Cytoskeletal analysis of human blastocysts by confocal laser scanning microscopy following vitrification

Katerina Chatzimeletiou1,*, Ewan E Morrison2, Yannis Panagiotidis3, Pierre Vanderzwalmen4, Nikos Prapas3, Yannis Prapas3, Basil C. Tarlatzis1, and Alan H. Handyside5

1Section of Reproductive Medicine, First Department of Obstetrics & Gynaecology, Aristotle University Medical School, Papageorgiou General Hospital, Thessaloniki 54603, Greece 2CRUK Clinical Centre at Leeds, St James’ University Hospital, Leeds LS9 7TF, UK 3Iakentro Advanced Medical Centre, Thessaloniki 542 50, Greece 4IVF Centre Prof. Zech, Bregenz, Austria 5The London Bridge Fertility Gynaecology and Genetics Centre, London SE1 9RY, UK

*Correspondence address. Tel: +30-231-332-3827; E-mail: katerinachatzine@hotmail.com

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BACKGROUND: Vitrification of human blastocysts is being used increasingly to cryopreserve supernumerary embryos following IVF. In this study, we investigate the effects of aseptic vitrification on the cytoskeleton and development of human blastocysts, by analysing survival rates and spindle and chromosome configurations by fluorescence and confocal laser scanning microscopy.

METHODS: A total of 55 fresh blastocysts and 55 day 5 dimethylsulphoxide/ethylene glycol vitrified blastocysts, which were allowed to remain in culture for 24 h post-warming, were rapidly fixed in ice cold methanol, and immunostained with an α-tubulin antibody to visualize microtubules in combination with antibodies against acetylated tubulin (to visualize spindles, poles and mid bodies), gamma tubulin (to identify spindle poles) and 4(6-diamidino-2-phenylindole) to visualize DNA.

RESULTS: In total, 213 spindles were analysed in the control (fresh) group of which 183/213 (85.9%) were normal, 20/213 (9.4%) were abnormally shaped, 9/213 (4.2%) were multipolar and 1/213 (0.5%) was monopolar. A total of 175 spindles were analysed in the vitrified group, of which 120/175 (68.6%) were normal, 39/175 (22.3%) were abnormally shaped, 10/175 (5.7%) were multipolar and 6/175 (3.4%) were monopolar. The incidence of multipolar spindles was similar in the two groups, but the level of abnormally shaped spindles, often associated with chromosome lagging, or congression failure, was significantly higher in the vitrified group compared with the fresh group (P < 0.05).

CONCLUSIONS: The high survival rate following thawing and the large proportion of normal spindle/chromosome configurations suggests that vitrification at the blastocyst stage on Day 5 does not adversely affect the development of human embryos and the ability of spindles to form and continue normal cell divisions. However, there was a significantly higher incidence of abnormal spindles in the vitrified group compared with the fresh group, notably of spindles with a focused and an unfocused pole as well as chromosome bridging and disorganized middle spindle fibres at telophase. Further investigation is warranted to elucidate the mitotic stages that are more vulnerable to damage during vitrification, the fate of the abnormal spindles and any potential effects that may be reflected on the chromosomal constitution of the developing blastocysts.

Key words: aseptic vitrification / chromosome bridging / confocal laser scanning microscopy / human blastocyst / spindle abnormalities

Introduction

Vitrification is an attractive ultrarapid cryopreservation technique which has gained support as an alternative promising substitute for slow freezing. It uses extremely high concentrations of cryoprotectants and allows the solidification of a solution below the glass transition temperature, without ice crystal formation (Liebermann, 2009; Vajta et al., 2009). Vitrification has been successfully applied to both cleavage and blastocyst stage embryos and clinical trials have shown high survival rates and promising implantation rates following transfer of thawed embryos at all stages (Liebermann and Tucker, 2006; Mukaida et al., 2006; Hong et al., 2009; Vanderzwalmen et al., 2009, 2003; Desai et al., 2010; Wikland et al., 2010). The data on the safety of vitrification in terms of obstetric and perinatal outcomes
are also reassuring (Mukaida et al., 2008; Liebermann, 2009; Noyes et al., 2009). However, blastocysts represent a unique challenge because of the difficulty in accomplishing the required level of dehydration and high viscosity evenly in all blastomeres, due to their multi-cellular structure and presence of the water-filled blastocele (Vanderzwalmen et al., 2002).

