Article

Oocyte cryopreservation: clinical outcome of slow-cooling protocols differing in sucrose concentration

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Abstract

Oocyte cryopreservation represents an important option for management of female fertility, avoiding the ethical concerns associated with embryo storage. This retrospective study evaluated the clinical outcome of two alternative slow freezing protocols involving different sucrose concentrations. From January 2004 to March 2006, spare oocytes from selected couples undergoing IVF or intracytoplasmic sperm injection were frozen using a slow-cooling protocol and thawed at a later stage. Patients were divided into two groups: group A (n = 65), whose oocytes were frozen with propane-1,2-diol (PrOH) and 0.1 mol/l sucrose; and group B (n = 66) whose oocytes were frozen with 0.3 mol/l sucrose. A total of 543 oocytes were thawed in group A and 601 in group B, achieving a survival rate of 24.3 and 71.2% respectively. Whilst fertilization rate (53.5 and 80.4% respectively) was higher in group B, enhanced results for group A were achieved over all (implantation rate per transferred embryos 12.2 versus 5.7%; pregnancy rate per transfer 16.7 versus 9.5%). Normal births and ongoing pregnancies have occurred in both groups. Although in slow-cooling methods higher sucrose concentration in the freezing mixture allows higher post-thaw survival and fertilization rates, overall this did not coincide with an improved clinical outcome.

Keywords: *cryopreservation, oocytes, pregnancy rate, slow freezing*

Introduction

Oocyte cryopreservation potentially represents an important option for management of female fertility, avoiding the ethical concerns associated with embryo storage and rescuing cases of failed semen production. Theoretically, it would also ensure safer oocyte donation, allowing appropriate screening of the biological material for viral infections. In Italy, both embryo freezing and gamete donation have been banned since March 2004, following the dictate of the new IVF law (40/2004). The same law, while forbidding the insemination of more than three oocytes, does not rule out the possibility of freezing spare oocytes. The technical approach most widely attempted is based on a slow-cooling/rapid-thawing method using propane-1,2-diol (PrOH) and sucrose as cryoprotectants (CPA), in line with the conditions originally developed for embryo freezing (Paynter and Fuller, 2004). However, initially these protocols have been shown to have limited efficiency in terms of survival (Gook *et al.*, 1993). Live births have been reported after intracytoplasmic sperm injection (ICSI) from oocytes cryopreserved using of PrOH + 0.3 mol/l sucrose (Fosas *et al.*, 2003), but it must be pointed out that the sample considered in that report was very small.

Replacement of sodium with choline in the cryoprotectant solution has been found to be beneficial for mature murine oocytes (Stachecki *et al.*, 1998). This modification was also tested with human oocytes with the aim of assessing the biological implications (Stachecki *et al.*, 2006) and the clinical potential (Quintans *et al.*, 2002; Boldt *et al.*, 2003, 2006), but it remains to be entirely evaluated. Success has also been reported recently following vitrification (Kuwayama *et al.*, 2005). Even though recent live births are very encouraging,

nonetheless the reproducibility of these protocols remains to be confirmed. Although fertilization, cleavage and implantation rates approach those of fresh oocytes in some studies (Porcu *et al.*, 2000), one of the main determining factors influencing the clinical outcome is the rate of survival, an aspect that clearly requires further improvement. In a recent paper, Chen and co-workers (2005) reported a very high survival rate after oocyte storage and pregnancy rates close to those of fresh embryos in a group of patients in which the oocytes were frozen using a slow-freezing protocol involving PrOH and 0.3 mol/l sucrose (Chen *et al.*, 2005); nevertheless, this result does not coincide with those of other authors (Borini *et al.*, 2006b; Levi Setti *et al.*, 2006).

The aim of this retrospective study was to evaluate the clinical outcome of two alternative slow-freezing protocols involving 0.1 and 0.3 mol/l sucrose, respectively, in the freezing mixture.

