Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration

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BACKGROUND: Recently, interest in oocyte cryopreservation has steadily increased. Newly developed protocols have dramatically improved survival rates, removing perhaps the major hurdle that has prevented this approach from becoming a fully established form of treatment. However, the clinical efficiency of these protocols has not been exhaustively explored and therefore remains controversial. METHODS: Morphologically normal oocytes displaying the first polar body were frozen—thawed with a slow cooling protocol that utilized 1.5 mol/l propane-1,2-diol (PrOH) and 0.3 mol/l sucrose. RESULTS: A total of 927 oocytes from 146 patients were frozen—thawed, achieving a 74.1% survival rate. Over 76% of microinjected oocytes displayed two pronuclei 16 h post-insemination, while the proportion of embryos at 44–46 h post-insemination was 90.2%. At this time point, the majority (68.3%) of embryos were at the two-cell stage, showing in most cases (78.7%) minimal or moderate fragmentation. Eighteen clinical pregnancies, three of which were twin, were observed, giving rise to rates of 12.3 and 9.7%, calculated per patient and per embryo transfer, respectively. The implantation rate was 5.2%. To date, four children have been born and three pregnancies resulted in spontaneous abortions, while the remaining pregnancies are ongoing. CONCLUSIONS: Our data indicate that although the combination of slow cooling and high sucrose concentration ensures high rates of oocyte survival, it is not sufficient to guarantee a high standard of clinical efficiency.

Key words: cryopreservation/fertility preservation/oocytes/pregnancy rate/slow cooling

Introduction

Cryopreservation of early cleavage embryos is a fundamental part of IVF programmes, allowing the deferred use of surplus embryos and thereby decreasing the risk of multiple pregnancies, maximizing the clinical efficiency for each cycle and limiting the need for repeated pharmacological stimulations. In principle, oocyte freezing would provide an even better alternative, by meeting the same demands of efficiency, but without the legal and ethical implications derived from the long-term storage of embryos. Unfortunately, despite initial attempts dating back as early as 1986 (Chen, 1986; Al-Hasani et al., 1987), data on the standards of success and safety of oocyte cryopreservation are either disappointing or numerically insufficient. The number of pregnancies achieved worldwide is probably <200. In most cases, studies have been episodic and, as such, clinically irrelevant. Only very few trials have been founded on relatively large populations of patients (Porcu et al., 2000; Borini et al., 2004), but even in those cases the rate of implantation calculated on the basis of the number of oocytes used has been generally ~2%, i.e. considerably less than the value guaranteed by embryo freezing. Traditionally, the slow cooling propane-1,2-diol (PrOH)-based protocol originally set up for cleaving embryos (Lassalle et al., 1985) has been the method most widely used. Until recently, the major factor responsible for the poor performance of oocyte freezing was the inability to achieve sufficiently high survival rates, and in some cases also fertilization rates (Al-Hasani et al., 1987; Tucker et al., 1998). In recent years, with the aim of improving the overall efficiency of oocyte cryopreservation, various modifications of this protocol have been tested. In 2001, Fabbri et al. (2001) succeeded in dramatically increasing the survival rate by raising the sucrose concentration from 0.1 to 0.3 mol/l in the freezing mixture, a condition that is believed to reduce the probability of intracellular ice formation by intensifying the extent of cell dehydration before cooling. Alternatively, with the aim of mitigating the toxic effect of the accumulation of solutes in the unfrozen fraction during ice formation, the so-called ‘solution effect’, Na+-depleted cryopreservation media have been tested, achieving survival rates of >80% (Quintans et al., 2002; Boldt et al., 2003; Stachecki et al., 2004). Such protocol modifications have been adopted for clinical treatment, giving rise to some pregnancies. The option of embryo cryopreservation has been banned by a recently introduced Italian IVF law. Our previous experience with oocyte storage has confirmed that the outcome of conventional slow cooling is clinically deficient, mainly due to very low survival rates (Borini et al., 2004). Therefore, with the intention of giving to our patients the best chances of achieving a pregnancy per
cycle of treatment, in this study we have applied a slow cooling protocol combined with use of high sucrose concentration (0.3 mol/l), and verified the clinical efficiency of this method.