It has been shown in animal models that inadequate intracellular delivery of cryoprotectants and insufficient dehydration of blastocysts can lead to a decreased survival rate and increased apoptosis (Kader et al., 2010, 2009a,b; Morató et al., 2010). Bettencourt et al. (2009) reported ultrastructural damage of mitochondria and cytoskeleton in ovine vitrified blastocysts and recent studies in mice have shown an alteration in methylation following vitrification (Wang et al., 2010). Most studies in human vitrified blastocysts concentrate on assessing the success of the procedure on survival rates post thaw and clinical outcomes following transfer. No studies have looked into the dividing cells and the effects vitrification may have on spindle structure, chromosome alignment and ability of spindles to form and continue normal cell division. Here, we have investigated the effects of vitrification on Day 5 on the cytoskeleton and development of human blastocysts, by analysing survival rates and spindle and chromosome configurations by fluorescence and confocal laser scanning microscopy.

Materials and Methods

Source of human preimplantation embryos

Surplus human blastocysts (n = 110) were donated for research from consenting couples undergoing IVF treatment in the Assisted Conception Unit of the Iakentro Advanced Medical Centre in Greece, where the embryos were vitrified (n = 55), fixed and labelled. The majority of the blastocysts originated from oocyte donation cycles. This work was approved by the ethical committee of the Aristotle University Medical School (License no. A 10570).

Ovarian stimulation

A standard protocol was used to induce superovulation and control timing of oocyte retrieval. Pituitary down-regulation was achieved by the administration of gonadotrophin-releasing hormone analogues (Suprefact; Hoechst Marnion Roussel, Arvekak; Ipsen, Cetrotide; Serono or Orgalutran; MSD), for the first 7–14 days, followed by ovarian stimulation with pure FSH (Metrodine; Serono, Middlesex, UK) or FSH and LH (Altermon; IBSA) or recombinant FSH (Puregon; MSD or Gonial-F; Serono) for 12–14 days. The patients were monitored regularly by ultrasound, and by measuring Estradiol levels, starting on Day 4 of gonadotrophin administration and when adequate follicular development had been demonstrated (≥3 follicles of 17 mm in diameter). 10 000 IU of hCG (Ovitrelle; or Pregnyl; MSD) was administered to trigger ovulation. Thirty-six hours after the hCG administration, transvaginal ultrasound guided egg retrieval was performed.

Oocyte retrieval

Oocytes were retrieved by flushing ovarian follicles with human tubal fluid medium (Sage) incubated in 5% CO₂ in air at 37°C and subsequently fertilized by conventional IVF or ICSI and cultured until the day of transfer in culture medium (SAGE or Vitrolife). Spare day 5 human blastocysts were either vitrified or processed fresh for cytoskeletal analysis. The blastocysts were assigned randomly in the fresh or vitrified group and they were of similar quality.

Human blastocyst vitrification

Fifty-five human blastocysts were vitrified on Day 5 under aseptic conditions according to Vanderzwalmen et al. (2009). After gradual exposure to dimethylsulphoxide/ethylene glycol (DMSO/EG) (5%/5%, 10%/10% and 20%/20%), the blastocysts were loaded into the gutter of a plug (Vitrisafe—MTG—Germany) before insertion into an outer protective straw (CryoBioSystème, France) that was welded and plunged into liquid nitrogen (LN₂). The vitrified blastocysts were warmed by immersing the tip of the straw (Vitrisafe) in 1, 0.5 and 0.25 M sucrose solutions and were allowed to recover in culture for 24 h before being treated for cytoskeletal analysis.

Human embryo fixation, immunolabelling and confocal imaging

Fifty-five blastocysts were used as controls (fresh) and were fixed and labelled on Day 5, 6 or 7 and 55 vitrified blastocysts were fixed and labelled 24 h post-warming (on Day 6).