Materials and methods

Source of oocytes

From January 2004 to March 2006, under written informed consent, spare oocytes from couples undergoing IVF or ICSI at the IVF Unit of S Raffaele Hospital in Milan, were stored and thawed at a later stage. One hundred and thirty-one couples (female aged 30–36) undergoing IVF or ICSI for tubal disease or medium/severe male factor were enrolled. Very severe male factors, such as oligoteratozoospermia with a total sperm count $<0.1 \times 10^6$/ml, as well as azoospermic patients, were excluded from the present study. With respect to female factors, stage IV endometriosis was considered an exclusion criterion. The study population represents 17% of the cycles including oocyte freezing, which in turn corresponds to 40% of the overall number of patients treated in the authors' centre.

Patients were then split into two groups according to the concentration of sucrose in the freezing mixture (0.1 versus 0.3 mol/l). Group A included 65 patients whose oocytes underwent slow cooling with PrOH and 0.1 mol/l sucrose; group B comprised 66 patients whose oocytes underwent slow freezing with 0.3 mol/l sucrose.

Ovarian stimulation was conducted according to a long protocol using gonadotrophin-releasing hormone agonist (Enantone®; Takeda, Osaka, Japan) and recombinant FSH (Puregon®; Organon; The Netherlands or Gonal F®; Serono, Geneva, Switzerland). Ovulation was triggered with human chorionic gonadotrophin (HCG) (Ovitrelle® or Gonasi®; Serono). Oocyte retrieval was performed transvaginally, under ultrasound guidance, 36 h after HCG injection using a CCD 17-gauge aspiration needle (CCD, Paris, France).

For those patients enrolled after March 10, 2004, according to the Italian IVF law, a maximum of three oocytes per patient were inseminated/injected while spare mature oocytes were cryopreserved, according to protocols described in previous studies (Borini *et al.*, 2004, 2006b). Oocyte and sperm preparation for conventional IVF, as well as the ICSI procedure, have been thoroughly described elsewhere (Van de Velde *et al.*, 1997). With respect to ICSI, briefly, cumulus and corona radiata cells were immediately removed after retrieval by a short exposure to HEPES-buffered medium (Quinn's Advantage HEPES Medium; Sage IVF Inc., Trumbull, CT, USA) containing 20 IU/ml hyaluronidase (Sage) and gentle aspiration in and out of a Pasteur pipette and mechanically cleaned from the remaining surrounding cumulus cells by aspiration using a denuding pipette (Denuding Flexi-Pet™; Cook, Australia) with a 170–130 μm diameter. The denuded oocytes were then assessed with respect to their meiotic maturation status. In preparation for ICSI, oocytes with an extruded first polar body (PBI), presumably at the metaphase II stage (MII), were selected (a maximum of three) both for the fresh and the cryopreservation cycle.

Freezing/thawing procedure

All cryopreservation solutions were prepared using Dulbecco's phosphate-buffered saline (PBS) (Gibco, Life Technologies Ltd, Paisley, Scotland) and a plasma protein supplement (PPS) (Baxter AG, Vienna, Austria). The two freezing solutions were constituted as follows: (a) 1.5 mol/l PrOH + 20% PPS in PBS and (b) 1.5 mol/l PrOH + 0.1 (or 0.3) mol/l sucrose + 20% PPS in PBS. The thawing solutions were prepared as follows: (1) 1.0 mol/l PrOH + 0.2 (or 0.3) mol/l sucrose + 20% PPS; (ii) 0.5 mol/l PrOH + 0.2 (or 0.3) mol/l sucrose + 20% PPS; (iii) 0.2 (or 0.3) mol/l sucrose + 20% PPS, (iv) PBS + 20% PPS.