Materials and methods

Source of oocytes
This clinical trial using cryopreserved oocytes had been approved by the local Institutional Review Board. Patients agreeing to the treatment signed an informed consent form. Oocytes were obtained from 286 couples undergoing assisted reproduction treatment from April 2004, following the enforcement of the Italian IVF law that has ruled out pronuclear stage and embryo cryopreservation.

The mean (±SD) age of patients was 34.9±4.2 years. Controlled ovarian stimulation was induced with a long protocol using leuprolerin (Enantone, Takeda, Rome, Italy) and rFSh (Gonal-F, Serono, Rome, Italy, or Puregon Organon, Rome, Italy). HCG at 10000 IU (Gonasi, Amsa, Rome, Italy) was administered when one or more follicles reached a maximum diameter of >23 mm. (Dal Prato et al., 2001).

After retrieval, oocytes were cultured in fertilization medium (Cook IVF, Brisbane, Australia) for at least 3 h. Complete removal of cumulus mass and corona cells was performed enzymatically using hyaluronidase (80 IU/ml; Sigma Aldrich Srl, Milan, Italy), and mechanically using fine bore glass pipettes. Only oocytes showing an extruded polar body 1 and presumably at the metaphase II stage were frozen after culture for a total period of time of ~4 h following retrieval.

Cryopreservation solutions
The oocytes were cryopreserved using a slow cooling method. All cryopreservation solutions were prepared using Dulbecco’s phosphate-buffered saline (PBS) (Gibco, Life Technologies Ltd, Paisley, UK) and a plasma protein supplement (PPS) (BAXTER AG, Vienna, Austria). The freezing solutions were (i) 1.5 mol/l PrOH + 20% PPS in PBS (equilibration solution) and (ii) 1.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS in PBS (loading solution), as described by Fabbri et al. (2001). The thawing solutions were (i) 1.0 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS; (ii) 0.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS; and (iii) 0.3 mol/l sucrose + 20% PPS.

Freezing procedure
Oocytes were washed in PBS supplemented with 20% PPS (10 mg/ml, final concentration). One or two oocytes were subsequently placed in an equilibration solution containing 1.5 mol/l PrOH + 20% PPS for 10 min. Afterwards, oocytes were transferred to the solution containing 1.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS, at room temperature for 5 min, then loaded into plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France) and placed into an automated Kryo 10 series III biological freezer (Planer Kryo 10/1.7 GB). The temperature was gradually lowered from 20 to –8°C at a cooling rate of 2°C/min. Manual seeding was induced during the 10 min holding ramp at –8°C. The temperature was then decreased to –30°C at a rate of 0.3°C/min and rapidly to –150°C at a rate of 50°C/min. The straws were finally plunged into liquid nitrogen and stored for later use.

Thawing procedure
The straws were rapidly air warmed for 30 s and then plunged into a 30°C water bath for 40 s. The cryoprotectant was removed at room temperature by stepwise dilution. Oocytes were expelled in the first thawing solution, 1.0 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS for 5 min, then equilibrated in 0.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS for an additional 5 min. Finally they were placed in 0.3 mol/l sucrose + 20% PPS for 10 min before final dilution in PBS + 20% PPS for 20 min (10 min at room temperature and 10 min at 37°C). The oocytes were placed in 20 μl drops of cleavage medium (Cook IVF, Brisbane, Australia) under warm mineral oil (Cook IVF, Brisbane, Australia) at 37°C in an atmosphere of 5% CO₂ in air for at least 3 h prior to insemination, according to our previous observations on the kinetics of reconstitution of the meiotic spindle (Bianchi et al., 2005b).