Blastocyst immunostaining was performed according to Chatzimeletiou et al. (2005a,b) using a primary rat monoclonal antibody specific for α-tubulin (Serotec) to visualize microtubules in combination with mouse monoclonal antibodies specific for gamma-tubulin (to identify spindle poles) or acetylated tubulin (to visualize spindle poles and mid bodies) (Sigma) and 4(6-diamidino-2-phenylindole) (DAPI) to visualize DNA. In brief, all blastocysts were rapidly fixed in ice cold methanol, washed in Ca²⁺/Mg²⁺ free phosphate-buffered saline (PBS; Gibco BRL) containing 2% bovine serum albumin (BSA; Sigma) and transferred into 10 μl drops of the primary antibodies (for antibody dilutions see Table I) under mineral oil (Sigma) and incubated at 4°C for 1 h. The blastocysts were then washed in PBS/BSA and transferred into 10 μl drops of the secondary antibodies [highly cross-absorbed Alexa Fluor 488 or 594 conjugates

<table>
<thead>
<tr>
<th>Table I</th>
<th>Dilution of the primary and secondary antibodies used for microtubule immunostaining.</th>
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<tbody>
<tr>
<td>Primary antibodies</td>
<td>Dilution in I × PBS/2% BSA</td>
</tr>
<tr>
<td>Rat anti-α-tubulin</td>
<td>1:800</td>
</tr>
<tr>
<td>Mouse anti-γ-tubulin</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse anti-acetylated tubulin</td>
<td>1:1000</td>
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Following 1 h incubation in the secondary antibodies, the blastocysts were washed in PBS/BSA and mounted on slides (BDH) in Vectashield antifade medium (Vector laboratories, CA, USA) under a coverslip. The coverslips were then sealed with nail varnish.

The blastocysts were examined using the Zeiss fluorescence microscope and/or the Leica TCS-SP laser scanning confocal microscope. Standard fluorescence images were captured using the ISIS software (Metasystems) and confocal image analysis was typically accomplished by capturing a z-series stack of 1 μm thick sections encompassing the entire blastocyst. The images were acquired sequentially to avoid bleed-through artefacts using the 488 nm line of an argon laser to image Alexa 488, the 568 nm laser line of a Kr laser to image Alexa 594 and an argon-UV laser to visualize DAPI staining of DNA. A 25x or a 100x UV-corrected oil immersion lens was used depending on whether low or high magnification of the blastocyst to be captured was desired.

Classification of spindle abnormalities

All interphase nuclei and metaphase/anaphase spindle and chromosome configurations were carefully examined and counted in each blastocyst. The criteria for classifying spindle abnormalities were as previously described by Chatzimeletiou et al. (2005a). A spindle with astral shaped or fusiform poles and with chromosomes aligned at the equator was classified as normal. A spindle with one or two poorly defined or apparently absent poles, generally with misaligned chromosomes was classified as having an abnormal shape. Spindles with more than two clearly defined astral poles and the characteristic "Y" or "X" shaped arrangement of DAPI-labelled chromosomes were classified as multipolar. Multipolarity was also confirmed in some cases by γ-tubulin labelling of centrosomes at the spindle poles. Finally, any DAPI-labelled presumptive chromosomes not aligned with the other chromosomes on the spindle were classified as lagging chromosomes (chromosome loss).

Statistical analysis

Statistical analysis of the results was carried out with the SPSS 9 (SPSS Inc., Headquarters, Chicago, IL, USA) statistical package for Windows. Fisher’s exact test was used to determine whether the incidence of spindle abnormalities differs in the fresh and vitrified blastocysts.