At 4 h after retrieval, oocytes were incubated at room temperature in a PBS solution containing 1.5 mol/l PrOH for 10 min and then in a solution of (group A) 1.5 mol/l PrOH + 0.1 mol/l sucrose or (group B) 1.5 mol/l PrOH + 0.3 mol/l sucrose, and subsequently loaded into plastic straws (paillettes souple 0.1 ml; Cryo Bio System, CBS, France). A maximum of three oocytes per straw was loaded. Each straw was sealed using a CBS High security sealer (CBS, France) and was expressly identified by permanent labelling. The straws were then placed in a Planer CRYO 360-1.7 (Sapio Life, Monza, Italy) programmable freezing device. The initial chamber temperature was 24°C. Then the temperature was slowly reduced to –8°C at a rate of –2°C/min. Ice nucleation was induced manually at –8°C. Finally, the straws were cooled slowly to –30°C at a rate of –0.3°C/min and afterwards rapidly to –150°C at a rate of –50°C/min. After 5 min of temperature stabilization, the straws were transferred into liquid nitrogen.

Thawing was conducted at room temperature, with a four-step procedure: 5 min in 1 mol/l PrOH + 0.2 mol/l sucrose (group A) or 0.3 mol/l sucrose (group B), 5 min in 0.5 mol/l PrOH + 0.2 mol/l sucrose (group A) or 0.3 mol/l sucrose (group B), 10 min in 0.2 mol/l sucrose (group A) or 0.3 mol/l sucrose (group B), 10 min in PBS. Both freezing and thawing solutions were supplemented with plasma protein solution (20%v/v). Oocytes were then replaced in culture medium at 37°C until further evaluation.

Insemination and embryo culture

Oocytes surviving immediately after thawing were cultured in cleavage medium (Quinn's Advantage Cleavage Medium;

Sage) for 3 h, reassessed for viability and then microinjected. Normal fertilization was defined as the presence of two pronuclei and two polar bodies 16–18 h later. All fertilized oocytes were transferred to fresh pre-equilibrated cleavage medium. Embryos were graded on day 2 according to the classification of Veeck (1999), with some modifications, considering also the presence of multinucleated blastomeres (MNB) (Meriano *et al.*, 2004); embryos were routinely transferred on day 2 using a soft catheter (K-SOFT 5000; Cook) without ultrasound guidance.

Endometrial preparation and embryo transfer

Endometrial thickness was supported with a steadily increasing dosage, 100–300 µg, of micronized 17β-oestradiol 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 180 mg micronized doses in gel (Crinone 8; Serono, Rome, Italy) via vaginal route was started on the day of oocyte thawing. Embryo transfers were performed on day 2 of progesterone administration. Endometrium thickness was checked and the cycle suspended if the lining was thinner than 8 mm or thicker than 12 mm. In 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.

Statistical analysis

Statistical analysis was performed using the Chi-squared test and a *P*-value ≤0.05 was considered as significant.

Results

Two alternative oocyte slow-cooling protocols involving the use of 0.1 (group A) or 0.3 (group B) mol/l sucrose in the freezing solution were applied for the treatment of 65 (group A) and 66 (group B) patients, for a total of 1144 oocytes cryopreserved subsequent to a single oocyte retrieval for each patient. All patients underwent one thawing cycle involving all or part of their stored oocytes, except two patients in group B who underwent two thawing cycles. Overall, 902 oocytes were thawed: 506 in group A and 396 in group B (derived from a pool of 543 and 601 stored oocytes respectively). In group A, 123 oocytes (24.3%) survived and 114 were microinjected, in group B 282 oocytes (71.2%) survived and 194 were used for ICSI. The survival rate of group A was significantly lower compared with group B ($P < 0.001$) (see **Table 1**). Not all surviving oocytes could be used for treatment because the Italian IVF law forbids the insemination of more than three oocytes. In some thawing cycles, especially in the 0.3 mol/l sucrose group, more than three surviving oocytes were obtained as a consequence of the practical need to loading oocytes in small groups in individual straws.

