CSIRO PUBLISHING

Reproduction, Fertility and Development, 2012, **24**, 323–326
http://dx.doi.org/10.1071/RD11061

Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies

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Abstract. Herein, we report the birth of two healthy babies to a woman following intracytoplasmic sperm injection (ICSI) using motile spermatozoa vitrified without permeable cryoprotectants. Spermatozoa (in a case of oligoasthenoteratozoospermia) were cooled in cut standard straws in human tubal fluid supplemented with 0.5% human serum albumin and 0.25 M sucrose. Sperm motility, capacitation-like changes, acrosome reaction and mitochondrial membrane potential (MMP) were compared in fresh and vitrified spermatozoa. Eight mature (MII) oocytes were microinjected with the vitrified–warmed motile spermatozoa. Although the motility of vitrified–warmed spermatozoa was markedly lower than that of fresh spermatozoa (60% *v.* 90%, respectively), there were no immediate visible differences in the percentages of capacitated and acrosome-reacted vitrified and fresh spermatozoa (10% *v.* 8% and 5% *v.* 8%, respectively). However, the MMP in vitrified spermatozoa was apparently adversely affected in the ejaculate used for ICSI compared with fresh spermatozoa (63% *v.* 96% spermatozoa with high MMP). Eighteen hours later, six oocytes showed signs of normal fertilisation. Two-pronuclear oocytes were cultured *in vitro* for 24 h and two four-blastomere embryos were transferred. Two healthy girls were born at term. Our findings suggest that permeable cryoprotectant-free vitrification can be applied successfully for some procedures in assisted reproduction, in particular in ICSI with motile vitrified spermatozoa, to achieve normal pregnancy and birth.

Additional keywords: acrosome, baby born, cryoprotectant free, intracytoplasmic sperm injection, mitochondrion, vitrification.

Received 26 February 2011, accepted 13 May 2011, published online 13 October 2011

Introduction

To avoid lethal intracellular ice formation (Mazur 1963), permeable cryoprotectants (such as glycerol, propylene glycol, ethylene glycol and dimethyl sulfoxide) are widely used for the cryopreservation of cells. However, the addition and, in particular, the removal of permeable osmotically active cryoprotective agents (permeable cryoprotectants) before cooling and after warming can induce lethal stress due to intracellular ice formation or so-called 'dilution effects'. Further problems include the chemical toxicity of cryoprotectants and their possible repercussions on the genetic apparatus of mammalian spermatozoa (Gilmore *et al.* 1997). Moreover, spermatozoa that survive the cryopreservation stress are likely to have undergone subtle functional changes associated with biophysical and biochemical factors influenced by cryoprotectants, which will affect their fertilising ability (Petrunkina 2007).

Earlier, we reported that human spermatozoa were cryopreserved successfully without the use of permeable cryoprotectants, preserving their relatively high motility levels and their ability to fertilise oocytes *in vitro* (Isachenko *et al.* 2004*a*, 2004*b*). No significant differences were noted between vitrified and conventionally frozen cells in parameters such as viability, recovery rate or percentage of morphologically normal spermatozoa with undamaged DNA. However, it was observed that the number of cryopreserved spermatozoa displaying features of the acrosome reaction was significantly different to that in freshly prepared swim-up spermatozoa (Isachenko *et al.* 2004*a*, 2004*b*, 2005, 2008).

Vitrification is now a widely applied technology in assisted reproduction; however, its focus has been primarily on the vitrification of embryos. Successful pregnancies and births have been reported following the use of vitrified oocytes and embryos, and vitrification protocols have started to form an important part of human reproductive medicine (Isachenko *et al.* 2007*a*). Although sperm vitrification techniques have been studied *in vitro*, neither a successful pregnancy nor a live birth after fertilisation with vitrified spermatozoa have been reported as yet.

Herein, we report here for the first time the birth of two healthy babies following intracytoplasmic sperm injection (ICSI) of motile spermatozoa after vitrification without permeable cryoprotectants.

Materials and methods

The permeable cryoprotectant-free vitrification of human spermatozoa was performed in Chile, with the clinical treatment approved by the University of Temuco Ethics Committees.

Unless stated otherwise, all chemicals were purchased from Sigma Chemical (St Louis, MO, USA).