Insemination and embryo culture

Insemination was routinely performed by ICSI as previously described (Borini et al., 1996). Normal fertilization was checked by the presence of two pronuclei and two polar bodies 16 h later. All fertilized oocytes were transferred to fresh cleavage medium (Cook IVF, Brisbane, Australia). Embryos were graded on day 2 on the basis of a I–IV scale (best–worst) based on the percentage (<5, 6–25, 26–50 and >50%, respectively) of anucleated fragments with respect to the overall cellular volume.

Endometrial preparation and embryo transfer

Endometrial growth was supported with a steadily increasing dosage, 100–300 μg of micronized 17β-estradiol in patches (Esclima, Schering, Milan, Italy) administered over a period of time varying from 10 to 18 days, depending on the patient (Borini et al., 1996). Progesterone supplementation either as injections of 100 mg in oil (Prontogest: Amsa, Rome, Italy) or as 180 mg micronized doses in a gel (Crinoline: Serono, Rome, Italy) via the vaginal route was started on the day of oocyte thawing (day 0). Embryo transfers were performed on day 2. Endometrial thickness was checked and the cycle suspended if the line was thinner than 8 mm or thicker than 12 mm. In the case of pregnancy, endometrial support treatment was continued for 60 days after transfer. Clinical pregnancy was defined as the presence of a gestational sac and fetal heart beat at ultrasound examination.

Results

A total of 2450 supernumerary oocytes with normal morphological characteristics from 286 patients were frozen following 304 stimulation cycles. So far, 51% of these patients (146) have undergone 201 thawing cycles, with a total of 927 oocytes thawed. At the time of submission of this manuscript, 199 oocytes of the patients who had undergone at least one thawing cycle had not been used. The number of surviving oocytes, showing no sign of darkened cytoplasm, vacuoles, swelling or cracks in the zona, was 687 (74.1%) (Table I). Microinjection was conducted 3 h after thawing on 589 oocytes. Ninety-eight surviving oocytes were not microinjected due to the fact that in some cases more than three viable oocytes were

| Table I. Post-thaw survival and fertilization of oocytes cryopreserved with a slow cooling PrOH-based protocol in a high sucrose concentration (0.3 mol/l) |
|---------------------------------|-----------------|
| Thawing cycles                  | 201             |
| Oocytes                         |                 |
| Thawed                          | 927             |
| Survived (%)                    | 687 (74.1)      |
| Microinjected                   | 589             |
| Fertilized                      |                 |
| 2PN (%)                         | 448 (76.1)      |
| 1PN or 3PN (%)                  | 34 (5.8)        |
| Degenerated                     | 27 (4.6)        |

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obtained after thawing and the requirement to comply with the Italian IVF law that virtually forbids the generation of more than three embryos for each transfer. A total of 448 (76.1%) oocytes displayed two pronuclei 16h post-insemination. The incidence of abnormally fertilized oocytes with 1–3 pronuclei was negligible (5.8% in total), while the rate of degeneration was 4.6%. Table II illustrates the embryo development data. The number of fertilized oocytes undergoing at least one cell division 44–46h post-insemination was 404 (90.2%). At this time point, the majority (68.3%) of embryos had not progressed beyond the two-cell stage. The proportion of embryos affected by minimal (grade I) or moderate (grade II) fragmentation was in total 78.7%. All embryos obtained were used in 185 transfers (Table III). The treatment of 16 patients was suspended before embryo transfer because of either fertilization or cleavage failure (six and 10 cases, respectively). In most cases, two or three embryos were available for transfer (37 and 41%, respectively, of the total number). Eighteen clinical pregnancies, three of which were twin, were observed, giving rise to rates of 12.3 and 9.7%, calculated per patient and embryo transfer, respectively, while the implantation rate was 5.2% (Table IV). So far, four children have been born. Three pregnancies resulted in spontaneous abortions; the others are still ongoing at 20 weeks or more.