A total of 61 oocytes (53.5%) were fertilized in group A, compared with 156 (80.4%) in group B ($P < 0.001$). In group A, 41 embryos were replaced, compared with 139 embryos in group B, corresponding to a transfer rate per patient of 46.2% and 95.5% respectively. This difference was statistically significant ($P < 0.001$) (see **Table 1**). Embryo quality was not substantially different in the two groups (see details in **Table 2**). With respect to group A, treatment outcome resulted in nine biochemical pregnancies (positive β-HCG test) and five clinical pregnancies, of which two were lost at week 9 of gestation. Concerning these losses, chromosome analysis was normal in one case, while a trisomy 7 was detected in the other one. The full-term pregnancies revealed a 46XY and a 46XX normal karyotype and resulted in the delivery of two

Table 1. Outcome of freeze/thawed cycles in the two study groups.

	Group A (0.1 mol/l sucrose)	*Group B (0.3 mol/l sucrose)*
No. of freeze/thawed cycles	65	68
No. of patients	65	66
Mean age of patient (years) ± SD	33.5 ± 2.9	33.9 ± 2.1
No. of oocytes frozen	543	601
No. of oocytes thawed	506	396
No. of oocytes surviving	123	282
Survival rate %	24.3[a]	71.2[a]
No. of oocytes injected	114	194
No. of oocytes fertilized	61	156
Fertilization rate %	53.5[b]	80.4[b]
Cleavage rate %	72.1[c]	91.0[c]
No. of embryos transferred	41	139
Percentage of embryos transferred %	93.2	97.9
No. of transfers	30	63
Transfer rate per patient (%)	46.2[d]	95.5[d]

[a,b,c,d] P<0.001

healthy babies. One pregnancy is still ongoing. With respect to group B, 10 biochemical and six clinical pregnancies (one twin) were obtained of which one corresponded to a normal 46XY fetus that developed to a full-term baby, three are currently ongoing and two (one single and one twin) ended in spontaneous abortions.

The implantation rates (IR) per thawed oocyte were 1.1% for group A and 2.6% for group B. These percentages represent the ratio of gestational sacs observed in oocytes thawed, adjusted for a value (survived/injected) that express the inability to inject all the survived oocytes due to law restriction. The pregnancy rate (PR) per transfer was 16.7% in group A and 9.5% in group B. These differences did not reach statistical significance. The PR per patient was 7.7 and 9.1% in groups A and B respectively. In terms of implantation rate per transferred embryos, the treatment outcome was 12.2% in group A and 5.7% in groups B, frequencies that appear different but that do not reach statistical significance (see **Table 3**).

In group A, the outcome did not seem to be related to the number of oocytes injected. In fact, four out of the five clinical pregnancies achieved came from thawing cycles where only one or two oocytes suitable for injection were obtained (see **Table 4**). A different trend was observed in group B, where most of the clinical pregnancies derived from the use of three viable oocytes after thawing (see **Table 4**).

Table 2. Characteristics of embryos derived from freeze/thawed oocytes in the two study groups.

	Group A (0.1 mol/l sucrose)	*Group B (0.3 mol/l sucrose)*
Embryo quality (%)		
A	29.3	27.0
B	53.7	56.6
C	17.0	16.4
No. cells (%)		
2	24.4	20.5
4	75.6	72.1
>4	0	7.4

Table 3. Clinical outcome following fertilization of freeze/thawed oocytes in the two study groups.

	Group A (0.1 mol/l sucrose)	*Group B (0.3 mol/l sucrose)*
No. of gestational sacs	5	7
Implantation rate per transferred embryo (%)	12.2	5.7
No. of pregnancies	5	6 (1 twin)
Pregnancy rate per transfer (%)	16.7	9.5
Pregnancy rate per patient (%)	7.7	9.1
No. of spontaneous abortions	2	2
No. of take home babies	2	1
Take home babies per transferred embryo (%)	4.9	0.72
No. of ongoing pregnancies	1	3

There were no statistically significant differences between the two groups.