Results

Spindle abnormalities in fresh blastocysts

Fifty-five fresh human blastocysts were processed for cytoskeletal analysis on Day 5 (n = 26), Day 6 (n = 22) and Day 7 (n = 7). All 55 fresh blastocysts had cells with mitotic spindles and were analysed. The range of mitotic spindles was 1–11 per fresh blastocyst. The mean cell number of the blastocysts analysed on Days 5, 6 and 7 was 80.4 ± 5.4, 172.8 ± 15.3 and 281.7 ± 29.1, respectively. Cytoskeletal analysis revealed that overall 37/55 (67.3%) fresh blastocysts had only normal spindles, while 18/55 (32.7%) had multipolar or abnormally shaped spindles in addition to normal spindles. In total, 213 spindles were analysed, of which 183/213 (85.9%) were normal, 20/213 (9.4%) were abnormally shaped, 9/213 (4.2%) were multipolar and 1/213 (0.5%) was monopolar (Table II).

Table II Spindle abnormalities in vitrified and fresh human blastocysts.

<table>
<thead>
<tr>
<th>Blastocyst classification</th>
<th>No. of blastocysts analysed</th>
<th>No. of blastocysts with abnormal spindles (%)</th>
<th>Total no. of spindles analysed</th>
<th>Normal spindles (%)</th>
<th>Abnormal spindles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 ≥ 1</td>
<td></td>
<td></td>
<td>Abnormal shape Multi-polar Mono-polar</td>
</tr>
<tr>
<td>Vitrified Day 6 total</td>
<td>47</td>
<td>14 (29.8)</td>
<td>175</td>
<td>120 (68.6)</td>
<td>42 (23.3) 10 (5.7)</td>
</tr>
<tr>
<td>Fresh Day 5 total</td>
<td>26</td>
<td>19 (73.1)</td>
<td>60</td>
<td>53 (88.3)</td>
<td>2 (3.3) 5 (8.3)</td>
</tr>
<tr>
<td>Fresh Day 6 total</td>
<td>22</td>
<td>14 (63.6)</td>
<td>106</td>
<td>90 (84.9)</td>
<td>13 (12.3) 2 (1.9)</td>
</tr>
<tr>
<td>Fresh Day 7 total</td>
<td>7</td>
<td>3 (4.7)</td>
<td>47</td>
<td>40 (85.1)</td>
<td>5 (10.6) 2 (4.3)</td>
</tr>
<tr>
<td>Fresh total</td>
<td>55</td>
<td>37 (67.3)</td>
<td>213</td>
<td>183 (85.9)</td>
<td>20 (9.4) 9 (4.2)</td>
</tr>
</tbody>
</table>

Blastocyst classification—Grade A: Top quality with well defined ICM and TE, Grade B: Medium quality, Grade C: poor quality.
Post-warming survival and spindle abnormalities in vitrified blastocysts

Fifty-one out of 55 (92.7%) human blastocysts that were vitrified on Day 5 survived post-warming and 47/51 vitrified blastocysts had cells with mitotic spindles and were analysed. The range of mitotic spindles was 1–10 per human vitrified blastocyst. The mean cell number of the blastocysts analysed 24 h post thaw, on Day 6, was 163.5 ± 9.3. Cytoskeletal analysis showed that 14/47 (29.8%) vitrified blastocysts had only normal spindles, 5/47 (10.6%) had only abnormal spindles and 28/47 (59.6%) had a mixture of normal and abnormal spindles, including abnormally shaped, monopolar and multipolar spindles, disorganized prometaphases, chromosome lagging, congression failure and chromosome bridging (Tables II and III, Figs. 1 and 2). A total of 175 spindles were analysed in total in the vitrified group, of which 120/175 (68.6%) were normal (Fig. 1A), 39/175 (22.3%) were abnormally shaped (Fig. 1F–H and Fig. 2I), 10/175 (5.7%) were multipolar and 6/175 (3.4%) were monopolar (Fig. 1C).