Discussion

Until relatively recently, a poor post-storage survival rate has been the major hurdle to the application of oocyte cryopreservation. Using the slow cooling method based on PrOH, Fabbri et al. (2001) reported a very high survival rate as a consequence of the increase of sucrose concentration to 0.3 mol/l in the cryopreservation mixture. In a regime in which embryo cryopreservation is forbidden, we tested the clinical outcome of such a protocol, speculating that high survival rates could coincide with an uncompromised state of viability. We obtained high survival and fertilization rates, as well as apparently undisturbed cleavage. However, in our experience, this high sucrose concentration protocol does not generate high pregnancy rates. In contrast, in the view of other authors, such a procedure can compete in efficiency with the storage of pronucleate stage eggs (Chen et al., 2005). This discrepancy leaves the question of the clinical efficiency of oocyte freezing unsolved, also because other studies claiming high success rates have shown an episodic character. Initial reports of pregnancies from cryopreserved oocytes were published almost 20 years ago (Chen, 1986; Al-Hasani et al., 1987). Some of the conditions applied in those studies may have affected the overall outcome, such as the use of poor quality or immature oocytes (Al-Hasani et al., 1987). However, it was soon appreciated that oocyte freezing would pose a formidable challenge. More recently, after a long period in which oocyte cryopreservation was not investigated, Porcu et al. (2000), using a 1.5 mol/l PrOH, 0.2 mol/l sucrose slow cooling protocol, obtained a moderately improved survival rate (54.1%). Despite the use of ICSI, applied with the intention of circumventing possible hardening of the zona, the fertilization rate remained suboptimal (57.7%). Again, this suggests that post-thaw survival cannot be assumed to be a reliable standard of viability, as confirmed by our study on the 1.5 mol/l PrOH, 0.1 mol/l sucrose protocol, in which we observed low fertilization rates (45.2%) in oocytes that appeared normal at the time of ICSI (Borini et al., 2004). Porcu et al. (2000) described the achievement of 16 pregnancies, two of which were twin, by far the largest clinical outcome achieved up to then. Disappointingly though, the rate of implantation calculated on the basis of the number of oocytes thawed was only 1.2%, a value approximately four times smaller than the rate applicable to embryo cryopreservation (Gook and Edgar, 1999). In more recent years, other clinical experiences have been gained, encouraged by attempts to develop more efficient protocols. Based on the assumption that during slow cooling increased dehydration caused by higher sucrose concentration reduces the risk of intracellular ice formation, thereby increasing the overall post-thaw viability, Fabbri et al. (2001) showed in effect that a high concentration of this sugar in the freezing mixture dramatically improves the survival rates to >80%, but they have not
followed up these findings with a clinical study. Using this method, Fosas et al. (2003) reported a survival rate of almost 90% and a pregnancy rate in excess of 50%. This remarkable outcome should be interpreted with caution. First, the size of the study is very small, involving only 88 oocytes recovered from seven women. Secondly, those women were donors not affected by pathologies interfering with their fertility. Therefore, on the basis of such data, the comment of the authors according to which the pregnancy from frozen oocytes was similar to that obtained using fresh oocytes from donors probably requires further confirmation. In addition, it should be noted that the number of embryos transferred on day 3 (n=26) was just half of the normally fertilized oocytes (n=58), a circumstance that, together with the authors’ choice to perform embryo transfer 3 days after thawing, indicates that transferred embryos were selected from a larger set. This measure, while perhaps partly explaining the high pregnancy rate, in some respects is in contradiction to the very essence of oocyte freezing, i.e. preservation of the female gamete’s reproductive potential.

A different approach has been applied by Stachecki et al. (1998) who showed that by replacing sodium with the less toxic organic ion choline, a measure believed to reduce the ‘solution effect’, it was possible to attain higher rates of post-thaw survival, fertilization and preimplantation development of mouse oocytes. This protocol has been adopted with apparent success for the treatment of IVF patients by Quintans et al. (2002). However, other modifications may have accounted for the observed improvements, such as stepwise exposure to PrOH, a slower cooling ramp (1.5°C/min) and higher seeding temperature (−6°C). From the clinical standpoint, considering that only 12 patients were treated, the reported pregnancy and implantation rates of 50 and 25%, respectively, are of relative value. In addition, the fact that three of the six pregnancies ended in spontaneous abortions deserves attention.