Table 4. Outcome based on number of oocytes injected per cycle.

Injected oocytes/cycle	*Group A (0.1 mol/l sucrose)*		*Group B (0.3 mol/l sucrose)*	
	Patients % (n)	*Gestational sacs %*	*Patients % (n)*	*Gestational sacs %*
0	18.5 (12)	-	–	–
1	24.6 (16)	40.0	–	–
2	20.0 (13)	40.0	14.7 (10)	14.3
3	36.9 (24)	20.0	85.3 (58)	85.7

Discussion

From a clinical point of view, oocyte cryopreservation represents a valid tool to maximize the success rate of those cycles in which oocytes are retrieved that cannot be inseminated, due to ethical concerns or legal restrictions. This is the case for patients treated under the Italian IVF law, for whom no more than three oocytes may be used in a fresh cycle. The choice of metaphase II (MII) oocytes selected for either the fresh or frozen treatment is problematic, because morphological characteristics are not sufficiently informative of developmental competence. For instance, oocytes with an extruded first polar body may have not reached meiotic arrest at MII (De Santis *et al*., 2005).

Oocyte storage presents some intrinsic limitations. Cells are very sensitive to intracellular ice formation or excessive dehydration during cooling and extracellular ice formation (Paynter and Fuller, 2004). Human oocytes tend to shrink more than murine oocytes and exhibit higher values per unit volume under similar conditions for permeabilities to both water and cryoprotectant (Paynter *et al*., 1999, 2001, 2005; Paynter, 2005). This means that close attention must be paid during addition and dilution of cryoprotectant (CPA) to avoid excessive osmotic stress (Trad *et al*., 1999; Paynter *et al*., 2005). Modifications of the 1.5 mol/l PrOH 0.1 mol/l sucrose slow-freeze, rapid-thaw protocol have been applied. Increasing the temperature during exposure to CPA and simultaneously shortening the time of exposure has shown some improvements (Yang *et al*., 1998). As demonstrated well by Paynter *et al*. (2005), differences in sucrose concentrations in the loading solutions correspond to different degrees of cell dehydration. In particular, a 30% cell shrinkage prior to the onset of cooling would appear to be beneficial to the survival of the oocyte, as shown by Fabbri and co-workers (2001), who reported higher survival when the sucrose concentration was increased to 0.2 mol/l and was even higher (up to 80%) when the sucrose concentration was 0.3 mol/l (Fabbri *et al*., 2001). Nonetheless, it was decided to also adopt a protocol with reduced amount of this CPA because at the time of beginning the present work, the clinical efficiency of these alternative approaches had not been ascertained, and in fact still remains intensely disputed (Porcu *et al*., 2000; Borini *et al*., 2004, 2006b; Levi Setti *et al*., 2006). Porcu and colleagues, reported results from different groups of patients, while the team of Borini and that of Levi Setti, presented data specifically on slow-cooling protocols involving either high or low sucrose concentration. In his recent paper using a slow-freezing protocol involving PrOH and 0.3 mol/l sucrose, Li reported high survival rates and clinical pregnancies (Li *et al*., 2005). Nonetheless, similar to other cases, this paper is far from being conclusive because of the small sample considered. As an alternative to slow cooling, vitrification has been described to provide high survival rates and clinical outcome (Kuwayama *et al*., 2005). These achievements, however, have not been independently confirmed and need further assessment. An interesting review has been recently published suggesting that application of the latest vitrification methods is more efficient and reliable than any version of slow freezing (Vajta and Nagy, 2006).