Abnormalities in fresh versus vitrified blastocysts: comparison of results

The embryos were randomly assigned to the fresh or the vitrified groups and they were of similar quality (Table II). Overall, the vitrified blastocysts showed excellent survival post-warming, but the cytoskeletal analysis detected specific spindle abnormalities that were not observed as frequently in fresh blastocysts. The incidence of multipolar spindles was similar in the two groups, but the level of abnormally shaped spindles, often associated with chromosome lagging or congression failure, was significantly higher in the vitrified group compared with the fresh group (P < 0.05). The most frequently observed type of spindle abnormality in the vitrified group was that of a spindle with one well-focused pole and another more rounded (unfocused) pole (Fig. 1G, and Fig. 2I). Some spindles also appeared more elongated (Fig. 1H) and occasionally had lagging chromosomes. Disorganized middle spindle fibres at telophase were also observed (Fig. 1B). It is of particular interest to note that these abnormalities were present even in good quality blastocysts, such as the blastocyst in Fig. 2 (Grade A), which survived in an excellent way after warming but (i) had the most frequently observed type of spindle abnormality with one well-focused pole and an unfocused pole and (ii) a spindle with chromosome bridging.

Distribution of acetylated and γ-tubulin

Acetylated tubulin antibodies strongly labelled spindle midbodies during telophase in both fresh and vitrified/thawed blastocysts. In some cases disorganized middle spindle fibres at telophase were also evident, which would inevitably result in binucleation. Acetylated tubulin also strongly labelled the spindle poles during both metaphase and anaphase and the tails of any extra sperm bound on the zona of IVF embryos (Fig. 1G and H). γ-tubulin was detectable at the spindle poles and confirmed bipolarity, multipolarity or the absence of poles in certain cases.

Discussion

This study provides the first cytoskeletal analysis of human vitrified blastocysts and compares the type and incidence of spindle abnormalities in those, to that observed in fresh blastocysts. The high survival rate following warming (92.7%) suggests that vitrification at the blastocyst stage does not adversely affect the development of human blastocysts.
embryos. This is in agreement with clinical trials that have shown high survival rates and promising implantation rates following transfer of thawed blastocysts (Liebermann and Tucker, 2006; Mukaida et al., 2006; Hong et al., 2009; Vanderzwalmen et al., 2009, 2002; Wikland et al., 2010).

Detailed cell count revealed no statistically significant difference in the total number of nuclei between the vitrified blastocysts, 24 h post-warming on Day 6 and fresh blastocysts on Day 6 (163 ± 9.3 versus 172.8 ± 15.3). The majority of mitotic spindles examined by confocal laser scanning microscopy in both groups had normal astral or fusiform shaped poles and were bipolar (68.6% vitrified versus 85.9% fresh). The level of multipolar spindles (tripolar and tetrapolar spindles with the characteristic ‘Y’ and cruciform ‘X’ shaped organization) was similar in vitrified and fresh blastocysts (5.7 versus 4.2%). However,
there was a significantly higher incidence of abnormally shaped spindles (lacking well-defined poles, and/or one or more chromosomes separate from the spindle resulting presumably either from congression failure or anaphase lag) in the vitrified group \( (P < 0.05) \), with more prominent the type of spindles with a well focused and an unfocused pole (Figs. 1G and 2I). For the first time, the present study also provides unique observations of chromosome bridging (Fig. 2G and H) which is associated with chromosome breakage and micronucleation and disorganized middle spindle fibres at telophase (Fig. 1B) which can lead to binucleation, extending our previous work into the mechanisms that lead to postzygotic chromosomal abnormalities in early human development (Chatzimeletiou et al., 2005a, b, 2008).

It is unclear whether the increase in spindle abnormalities following vitrification is due to toxicity or mechanical stress following exposure to the high concentrations of cryoprotectants or due to the submersion into liquid nitrogen. However, our recent pilot data (Chatzimeletiou et al., 2010) suggested that it is the exposure to the cryoprotectants, which causes the cells to shrink, that gives rise to the abnormally shaped spindles observed. This was concluded by the fact that when human blastocysts were analysed cytoskeletally, 4 h following exposure to the cryoprotectants, but without being submerged into LN2, they had the same type of abnormally shaped spindles as those that were vitrified and submerged in LN2 (Chatzimeletiou et al., 2010 and unpublished data). It is possible that if cells are caught at a vulnerable stage of mitosis during vitrification, spindle damage will be inevitable. Whether it is the metaphase/anaphase stage that is more vulnerable to damage than the point of centrosome duplication and movement to opposite poles, or any other mitotic stage warrants further investigation.