The use of Na⁺-depleted freezing solutions has also been adopted by Boldt et al. (2003). High survival and acceptable fertilization rates were obtained (74.4 and 59.0%, respectively). Following transfer of 11 embryos, pregnancy rates per thaw cycle and per embryo transfer were 25.0 and 36.4%, respectively. Again, while it is difficult to assess the relative merit of the use of choline because of the coincident adoption of other protocol changes, the treatment of only 15 patients poses inevitable reservations on the clinical significance of this study.

As an alternative to slow cooling, Yoon et al. (2003) vitrified 474 oocytes recovered from 34 patients, attaining a survival rate of 68.6% at thaw. However, only 41% of the original number of thawed oocytes appeared suitable for microinjection. The pregnancy rates per transfer and implantation rate were 21.4 and 6.4%, respectively, while the rate of implantation in relation to the number of oocytes thawed was only 1.6%. On the basis of this experience, the largest of its kind, the claims that current vitrification protocols may be a more efficient cryopreservation method (Kuleshova and Lopata, 2002) remain unsubstantiated.

Very recently, the performance of the slow cooling procedure has been assessed further in 21 freezing–thawing cycles by Chen et al. (2005), who applied the high sucrose protocol described by Fabbri et al. (2001). Seventy-five percent of 159 oocytes were found to be intact after thawing. Fertilization and cleavage were within the normal ranges. Pregnancy and implantation rates were considerable (33 and 11%, respectively). Perhaps even more significant was the rate of implantation per oocyte thawed (5%). Such an outcome led Chen et al. to consider that the efficiency of oocyte freezing is comparable with that of embryo freezing. Our data argue against this conclusion. Using essentially the same protocol, we thawed a much larger number of oocytes, confirming previous findings that a high sucrose concentration ensures elevated survival and fertilization rates, while degeneration rates after ICSI were within the normal range. We also observed a high rate of cleavage at 44–46h post-insemination. At such a time point, the fraction of embryos that had progressed beyond the first cell cleavage was small. This could be a manifestation of delayed development and perhaps compromised viability. In effect, in a separate pilot study, we have recently observed that the incidence of early cleavage at 25h post-insemination is only 7.1% in embryos developed from oocytes frozen with the same 0.3 mol/l sucrose protocol, as opposed to a frequency of 59.5% in embryos from fresh sibling oocytes (Bianchi et al., 2005a). However this initial finding needs to be expanded with larger numbers. From our analysis, it also emerges that the large majority of embryos displayed minimal or moderate cell fragmentation. While this observation is important per se, it cannot be compared with other published studies because, with the exception of the work of Boldt et al. (2003), the quality of embryos derived from frozen oocytes has rarely, if ever, been described. Clinically, it is important to note that the treatment of >10% of the patients whose oocytes were thawed was interrupted because of either fertilization or cleavage failure.

Our pregnancy and implantation rates are considerably lower than those published by Chen et al. (2005). We do not have obvious explanations for this inconsistency. We can only comment on the fact that we thawed a much larger number of oocytes, a fact that probably makes our data less prone to the risk of variability related to the sample size. In effect, our rates of laboratory and clinical efficiency, although lower, have been reasonably steady throughout the period of the study (data not shown). Moreover, because the Italian IVF law prohibits the generation of more than three embryos regardless of the treatment type, in each thawing cycle we thawed the smallest possible number of oocytes in an attempt to reduce the likelihood of recovering more than three injectable oocytes. While on the one hand, thawing oocytes in small groups maximizes the overall chances of achieving a pregnancy per stimulation cycle, on the other hand, the impossibility of generating supernumerary embryos and performing embryo selection may have affected our clinical outcome in terms of pregnancy rate per thawing cycle.