As shown by the comparison between groups A and B, the data are in line with previous reports showing that survival after slow cooling can be considerably improved by using high sucrose concentration in the freezing solution. Routinely, the 0.1 mol/l sucrose freezing protocol implies the use of 0.2 mol/l sucrose in the dilution solutions while the 0.3 mol/l protocol was originally designed by maintaining the same sucrose concentration during rehydration. For this reason, these protocols were applied without any change. However, a factor for consideration is the use of higher sucrose concentrations in the thawing solutions that could assist in the control of post-thaw osmotic stress. A recent study appeared to confirm the validity of this approach (Boldt *et al*., 2006). The findings validate previous data that indicate that the 0.1 mol/l sucrose protocol, apart from resulting in low survival rates, compromises oocyte fertilization ability. Since frozen–thawed oocytes are routinely microinjected, it may be presumed that poor fertilization associated with this protocol is secondary to cell function alterations other than zona pellucida–sperm interaction. In fact, it has been widely discussed that morphological normality after thawing does not always reflect overall viability (Coticchio *et al*., 2005). It is not surprising that in the low sucrose group the dramatic loss in viable oocytes after thawing, in association with reduced fertilization rates, gave rise to a low transfer rate per patient. This effect was exacerbated by the restrictions under which treatments were provided. The thawing procedure was, in fact, designed in order to obtain no more than three viable oocytes, to optimize the use of the stored material while complying with the Italian law. However, some oocytes that appeared healthy immediately after thawing were found non-viable a few hours later. Nevertheless, they were not replaced because this would

have implied ageing *in vitro* of the viable oocytes that had been thawed a few hours before. Therefore, in some thawing cycles fewer than three oocytes were available for microinjection. This had a downstream effect on the number of normally fertilized oocytes and embryos available for transfer. Such circumstances also limited the outcome of group B, where on the other hand higher survival and fertilization rates allowed the achievement of much higher transfer rates. It is paradoxical, though, that in the group with the lower transfer rate per thawing cycle (0.1 mol/l sucrose), considerably higher implantation and pregnancy rates per transfer were achieved. This suggests that while the 0.1 mol/l protocol severely compromises the survival rate, at the same time it is able to better preserve the developmental potential of survived oocytes, a finding previously reported by other authors (Borini *et al.*, 2004). It is not known why the oocytes that survive after storage with the 0.3 mol/l protocol generate embryos with reduced ability to implant, but preliminary evidence suggests that this may be caused by cell damage detectable via ultrastructural analysis (Nottola *et al.*, in press). On the other hand, confocal microscopy observation suggests that the different developmental ability of oocytes stored with the two protocols may not be explained by meiotic spindle damage. In fact, oocytes cryopreserved with the 0.3 mol/l protocol exhibit a frequency of normal spindle morphology comparable to fresh controls (Coticchio *et al.*, 2006). Meiotic spindle alterations after freezing have also been ruled out in the case of the choline-based method (Stachecki and Cohen, 2004). The observations suggest that possible spindle alterations are not related to embryo morphological quality assessed by standard criteria, given that the proportion of class A embryos was not reduced in group B.

However, careful observation of embryos derived from frozen–thawed oocytes may reveal impairment of their developmental potential, as suggested by a reduced pace of cell division compared with sibling fresh oocytes (data not shown). This point is still a matter of debate, considering that a recent paper reported unaltered fertilization rate but impaired cleavage in embryos from frozen oocytes compared with fresh controls (Chamayou *et al.*, 2006). In both groups, a noticeable difference between positive β-HCG tests and clinical pregnancies suggests significant post-implantation embryo loss. This trend is in agreement with the pronounced incidence of spontaneous abortions. While these observations require cautious interpretation because of the small figures in this study, the hypothesis that the clinical outcome of oocyte cryopreservation is jeopardized by compromised embryo viability should be carefully tested in future investigations. In terms of implantation efficiency, the two protocols do not seem appreciably different. In fact, when they are compared, taking into account the number of thawed oocytes, similar rates are obtained. These low implantation rates were determined not only by the limited efficiency of the technique, but also by the fact that all embryos deemed viable were transferred, any possibility of selection being denied in principle. By comparison, the implantation rate of frozen embryos calculated on the basis of the number of oocytes from which they are obtained has been estimated to correspond to about 4% (Gook and Edgar, 1999). Because of the limitation on the number of embryos (and therefore oocytes) that could be used in each frozen cycle, the data can hardly be compared with those of others. It is not surprising that in cases in which embryo selection was allowed, higher implantation and pregnancy rates were reported (Boldt *et al.*, 2003; Fosas *et al.*, 2003). Regardless, studies with very low numbers of patients are problematic to appraise. The estimation of clinical efficiency is still a matter of debate (Min *et al.*, 2004), but, especially in cases like that described in the present study, it is probable that this will reflect the amount of implantations achieved per stimulation cycle, including the stored material (Borini *et al.*, 2006a). Bearing in mind that in this study only some of the frozen oocytes were thawed, it is not yet possible to work out the overall efficiency of the interventions, but the oocytes that are still stored are expected to contribute to more pregnancies.

