Developmental rate and ultrastructure of vitrified human pronuclear oocytes after step-wise versus direct rehydration

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BACKGROUND: This study compared the viability of human pronuclear oocytes subjected to vitrification followed by post-thaw step-wise removal of cryoprotectants versus direct rehydration, in terms of their subsequent in vitro survival and ultrastructural features. METHODS: A total of 115 three-pronuclei stage oocytes were cryopreserved in super-open-pulled straws by vitrification in 40% ethylene glycol + 0.75 mol/l sucrose for either 1 min or 10 s at 38°C, followed by direct plunging into liquid nitrogen. After thawing, oocytes vitrified for 1 min (group 1) or 10 s (group 2) were expelled into a graded series of sucrose solutions (1.0, 0.75, 0.5, 0.25 and 0.12 mol/l) for removal of the cryoprotectant in five 2.5 min steps. A second batch of oocytes vitrified for either 1 min (group 3) or 10 s (group 4) were directly expelled into culture medium at 38°C after thawing. Finally, the ultrastructural changes occurring in oocytes in each of the treatment groups were evaluated. RESULTS: Oocyte development (division to two-blastomere stage) rates after in vitro culture were 82, 83, 0 and 0% for groups 1, 2, 3 and 4, respectively. The harsh osmotic process involved in direct rehydration provoked ultrastructural changes, including the disruption of cytoplasmic and pronuclear membranes as well as intracellular organelles. CONCLUSION: The direct post-thaw rehydration of human pronuclear oocytes has lethal osmotic effects, such that protocols for vitrifying human pronuclear oocytes should include the step-wise removal of the cryoprotectant.

Key words: damage/direct rehydration/human oocytes/ultrastructure/vitrification

Introduction

Since in some countries it is illegal to cryopreserve an oocyte after fusion of the pronuclei (PN), there is currently much interest in developing a refined method of cryopreserving human pronuclear oocytes.

Conventional (slow) freezing of human pronuclear oocytes has been the most widely used method of storage up until now (Mohr et al., 1985; Siebzehnrübl et al., 1986; Testart et al., 1987; Veeck et al., 1993; Van den Abbeel et al., 1997; Al-Hasani et al., 1999; Damario et al., 1999). However, there have been several recent reports of the successful cryopreservation of human pronuclear oocytes by direct plunging into liquid nitrogen (vitrification) (Park et al., 2000; Liebermann et al., 2002a,b; Selman and El-Danasouri, 2002). This method is now an object of intensive investigation in a number of laboratories, taking into account that the protocol of vitrification includes two major benefits: the complete process can be completed in only minutes in contrast to a long time for the conventional method, and this method does not require specialist equipment, in contrast to conventional freezing techniques.

Prior to the successful vitrification of human pronuclear oocytes, an effective protocol for the vitrification of mouse oocytes at this stage was developed, which involved direct plunging into liquid nitrogen (Van der Auwera et al., 1990; Shaw et al., 1991; Nowshari et al., 1995). Since then, several publications on the vitrification of animal oocytes at the pronuclear stage have emerged, in which the ability of cells of transgenic mice (Tada et al., 1995; Bagis et al., 2002) and rabbits (Hochi et al., 2001) to develop after cryopreservation was evaluated. Subsequent protocols for the vitrification of human pronuclear oocytes were based on the data provided by these studies.

Today, human pronuclear oocytes can be cryopreserved successfully by vitrification (Park et al., 2000; Jelinkova et al., 2002; Liebermann and Tucker, 2002; Liebermann et al., 2002a;
Selman and El-Danasouri, 2002). According to these protocols, the human oocytes, once thawed, are placed into hypertonic disaccharide solution (normally sucrose) to remove the permeable cryoprotectants before transferring the cells to an isotonic culture medium. This rehydration process is generally conducted by gradual dilution of the permeable cryoprotectants through exposure in three steps to 0.5, 0.25 and 0.125 mol/l sucrose (Park et al., 2000; Liebmann et al., 2002a) or to 1.0, 0.5 and 0.25 mol/l sucrose (Selman and El-Danasouri, 2002). Four-step dilution protocols involving exposure to 1.0, 0.5, 0.25 and 0.125 mol/l sucrose have also been described (Jelinkova et al., 2002; Liebmann and Tucker, 2002). In contrast, recently it has been possible to rehydrate vitrified ovine and bovine oocytes directly after thawing without the need for gradual dilution of the cryoprotectants (Isachenko et al., 2000; Papis et al., 2000).

