et al. (2013), Reis Soares et al. (2003) and Munné et al. (2006) analysed one or two blastomeres on Day 3 embryos from young oocyte donors (27.0 and 25.6 years, respectively) by FISH. Both groups reported high rates of chromosomal abnormalities of 56.5 and 57%, respectively, but they also included mitotic errors which were not detected in our study. In the study of Forman et al. (2012), fresh and vitrified sibling oocytes were tested for aneuploidy by trophectoderm biopsy on Day 5. They found a similar aneuploidy rate (meiotic and mitotic errors) in the transferable blastocysts between fresh and vitrified oocytes (16.4 and 17.2%, respectively).

Interestingly, for donor 6, two fresh and three vitrified zygotes were analysed and only one vitrified zygote appeared to be euploid. These results suggest that chromosomal constitution errors are likely inherent to the individual rather than to any of the ART procedures used here.

A possible shortcoming in our study is the choice to perform a simultaneous instead of a sequential PB biopsy. When a simultaneous PB biopsy is performed, both PBs are distinguished based on their size. It has been shown that morphological grading of the PBs is only 63% consistent (Treff et al., 2012). On the other hand, single-nucleotide polymorphism (SNP) microarray heterozygosity analysis of PBs was proven to be 94% predictive. A diploid cell, like the first PB, will be either homozygous or heterozygous at any given SNP, while the second PB, containing only one copy of each chromosome, will be homozygous for every SNP. Therefore, the first PB will be the one with the greatest heterozygosity. In our study, the results of the first and second PB showed that both PBs were euploid in 72.7% of the zygotes analysed. Therefore, the additional information of the SNP analysis was not needed in this study.

In conclusion, although only a small set of oocytes was tested, our results show that most of the vitrified oocytes from young donors are euploid after vitrification and, thus, the second meiosis is probably not affected by the procedure.

### Embryonic developmental kinetics

In our study, the average number of cells in Day 3 embryos developed from fresh and vitrified oocytes after ICSI was 8.5 and 8.1, respectively, and this was not significantly different between the two groups. The same parameter was analysed by Cobo et al. (2008), Almodin et al. (2010) and Forman et al. (2012) for fresh and vitrified oocytes. Cobo et al. (2008), using Cryotop, found an average cell number on Day 3 of 6.9 for fresh and 6.9 for vitrified oocytes. However, Almodin et al. (2010), using Vitr-in, found a significant difference between the number of blastomeres obtained on Day 3 between fresh 6.86 and vitrified 6.35 oocytes. Also Forman et al. (2012), using Cryotop, observed a significant difference in the blastomere numbers between embryos from fresh (7.3) and vitrified (6.6) oocytes. However, in those reports, more embryos were analysed and different vitrification devices were used. The absence of a significant difference in our study could be due to the smaller sample size.

Time-lapse imaging has gained a lot of interest during the last decade and has been used to identify cleavage timings, before embryonic genome activation, that correlate with embryo quality and their ability to form blastocysts (Wong et al., 2013). The nine time points that we were able to analyse were not significantly different between fresh and vitrified oocytes. Based on those nine time points, we tested five of the six predictive morphokinetic time intervals as reported by Wong et al. (2013). Those time intervals were not significantly different.

### Table I DNA methylation analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fresh oocytes</th>
<th>Vitrified oocytes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 5mC</td>
<td>1.0 ± 0.49</td>
<td>0.83 ± 0.41</td>
<td>0.299</td>
</tr>
<tr>
<td>Mean 5hmC</td>
<td>1.0 ± 0.40</td>
<td>0.81 ± 0.36</td>
<td>0.189</td>
</tr>
<tr>
<td>Mean 5mC/5hmC</td>
<td>1.0 ± 0.18</td>
<td>1.05 ± 0.28</td>
<td>0.543</td>
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The mean 5mC, 5hmC and 5mC/5hmC levels of the Day 3 embryos obtained from fresh and vitrified oocytes after ICSI are given: no significant differences were observed between the two groups.

**Figure 3** DNA (hydroxymethyl)ation analysis. Representative image after double immunofluorescence staining on human preimplantation embryos derived from injected fresh (left panel) and vitrified (right panel) sibling oocytes. For each embryo, the transmission image, 5mC staining (shown in red), 5hmC staining (shown in green) and the merge of 5mC and 5hmC are shown. Note the presence of ananucleated blastomere in the fresh embryo (arrow).
between the embryos derived from fresh versus vitrified oocytes. Overall, the embryonic development up to the five-cell stage of vitrified oocytes showed the same morphokinetic timings as their fresh sibling oocytes. The time at which the second PB was extruded was a very important time point for us, because it gives an indication on the time the oocyte needs to recover completely after the vitrification and warming procedure. As injection was performed 2 h after warming, this period seems to be sufficient for the normal restoration of the cytoskeletal structure and function.

The first time interval we investigated is the time from injection to pronuclear abuttal, initially described by Payne et al. (1997). Unfortunately, we were unable to analyse this time interval.

The duration of the first cytokinesis (second time interval) for the embryos developed from injected fresh and vitrified oocytes in our study was 29 and 30 min, respectively. According to Wong et al. (2010), probably most of our embryos would have reached the blastocyst stage (duration <33 min). Caution should be taken however, since Wong et al. (2010) investigated cryopreserved zygotes (although these did not develop significantly differently to 10 fresh injected oocytes) in their study and the first cytokinesis was the first division after zygote warming.