A couple, both 39 years old, underwent assisted reproduction due to severe endometriosis and oligoasthenoteratozoospermia. Cryopreserved spermatozoa were used because the woman's partner was absent during the oocyte retrieval procedure.

Ejaculate from the patient was obtained by masturbation after 48 h of sexual abstinence. The ejaculate contained 13×10^6 motile spermatozoa mL^{-1}. Sperm analysis was performed according to the published guidelines of the World Health Organisation (1999). Of these spermatozoa, 42% had progressive motility and 8% were morphologically normal.

The standard swim-up technique was used for sperm preparation in culture medium (human tubal fluid (HTF); Irvine Scientific, Santa Ana, CA, USA; Quinn *et al.* 1985) supplemented with 1% human serum albumin (HSA; Irvine), henceforth referred as HTF + 1% HSA. Spermatozoa prepared using the swim-up technique were centrifuged at 340*g* for 5 min and then diluted with the same medium to achieve a final concentration of 5×10^6 spermatozoa mL^{-1}. This cell suspension was then diluted (1 : 1) with a vitrification solution composed of HTF + 1% HSA supplemented with 0.5 M sucrose. The 0.5 M sucrose was prepared in double-distilled water, filtered and frozen until use. After dilution, the cell suspension was maintained at 37°C in 5% CO_2 for 5 min before the cooling procedure (direct plunging into liquid nitrogen) was performed.

Cooling of spermatozoa was performed in cut standard straws (CSS; Isachenko *et al.* 2007*b*). A CSS was made from a standard insemination 0.25-mL straw cut at an angle approximately 45°. A 10-μL aliquot of spermatozoa suspension was deposited on the end of the inner part of the CSS. Then, the CSS was placed inside a sterile 0.5-mL insemination straw, which was sealed hermetically with a hand-held sealer, and plunged into liquid nitrogen. The spermatozoa were kept frozen in liquid nitrogen (at −196°C) for 7 months.

For warming, the packaged CSS was partly removed from the liquid nitrogen and the upper part of the 0.5-mL straw was cut off. Then, the CSS was removed from the packaged straw and quickly immersed in 1.8 mL HTF + 1% HSA that had been prewarmed to 37°C in a 2-mL Eppendorf tube. In this way, a very fast warming of spermatozoa (~30 000°C min^{-1}) was achieved.

Next, the warmed, diluted spermatozoa were concentrated by centrifugation at 340*g* for 5 min and the resulting pellet was resuspended in 10 μL of the same medium and used for further culture, evaluation and ICSI.

The freshly prepared swim-up and thawed vitrified spermatozoa were assessed for changes in the following physiological and morphological parameters: progressive motility, capacitation-like membrane changes, acrosome reaction and mitochondrial membrane potential (MMP).

Acrosome reaction and 'capacitation' were assessed in viable spermatozoa using fluorescence microscopy by double fluorescence chlortetracycline (CTC)–Hoechst 33258 staining (Smiley *et al.* 1991). To evaluate mitochondrial function, changes in the MMP ($\Delta\Psi_m$) were detected using the fluorescent cationic dye JC-1 according to the methods of Smiley *et al.* (1991). This test was performed with the Mitochondrial Permeability Detection Kit AK-116 (MIT-E-ΨTM; BIOMOL International, Plymouth Meeting, PA, USA) and the $\Delta\Psi_m$ was determined essentially as described by Isachenko *et al.* (2008).

To induce superovulation, pituitary suppression was achieved using a long protocol with a microdose of a gonadotrophin-releasing hormone (GnRH) analogue (Leuprolide acetate; Lupron; Abbott, Santiago, Chile), followed by ovarian stimulation with recombinant FSH (Gonal F; Merck Serono Laboratories, Gibbstown, NJ, USA) and human menopausal gonadotrophin (hMG; Ferring, Copenhagen, Denmark).

Ten cumulus–oocyte complexes were retrieved 36 h after the administration of 10 000 IU human chorionic gonadotrophin (hCG; Pregnyl; Organon, Bloomington, IN, USA). Oocytes were denuded in 80 IU mL^{-1} hyaluronidase (Hyaluronidase; Hyaluronidase, Sage, Tuebingen, Germany). Eight mature (MII) oocytes were microinjected with the vitrified–warmed spermatozoa. Eighteen hours later, six oocytes showed signs of normal fertilisation. Two-pronuclear oocytes were cultured *in vitro* (Global Culture Media; LifeGlobal) for 24 h and two four-blastomere embryos (Grades a (4a) and b (4b); Steer *et al.* 1992) were transferred to the uterine cavity under ultrasonographic guidance (Frydman Ultra Soft Catheter with Echo Tip; C.C.D., Paris, France).