The present study was designed to assess the viability of vitrified human pronuclear oocytes after step-wise removal of cryoprotectants versus direct rehydration. Oocyte viability was established by their subsequent in vitro survival and ultrastructural features.

Materials and methods

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St Louis, MO). Written informed consent was obtained from the participating couples for the cryopreservation, culture and fixing of oocytes for transmission electron microscopy (TEM). The study, performed in Italy and Germany, was approved by the University Review Board (Italy) and University Ethics Committees (Germany).

Patients with unexplained infertility were selected for IVF-ICSI with triptorelin (Decapeptyl®, Ferring, Kiel, Germany and Ipsen SPA, Milan, Italy) and recombinant FSH (Gonal F®, Serono, Unterschleissheim, Germany and Pharma, Rome, Italy) according to the ‘long’ protocol. Ovulation was induced by the administration of 10 000 IU HCG (Pregnin®, Organon, Oss, The Netherlands). Oocytes were retrieved 34–36 h later and inseminated with the husband’s sperm through conventional IVF or ICSI techniques. Fertilization was assessed 14–18 h after insemination. Abnormally fertilized oocytes (at the 1PN or 3PN stage) were used for vitrification and subsequent TEM studies.

Vitrification, thawing and culture

In a first series of experiments performed in Italy, 68 patients ranging in age from 20 to 36 years [mean (±SD) age 25.7 ± 4.1 years] volunteered to have their oocytes cryopreserved by vitrification at the 3PN stage.

The oocytes (n = 81) obtained from the patients were randomly assigned to a control (n = 10) and four experimental groups (see Figure 1 for a diagram of the experimental design). The oocytes in these experimental groups were cryopreserved (vitrified) by direct plunging into liquid nitrogen. Vitrification was performed according to a previously described method (Isachenko et al., 2001) with two different modes of removal of the cryoprotectant: step-wise and direct rehydration. All the cryopreservation solutions were prepared in TCM-199 supplemented with 20% fetal calf serum. Oocyte cryopreservation was performed in super-open-pulled-straws (SOPS) (Medical Technology GmbH, Altendorf, Germany) (Vajta et al., 1998) by 1 min or 10 s pre-cooling exposure in 40% ethylene glycol + 0.75 mol/l sucrose at 38°C and plunging into liquid nitrogen. After thawing, oocytes with 1 min (group 1, n = 17) or 10 s (group 2, n = 18) exposures in vitrification medium were expelled into a graded series of sucrose solutions (1.0, 0.75, 0.5, 0.25 and 0.12 mol/l) for gradual removal of the cryoprotectant in five 2.5 min steps. Roughly half of the oocytes with 1 min pre-cooling exposure (group 3, n = 18) or 10 s (group 4, n = 18) were directly expelled into the culture medium at 38°C after thawing (Figure 1). All the oocytes were then cultured and allowed to develop through multiple cleavage division to the blastocyst stage for an additional 6 days in 5% CO₂ at 37°C in Universal IVF Medium (I and II) (Medicult, Redhill, Surrey, UK).

Vitrification, thawing and ultrastructural analysis

A second series of experiments was performed to provide oocytes for ultrastructural analysis after vitrification. 1PN and 3PN stage oocytes (n = 34) from 25 patients ranging in age from 29 to 40 years (mean age 36.7 ± 3.1 years) were divided into two groups and vitrified as described above, followed by thawing and step-wise removal of cryoprotectants (n = 14) or thawing and direct rehydration (n = 14). The 14 step-wise rehydrated oocytes and 10 of the directly rehydrated oocytes were subjected to electron microscopy after 1 h of exposure to culture medium in a CO₂ incubator. Some of the directly rehydrated oocytes (n = 4) were fixed for TEM observation just after thawing. Fresh oocytes (n = 6) were used as controls.

After rehydration, the oocytes were fixed in 3% glutaraldehyde for 5 min at 22°C, and then stored in fixative for 1–30 days at 4°C prior to embedding in resin. This involved post-fixing the oocytes in 1% osmium tetroxide (OsO₄; Plano, Wetzlar, Germany) for 1 h at 22°C, rapid dehydration in a graded ethanol/acetone series, and embedding in Durcupan® resin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 24 h at 45°C followed by polymerization for 48 h at 60°C. Ultrathin sections were cut on a Reichert microtome and mounted on 100 mesh copper grids. These sections (70–100 nm) were stained with uranyl acetate (30 min) and Reynold lead citrate (15 min), examined using a Zeiss TEM microscope at 80 kV, and photographed on Kodak film. Ultrastructural analysis was performed using data of Sousa and Tesarik (1994), Sathananthan et al. (1997) and El Shafei et al. (2000).