The third time interval we analysed is the appearance of the nuclei after the first cleavage. Lemmen et al. (2008) initially reported that this appearance at the two-cell stage was within 30 h for greater than or equal to four-cell stage embryos and around 35 h for less than four-cell stage embryos. In addition, they reported that the synchrony in appearance of nuclei after the first cleavage was associated with pregnancy success: the appearance of both nuclei was around 10 min in embryos of pregnant patients and around 100 min in embryos of non-pregnant patients. For the fresh and vitrified oocytes in our study, this was 58 and 44 min, respectively. Although this interval seemed longer, it can be partly explained by the fact that we only took pictures every 20 min making the interval in which the nuclei can appear much bigger.

The fourth time interval we analysed is the time between the first and second mitosis, which should be between 7.8 and 14.3 h according to Wong et al. (2010) or faster than 11.9 h according to Meseguer et al. (2011), where fresh donor oocytes were cultured in Sage media and pictures were taken every 20 min. We found similar averages for the time to reach the five-cell stage within this period, which was only 1.37 h for fresh and 0.86 h for vitrified oocytes in our study compared with 2.19 h in the study reported by Basile et al. (2013). In the study of Basile et al. (2013), the average time at the three-cell stage is much longer as compared with a similar study of the same group (0.76 h) (Meseguer et al., 2011). The focus of the latter study was on the relationship between morphokinetic parameters and implantation while the study of Basile et al. (2013) showed that embryo morphokinetics is not affected by the type of culture media. To conclude, the developmental kinetics up to the five-cell stage is very similar for embryos obtained from fresh or vitrified oocytes and their cell numbers on Day 3 are comparable.

**DNA (hydroxy)methylation analysis**

There is an increasing awareness of the stressors to which the embryo has to adapt during in vitro culture and the possible epigenetic changes...
they can cause. At the moment, the possible contribution of oocyte vitrification to those epigenetic changes is unknown. It has been shown that the spatial and temporal expression patterns of DNA methyltransferase enzymes are disturbed in embryos cryopreserved by slow freezing on Day 3 (Petrussa et al., 2014). This corresponds well with the finding that DNA methylation and hydroxymethylation levels from Day 3 and Day 4 embryos differed significantly between good and poor quality embryos (Petrussa et al., unpublished data). Santos et al. (2010) correlated inappropriate global DNA methylation with abnormal chromatin and poor embryo quality. Development up to the blastocyst stage was consistent with normal DNA methylation and chromatin organization. Therefore, we investigated the effect of oocyte vitrification on the methylation and hydroxymethylation patterns by quantifying the relative 5mC and 5hmC fluorescence intensity levels in Day 3 embryos. No significant differences were observed between the embryos obtained from fresh and vitrified oocytes.

Since 5hmC is far less abundant than 5mC (Tahiliani et al., 2009), it is impossible to make a quantitative comparison between both modifications by immunocytochemistry, but each modification can be compared between embryos from the same staining experiment. A large intra-embryonic but also inter-embryonic variability was seen in embryos of both groups when comparing the levels of 5mC and 5hmC between different nuclei. The methylation on the genomic DNA is actively redistributed during early development (Arányi and Páldi, 2006). This dynamic redistribution is sensitive to environmental influences and may generate a high variation in DNA methylation patterns, as we observed in our embryos. This internuclear variability is probably inherent to the DNA methylation re-programming process. In addition, it was noted that a high proportion of the blastomeres were binucleated or anucleated: 2.6 and 8.4%, respectively, for embryos from fresh oocytes and 7.2 and 7.2%, respectively, for embryos from vitrified oocytes. Embryos were counterstained with PI to verify the absence of DNA in the anucleated blastomeres. Also, reciprocal patterns were not necessarily present; one embryo, for example, contained three anucleate blastomeres but no binucleate blastomeres. This phenomenon is well known and has been described in several reports. Different explanations for this nuclear state have been proposed, but no clear answer has been provided so far (Winston et al., 1991; Hardy et al., 1993; Levy et al., 1998a, b; Moriwaki et al., 2004; Chatzimeletiou et al., 2005; Xanthopoulou et al., 2011).

No differences in global methylation or hydroxymethylation levels could be found in our study, suggesting that embryos derived from vitrified oocytes do not carry abnormal global DNA methylation epigenetic patterns. Nevertheless, this does not exclude localized or gene-specific differences that may have a profound effect on gene expression and therefore needs further testing. The fact that good quality embryos from vitrified oocytes have similar global methylation and hydroxymethylation levels as compared with good quality control embryos from fresh oocytes is reassuring.

**Conclusion**

By analysing three important safety aspects of oocyte vitrification at different time points during human preimplantation development, we found no differences between fresh and vitrified oocytes from young donors. Data on the aneuploidy rate (from aCGH on PBs), developmental kinetics to the five-cell stage from time-lapse analysis and global DNA methylation patterns on Day 3 embryos are all reassuring with respect to the safety of the oocyte vitrification technique. Other studies have also shown that the major oocyte characteristics, viability and ultrastructure are preserved after vitrification. Although generally the data so far are encouraging, further analysis in young and older sub-fertile/infertile patients and follow-up studies of children born after oocyte cryopreservation remain necessary.

**Supplementary Material**

Supplementary Material is available at http://molehr.oxfordjournals.org/ online.

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**Authors’ roles**

N.D.M. and L.P. were involved in the study design, sample preparation, experimental procedures, data collection and analysis and manuscript preparation; G.V., K.J. and D.S. were involved in manuscript preparation; C.S was involved in data analysis and manuscript preparation; Y.V., J.S. and G.B. were involved in sample preparation and experimental procedures; and M.D.R. and H.V.D.V. were involved in the study design, data analysis and manuscript preparation. All authors approved the final version.

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**Conflict of interest**

All authors have completed the conflict of interest disclosure form, and have no conflicts to declare.

**References**

Albertini DF. When looks are deceiving—the challenge facing embryo quality prognosticators. *J Assist Reprod Genet* 2014;31:249–250.