From a methodological standpoint, our cryopreservation conditions are very similar, if not identical, to those used by Chen et al. (2005). The only difference consists of the fact that these authors used patient serum as a protein supplement, rather than albumin or albumin-enriched protein solution, in their cryopreservation mixtures. Experiments involving the cryopreservation of mouse oocytes have suggested that serum
may possess a cryoprotective effect (Stachecki et al., 1998). An improvement in the survival rate as a result of the inclusion of serum in the cryopreservation mixtures has also emerged from studies aiming at optimizing the slow freezing of biopsied human embryos (Jericho et al., 2003). However, it appears rather speculative to presume that the presence or absence of this component can explain the different success rates reported in our study and that of Chen et al. (2005).

Another possible technical aspect of our study open to discussion is the relatively low seeding temperature (–8°C). In effect, it has been suggested that higher temperatures (up to –4°C) can give rise to higher survival rates (Trad et al., 1999). However, these findings have been generated with immature, failed fertilized and polypronuclear human eggs, material that is likely to have different osmotic properties and therefore would respond differently to freezing conditions compared with fresh mature oocytes. Furthermore, it is worth noting that the study of Chen et al. (2005) has been conducted by applying a seeding temperature of –7°C, i.e. one degree higher than that used in our experience, a difference that in practical terms is unlikely to generate significant consequences, considering the limited accuracy in temperature measurement of available slow cooling devices. Nevertheless, we consider that the issue of the seeding temperature warrants further investigation.

Other factors may compromise oocyte viability. In an osmotic response study, we have shed some light on the diverse conditions to which oocytes are exposed during treatment with varying sucrose concentrations (Paynter et al., 2005). In fact, we have shown that when the oocyte is treated with a concentration of 0.3 mol/l, dehydration, and therefore osmotic stress, is very intense and rapid. A difference of just a minute or two in the duration of exposure to a cryoprotectant can result in extreme differences in the volume of the cell and is likely to have drastic consequences during the subsequent freezing and thawing.

On the strictly clinical front, we can rule out that our low implantation rates associated with the 0.3 mol/l sucrose freezing protocol are secondary to inappropriate endometrial preparation and/or a poor embryo transfer technique. Before the approval of the IVF law, for over a decade we had routinely applied embryo freezing, using the same endometrial preparation and embryo transfer procedures adopted in this study and achieving pregnancy rates per transfer and (classically defined) implantation rates as high as 24.1 and 11.9%, respectively (Dal Prato et al., 2002). These frequencies are more than double compared with those presented here in relation to oocyte freezing.

Considering that legal restrictions prevented us from using all the surviving oocytes, our theoretical rate of implantation per oocyte thawed corresponds to 2.6%, well below the rate reported by Chen et al. (2005) and comparable with the 2.3% rate described in our previous study on the clinical outcome of the conventional 1.5 mol/l PrOH, 0.1 mol/l sucrose slow cooling protocol (Borini et al., 2004). A more detailed comparison between the 0.1 and the 0.3 mol/l sucrose protocols suggests that in our hands the latter, in addition to generating much higher survival rates, decisively improves the fertilization rate (45.4 versus 76.1%), while cleavage rates appear similar (86.3 versus 90.2%). Nevertheless, the implantation ability of the many embryos obtained with the 0.3 mol/l sucrose protocol is apparently lower compared with that of the fewer embryos derived with the 0.1 mol/l sucrose protocol (5.2 and 16.4%, respectively). Therefore, we cannot confirm that the slow cooling/high sucrose concentration protocol ensures a high standard of clinical efficiency. At present, it is not clear why oocytes stored with this protocol survive, fertilize and develop with high rates, but may implant with poor frequencies. It has long been suspected that disruption of the meiotic spindle could compromise the developmental ability of frozen–thawed oocytes. While the overall evidence in this respect is not conclusive, on the other hand recent studies have denied such a hypothesis (Stachecki et al., 2004). Through confocal and electron microscopy, we are currently investigating various cellular characteristics in an attempt to identify conditions that may explain the reduced implantation of frozen–thawed oocytes.

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