Statistics

Data were analyzed using the software program SigmaStat (Jandel Statistics, San Rafael, CA, USA. P < 0.005 was considered statistically significant.

Results

Vitrification, thawing and culture

All 10 fresh 3PN oocytes, were viable, developing to the blastocyst stage. This high development rate was taken as a
benchmark against which the success of vitrification and culture under routine laboratory IVF conditions was judged.

After thawing followed by culture, the mean (±SD) rates of formation of the 2-cell embryo of 82.4 ± 9.5 and 83.3 ± 9.0% were recorded for the oocytes in groups 1 and 2 (vitrification/thawing followed by step-wise rehydration), respectively (Figures 1 and 2). In the 36 oocytes of groups 3 and 4 (vitrification/thawing followed by direct rehydration), the cytoplasm showed a dark, granular appearance; none of these oocytes developed to the two-blastomere stage \((P < 0.005\) for groups 3 and 4 versus groups 1 and 2). A proportion of oocytes in groups 1 and 2 (70.1 ± 10.1 and 70.4 ± 11.0%, respectively) developed to the 7- to 8-cell stage, whilst approximately one-third of the surviving oocytes in groups 1 and 2 went on to form blastocysts: 29.4 ± 12.9 and 27.8 ± 11.4%, respectively.

Figure 3 shows the appearance of the vitrified human pronuclear oocytes subjected to the post-thaw step-wise removal of cryoprotectants or direct rehydration.

**Vitrification, thawing and ultrastructural analysis**

The ultrastructure of both fresh and vitrified/step-wise rehydrated pronuclear oocytes was similar to our previously published descriptions (Isachenko et al., 2003b). The smooth endoplasmic reticulum (SER) of a fresh pronuclear oocyte is associated with mitochondria (see figure 3 in Isachenko et al., 2003b). The presence of small vesicles not associated with mitochondria was noted. After vitrification, the thawed human pronuclear oocytes subjected to the post-thaw step-wise removal of cryoprotectants or direct rehydration.

The dark pulp is enveloped by membrane and is characteristic of the deformation and destruction provoked by the intense osmotic effects of direct rehydration. The cytoplasm shows transparent (low density) spots which are probably the result of the disruption of lysosomes that release their proteolytic contents, inducing lysis of the cytoplasmic matrix. Figure 4 also shows oocytes that were fixed for TEM just after thawing. The image shows the disruption of the pronucleus followed by rupture of first the membranes and later the nucleoli.

**Discussion**

Once thawed, the vitrified human oocytes need to be separated from the permeable protective agents used in the cryopreservation process, and this is usually achieved by immersion in a graded series of sucrose solutions until isotonic conditions are met. This gradual replacement of cryoprotectants with sucrose solution serves to rehydrate the thawed oocytes. Typical rehydration protocols vary in the number of dilution steps involved and the concentrations of sucrose solutions. Thus, several three- or four-step procedures have been described.
using 0.5–0.25–0.125 mol/l (Park et al., 2000; Liebermann et al., 2002a), 1.0–0.5–0.25 mol/l (Selman and El-Danasouri, 2002) and 1.0–0.5–0.25–0.125 mol/l (Jelinkova et al., 2002; Liebermann and Tucker, 2002) sucrose solutions.

In the present study, we used a vitrification/direct post-thaw rehydration protocol that had been tested previously in our laboratory on germinal vesicle (GV) ovine oocytes (Isachenko et al., 2001) and, after slight modification, in rat early morulas, early blastocysts and expanded blastocysts (Isachenko et al., 2003a). With the subsequent step-wise dilution of cryoprotectants, the same vitrification protocol was found to be highly efficient for human pronuclear oocytes, yet was completely ineffective when oocytes were directly rehydrated after thawing.