In conclusion, it remains unproven that the challenge of oocyte freezing has been met by a protocol that is able to generate high survival rates. Although in slow-cooling methods higher sucrose concentration in the freezing mixture leads to higher post-thaw survival and fertilization rates, this may not coincide with an overall improved clinical outcome. More has to be done to achieve high survival rates while preserving oocyte viability.

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References

Boldt J, Tidswell N, Sayer A *et al.* 2006 Human oocyte cryopreservation: 5-year experience with a sodium-depleted medium slow freezing method. *Reproductive BioMedicine Online* **13**, 96–100.

Boldt J, Cline D, McLaughlin D 2003 Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. *Human Reproduction* **18**, 1250–1255.

Borini A, Lagalla C, Bonu MA *et al.* 2006a Cumulative pregnancy rates resulting from the use of fresh and frozen oocytes: 7 years' experience. *Reproductive BioMedicine Online* **12**, 481–486.

Borini A, Sciajno R, Bianchi V *et al.* 2006b Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration. *Human Reproduction* **21**, 512–517.

Borini A, Bonu MA, Coticchio G *et al.* 2004 Pregnancies and births after oocyte cryopreservation. *Fertility and Sterility* **82**, 601–605.

Borini A, Bafaro MG, Bianchi L *et al.* 1996 Oocyte donation programme: results obtained with intracytoplasmic sperm injection in cases of severe male factor infertility or previous failed fertilization. *Human Reproduction* **11**, 548–550.

Chamayou S, Alecci C, Ragolia C *et al.* 2006 Comparison of in-vitro outcomes from cryopreserved oocytes and sibling fresh oocytes. *Reproductive BioMedicine Online* **12**, 730–736.

Chen SU, Lien YR, Chen HF *et al.* 2005 Observational clinical follow-up of oocyte cryopreservation using a slow-freezing method with 1,2-propanediol plus sucrose followed by ICSI. *Human Reproduction* **20**, 1975–1980.

Coticchio G, De Santis L, Rossi G *et al.* 2006 Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation. *Human Reproduction* **21**, 1771–1776.

Coticchio G, Bonu MA, Bianchi V *et al.* 2005 Criteria to assess human oocyte quality after cryopreservation. *Reproductive BioMedicine Online* **11**, 421–427.

De Santis L, Cino I, Rabellotti E *et al.* 2005 Polar body morphology and spindle imaging as predictors of oocyte quality. *Reproductive BioMedicine Online* **11**, 36–42.