Results

Thirty minutes after thawing, the vitrified spermatozoa displayed 60% progressive motility (*v.* 90% in freshly prepared swim-up spermatozoa); 10% were identified as displaying a 'capacitation' CTC pattern and 5% were identified as displaying an 'acrosome reaction' pattern, compared with 8% and 5%, respectively, of freshly prepared swim-up spermatozoa. Sixty-three per cent of spermatozoa were classified as having high MMP (*v.* 96% of freshly prepared spermatozoa).

Whether these figures are representative and whether the apparent detrimental effect on $\Delta\Psi_m$ constituted a phenomenon coincident with vitrification requires further investigation and

analysis, involving advanced flow cytometric and computer-assisted techniques on a large number of samples.

Fifteen days after embryo transfer, the maternal β-hCG level was 360 IU L^{-1}. Two healthy girls with bodyweights of 2.910 g and 2.140 g were born after 37 weeks of pregnancy on 24 November 2009.

Currently, there are another two pregnancies following ICSI with thawed vitrified spermatozoa and another two following intrauterine insemination with vitrified spermatozoa in a large suspension volume (0.5 mL; Sanchez *et al.* 2012).

Discussion

Cryopreservation induces extensive damage to cells during both freezing and thawing. According to present knowledge, the effective induction of anabiosis in cells at very low temperatures (e.g. in liquid nitrogen at −196°C) can be achieved by optimising the multifactorial freezing process (Lozina-Lozinski 1982), usually with permeable cryoprotectants (Levin 1982). By depressing the freezing point and binding intracellular water, the permeable and non-permeable cryoprotectants help prevent ice formation and thereby reduce cryodamage (Andrews 1976; Franks 1977).

However, the use of most common cryoprotectants (e.g. ethylene glycol, propylene glycol, glycerol and dimethyl sulfoxide) implies that, in principle, the cells frozen with these cryoprotectants cannot be lyophilised (freeze dried). Moreover, permeable cryoprotectants have been shown to have numerous negative effects on spermatozoa, including damaging the cytoplasm, functional destabilisation and mutagenesis (Hammerstedt and Graham 1992; Petrunkina 2007).

Successful application of the vitrification technology supports the notion that: (1) cells can be frozen effectively without toxic permeable cryoprotectants; and (2) such frozen material could, in principle, be lyophilised. However, it is critical to ensure that freeze drying is not associated with the genetic and developmental abnormalities that have been observed after fertilisation with mouse freeze-dried spermatozoa (Ward *et al.* 2003).

Cryopreservation is normally achieved through a tertiary combination of cells, permeable cryoprotectants and a low-temperature environment. In contrast, our cryopreservation protocol can be considered as a simplified binary combination of cells (in a simplified medium containing sucrose as a natural cryoprotectant) and a cold environment. The birth of two healthy babies using this IVF technique is not only the first report of successful fertilisation using vitrified spermatozoa (which has obvious practical advantages for ART), but also demonstrates that highly organised cells (human spermatozoa) may be effectively freeze dried (lyophilised) with the recovery of their most important physiological function after thawing, namely the propagation of genetic hereditary information and the subsequent birth of new individuals. Of course, it would need to be proved in a large number of ejaculates that the damage produced by vitrification does not exceed the damage produced by conventional freezing and that there are no deleterious effects on the genetic integrity of the spermatozoa after vitrification (Ward *et al.* 2003). Although they are important, these aspects are beyond the scope of the present report.

In conclusion, our findings suggest that a permeable cryoprotectant-free vitrification technique can be used successfully for some procedures in assisted reproduction: the technique represents a platform for future research. In particular, ICSI with vitrified spermatozoa can achieve normal pregnancy and birth. The adoption of this technology for routine medical practice would require further investigation and comparative clinical trials.

Acknowledgements

The authors thank Dr R. A. P. Harrison for reading the manuscript and help with English revision of the paper.

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