We used 3PN oocytes for experimental cryopreservation, thawing and culture, and oocytes at the 1PN or 3PN stage for vitrification, thawing and ultrastructural analysis, given that the latter are the model closest to normal fertilized oocytes (2PN) and also show a similar developmental potential (Liebermann et al., 2002a). In several cryopreservation experiments conducted on 2PN oocytes, similar development rates were achieved using 3PN oocytes (Liebermann and Tucker, 2002; Liebermann et al., 2002a; Isachenko et al., 2003c). Moreover, high pregnancy rates after the transfer of embryos derived from vitrified 2PN oocytes were recorded using a vitrification protocol tested on oocytes at the 1PN and 3PN stage (Liebermann and Tucker, 2002; Liebermann et al., 2002a; Selman and Al-Danasouri, 2002; Isachenko et al., 2003b).

Our comparison of vitrification protocols involving the step-wise removal of cryoprotectants as opposed to direct rehydration was based on the following developments in the cryopreservation of mammalian embryos and oocytes by direct plunging into liquid nitrogen.

Initially, Leibo and Oda (1993) proposed that it was unnecessary to fully bind intracellular water using cryoprotective agents before cooling. Thus, several of the protocols developed around that time included a short time of exposure to cryoprotectants before plunging the oocytes into liquid nitrogen.

**Figure 4.** Electron micrograph of vitrified pronuclear oocytes after thawing followed by direct rehydration. Note the deformation and disruption of the cytoplasmic membranes (M). RPN = remains of the pronucleus; S = low density spots; C = cavity; P = dark pulp; V = vesicle; N = nucleus; arrowheads = pronuclear membrane remnants (envelope). Bar = 1 μm.
nitrogen (Nakagata, 1989). This was followed by reports of the direct post-thaw rehydration of vitrified embryos (Ishimori et al., 1993; Vajta et al., 1995; Isachenko et al., 1997, 2003a). Finally, among the latest developments in the field are the ultra-rapid freezing of rat embryos with ultra-rapid (4 s) exposure to the vitrification solution followed by direct dilution of permeable cryoprotectants in isotonic medium immediately after thawing (Isachenko et al., 2000).

Thus, it would appear that the ideal protocol for embryo vitrification plus direct rehydration may be a compromise between the two conditions: maximal binding of intracellular water by permeable cryoprotectants, and the presence of minimal post-thaw quantities of these cryoprotectants in the cytoplasm. Prolonged exposure to the cryoprotectant seems to require extra-rehydration due to the increased quantity of intracellular cryoprotectant. In contrast, an insufficient amount of intracellular cryoprotectant during pre-cooling exposure may result in insufficient binding of intracellular water. The step-wise dilution procedure used here after thawing indicates that both 10 s and 1 min exposure of the oocytes to the permeable cryoprotectant is sufficient for intracellular water binding. We, therefore, also tested these two time periods of vitrification when the vitrified oocytes were directly rehydrated after thawing.

Our ultrastructural analysis revealed changes that could be attributed to osmotic damage to cytoplasmic membranes as well as to the membranes enveloping intracellular organelles. We suggest that the specificity of human pronuclear oocytes, reflected by their high sensitivity to osmotic processes, is related to the specificity of both intracellular lipids and those of the cytoplasmic and organelle membranes. Lipids are the main cellular compounds, the cooling of which is connected with a number of negative effects (Lieberman et al., 2002b). One of the main types of damage is lyotropic phase transition in lipids, which occurs during cooling (Singer and Nicolson, 1972) due to thermotropic phase transition of polar lipids and consequent lateral phase separation of membrane lipids and proteins (Quin, 1989) and exclusion of the proteins from the bilayer. Given the detrimental fate of lipids during cryopreservation, the ‘lipid cryostability’ yet ‘osmotic non-stability’ of pronuclear human oocytes is still far from being fully understood.

The need for step-wise dilution of cryoprotectants after thawing vitrified human oocytes is supported by empirical experiments. Hong et al. (1999) compared the use of 2.5 and 5 min intervals in two four-step dilution regimes after vitrification and concluded that the 2.5 min interval was more effective in terms of subsequent preimplant embryo development. We used this time regime in our experiments.

The chemical and physical properties of cellular lipids in human pronuclear oocytes are the same as those in the embryo. Thus, it may be speculated that the gradual step-wise dilution of the cryoprotectant would be desirable for oocytes at any stage of development. Indeed, it has been shown recently that six-step dilution provides improved results over two-step dilution in the vitrification of human blastocysts (Cho et al., 2002).

In conclusion, the method of direct post-thaw rehydration induces lethal osmotic effects in human pronuclear oocytes. Accordingly, the vitrification protocol for these oocytes must include the step-wise dilution of the cryoprotectant.

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