Fabbri R, Porcu E, Marsella T *et al.* 2001 Human oocyte

cryopreservation: new perspectives regarding oocyte survival. *Human Reproduction* **16**, 411–416.
Fosas N, Marina F, Torres PJ *et al*. 2003 The births of five Spanish babies from cryopreserved donated oocytes. *Human Reproduction* **18**, 1417–1421.
Gook DA, Edgar DH 1999 Cryopreservation of the human female gamete: current and future issues. *Human Reproduction* **14**, 2938–2940.
Gook DA, Osborn SM, Johnston WI 1993 Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. *Human Reproduction* **8**, 1101–1109.
Kuwayama M, Vajta G, Kato O *et al*. 2005 Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online* **11**, 300–308.
Levi Setti PE, Albani E, Novara PV *et al*. 2006 Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Human Reproduction* **21**, 370–375.
Li XH, Chen SU, Zhang X *et al*. 2005 Cryopreserved oocytes of infertile couples undergoing assisted reproductive technology could be an important source of oocyte donation: a clinical report of successful pregnancies. *Human Reproduction* **20**, 3390–3394.
Meriano J, Clark C, Cadesky K *et al*. 2004 Binucleated and micronucleated blastomeres in embryos derived from human assisted reproduction cycles. *Reproductive BioMedicine Online* **9**, 511–520.
Min JK, Breheny SA, MacLachlan V *et al*. 2004 What is the most relevant standard of success in assisted reproduction? The singleton, term gestation, live birth rate per cycle initiated: the BESST endpoint for assisted reproduction. *Human Reproduction* **19**, 3–7.
Nottola SA, Macchiarelli G, Coticchio G *et al*. Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations. *Human Reproduction* (in press).
Paynter SJ 2005 A rational approach to oocyte cryopreservation. *Reproductive BioMedicine Online* **10**, 578–586.
Paynter SJ, Fuller B 2004 Cryopreservation of the female reproductive cells: currents concepts and controversies. *2PN Attualità Scientifica in Biologia della Riproduzione* **1**, 1–27.
Paynter SJ, Borini A, Bianchi V *et al*. 2005 Volume changes of mature human oocytes on exposure to cryoprotectant solutions used in slow cooling procedures. *Human Reproduction* **20**, 1194–1199.
Paynter SJ, O'Neil L, Fuller BJ *et al*. 2001 Membrane permeability of human oocytes in the presence of the cryoprotectant propane-1,2-diol. *Fertility and Sterility* **75**, 532–538.
Paynter SJ, Fuller BJ, Shaw RW 1999 Temperature dependence of Kedem–Katchalsky membrane transport coefficients for mature mouse oocytes in the presence of ethylene glycol. *Cryobiology* **39**, 169–176.
Porcu E, Fabbri R, Damiano G *et al*. 2000 Clinical experience and applications of oocyte cryopreservation. *Molecular and Cellular Endocrinology* **169**, 33–37.
Quintans CJ, Donaldson MJ, Bertolino MV *et al*. 2002 Birth of two babies using oocytes that were cryopreserved in a choline-based freezing medium. *Human Reproduction* **17**, 3149–3152.
Stachecki JJ, Cohen J 2004 An overview of oocyte cryopreservation. *Reproductive BioMedicine Online* **9**, 152–163.
Stachecki JJ, Cohen J, Garrisi GJ *et al*. 2006 Cryopreservation of unfertilized human oocytes. *Reproductive BioMedicine Online* **13**, 222–227.
Stachecki JJ, Cohen J, Willadsen SM 1998 Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. *Cryobiology* **37**, 346–354.
Trad FS, Toner M, Biggers JD 1999 Effects of cryoprotectants and ice-seeding temperature on intracellular freezing and survival of human oocytes. *Human Reproduction* **14**, 1569–1577.
Vajta G, Nagy ZP 2006 Are programmable freezers still needed in the laboratory? Review on vitrification. *Reproductive BioMedicine Online* **12**, 779–796.
Van de Velde H, Nagy ZP, Joris H *et al*. 1997 Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. *Human Reproduction* **12**, 2246–2250.
Veeck LL 1999 *Atlas of Human Gametes and Conceptuses: an Illustrated Reference for Assisted Reproductive Technology*. Parthenon Publishing Group, New York.
Yang DS, Blohm PL, Winslow KL 1998 A twin pregnancy after microinjection of human cryopreserved oocytes with a specially developed oocyte cryopreservation regime. *Fertility and Sterility* **70** (Suppl. 1), S239.

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