Further analysis is also needed to establish if the morphological change in the structure of the spindle, that is so frequently observed in the vitrified group, has a functional effect on the way the spindle progresses into mitosis. If this abnormality in morphology is superficial and these abnormally shaped spindles complete mitosis normally it is likely that the derivative cells will be normal. However, if this abnormality in the shape of the spindle is conjugated with a function abnormality, it is possible that the spindle will either fail to progress further or if it progresses through mitosis the derivative cells may have an abnormal chromosomal constitution. This is certainly the case for the spindles that are associated with chromosome lagging, in which the derivative cells will be affected by chromosome loss, and consequently will become monosomic.

Although cell cycle checkpoints are thought not to operate during cleavage before global activation of the embryonic genome, a functional spindle assembly checkpoint at the blastocyst stage, should minimize any deleterious effects by arresting mitosis until the defect is
correction or by eliminating mitotically arrested cells by apoptosis (Rieder and Palazzo, 1992; Musacchio and Hardwick, 2002). In vertebrate somatic cells and sea urchin zygotes, mitosis is prolonged 2–3-fold when monopolar spindles assemble, but not when multipolar spindle assemble, suggesting that the checkpoint control for metaphase-anaphase transition does not monitor excess spindle poles or bipolar spindle symmetry. (Sluder and Begg, 1983; Wang et al., 1983; Sluder et al., 1997). Therefore tripolar and tetrapolar spindles are likely to progress, giving rise to cells with chaotic chromosomal constitutions, as the segregating chromosomes would be pulled in three or four directions respectively.

The effects spindle abnormalities may have on the development of human blastocysts will depend on the cell lineage they occur, the proportion of cells affected and the potential for further division. The total number of spindles analysed per blastocyst ranged from 1 to 10 and although several of them were in the inner cell mass (ICM) the majority of the spindles were in the trophoderm (TE) (Table III). It is therefore possible that even if these abnormal spindles are functional and produce chromosomally abnormal cells, if they are located in the TE, there will be no detrimental effect, as TE cells only give rise to the placenta. Even in the case of abnormal spindles progressing into mitosis in the ICM, there may be no detrimental effect if the abnormal cells produced give rise to the extrafetal membranes. The only case in which a detrimental effect may occur is if the abnormal cells give rise to the fetus. However, low level mosaicism localized in specific tissues of the fetus may remain phenotypically invisible in adulthood.

In conclusion, the high survival rate following warming (92.7%) and the large proportion of normal spindle/chromosome configurations observed in the human blastocysts that were vitrified in the present study suggest that vitrification at the blastocyst stage does not adversely affect the development of human embryos and the ability of spindles to form and continue normal cell divisions. However, detailed examination by confocal laser scanning microscopy revealed a significantly higher incidence of spindle abnormalities in the vitrified group compared with the fresh group, notably of spindles with a focused and an unfocused pole and uniquely identified various other abnormalities, reflecting mechanisms that can lead to chromosomal mosaicism in early human development. Further investigation is warranted to elucidate the mitotic stages that are more vulnerable to damage during vitrification, the fate of the abnormal spindles and the potential effects these may have on the chromosomal constitution of the developing blastocysts. It is suggested that vitrification is always applied with caution to avoid any potential risks arising from abnormal divisions of affected spindles, which if in the ICM, may lead to mosaicism and affect embryo development.

Authors’ roles

K.C., P.V. and A.H.H. designed the study. K.C., Y.P. and P.V. performed blastocyst vitrification/warming, K.C. performed antibody labelling of both fresh and vitrified blastocysts, K.C. and E.E.M. performed analysis by confocal and fluorescence microscopy, image capturing and statistical interpretation of the results. All authors (K.C., E.E.M., Y.P., P.V., N.P., Y.P., B.C.T. and A.H.H.) contributed to manuscript drafting and critical discussion and approved of the final version of the manuscript.

Conflict of interest

B.C.T. has received Merck Serono Unrestricted Research grants, travel grants and Honorarium, Merck sharp and Dohme Unrestricted research grants, travel grants and Honorarium, BSA & Ferring Travel grants and Honorona.

References


