Article

Evidence-based clinical outcome of oocyte slow cooling

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Abstract

In the last few years, there has been a significant improvement in oocyte cryopreservation techniques. To investigate the clinical significance of oocyte freezing, an assessment of the cumulative pregnancy rate per started cycle derived from the use of fresh and frozen–thawed oocytes was performed. Between 2004 and 2006, 749 cycles were carried out, in which no more than three fresh oocytes were inseminated either by standard IVF or microinjection. Supernumerary mature oocytes were cryopreserved by slow cooling. Cryopreservation of fresh embryos was performed in rare cases to prevent the risk of ovarian hyperstimulation syndrome using a standard embryo freezing protocol. Fresh embryo transfer cycles totalled 680, 257 of which resulted in pregnancy. The pregnancy rates per patient and per transfer were 34.3% and 37.8% respectively. When frozen–thawed oocytes were used, following 660 thawing cycles, 590 embryo transfers were performed in 510 patients. Eighty-eight pregnancies were achieved with embryos from frozen oocytes, with a success rate of 17.2% per cycle. When fresh and frozen–thawed cycles were combined, the number of pregnancies was 355, giving a cumulative pregnancy rate of 47.4%. Oocyte cryopreservation can contribute considerably to the overall clinical success, ensuring a cumulative rate approaching that achievable with embryo storage.

Keywords: *cryopreservation, cumulative pregnancies, oocytes, pregnancy rate, slow cooling*

Introduction

Embryo cryopreservation is a well-established and successful procedure that is able to maximize the reproductive potential of a cohort of oocytes derived from a single recovery. Therefore, it has been argued that the cumulative delivery rate per cycle, including pregnancies generated from both fresh and frozen embryos, should be adopted as a more objective method to express IVF success rate (Jones *et al*., 1995; Tiitinen *et al*., 2004). In effect, the contribution of frozen embryos to the clinical outcome can be very important, raising overall success to rates as high as 60–70% (Ubaldi *et al*., 2004). In principle, the same concept could be applied to oocyte cryopreservation, but until recently such a form of storage has been attempted sporadically. In effect, for many years post-thaw oocyte survival rates remained unacceptably low (Borini *et al*., 1998; Tucker *et al*., 1998; Porcu *et al*., 2000), negatively affecting the overall clinical outcome. Under such conditions, oocyte cryopreservation could not be proposed as a viable treatment option and its application was limited mainly to rare cases in which embryo storage was not applicable. As a consequence, many studies included small or negligible numbers of patients (Boldt *et al*., 2003; Fosas *et al*., 2003; Chen *et al*., 2005), making difficult, if not impossible, an objective appraisal of the clinical outcome. In the last few years, though, novel slow cooling (Fabbri *et al*., 2001; Boldt *et al*., 2006) and vitrification (Kuwayama *et al*., 2005) protocols have been able to significantly improve the rates of survival and fertilization of frozen–thawed oocytes. Recent studies suggest that oocyte cryopreservation can

be applied in a reproducible fashion (Borini *et al.*, 2006b; Levi Setti *et al.*, 2006), in some cases achieving high implantation rates (Boldt *et al.*, 2006; Bianchi *et al.*, 2007). This has made stronger the argument for the routine use of stored oocytes in a clinical scenario. In effect, increasingly higher numbers of patients have been treated with frozen oocytes, especially in Italy where embryo storage is not allowed. In this country, a legislation introduced in 2004 prohibits, in fact, the insemination of more than three oocytes. Therefore, appraising the possible contribution of 'supernumerary' frozen oocytes to the overall clinical outcome has become an essential and urgent issue, also for making patients more aware of their overall chances of success. Data in this respect are lacking. It is believed that to date only one study, which was published recently by our group, has addressed this issue (Borini *et al.*, 2006a). In the present manuscript, the authors describe an extension of their clinical experience in oocyte cryopreservation over a period starting with the introduction of the Italian IVF law, reporting in particular the cumulative pregnancy rate that was derived from both fresh and cryopreserved oocytes, adopting one of two available slow cooling protocols. Results were obtained in different centres for reproductive health: Tecnobios Procreazione and some affiliated centres in different cities.

Materials and methods

Patients and oocytes

This study included 749 patients treated for infertility at Tecnobios Procreazione and affiliated centres, Italy, over the period April 2004 to December 2006, following the enforcement of the Italian IVF law that has ruled out pronucleate stage and embryo cryopreservation. Treatment using cryopreserved oocytes had been preliminarily approved by the local Institutional Review Board. Patients agreeing to the treatment signed an informed consent form. Ovarian stimulation was induced with a long protocol using leuprorelin (Enantone; Takeda, Rome, Italy) and recombinant FSH (rFSH; Gonal-F, Serono, Rome, Italy; or Puregon, Organon, Rome, Italy). A dose of 10,000 IU human chorionic gonadotrophin (HCG; Gonasi, Amsa, Rome, Italy) or one ampoule of recombinant HCG (rHCG; Ovitrelle, Serono, Rome, Italy) was administered when one or more follicles reached a maximum diameter of >23 mm. (Dal Prato *et al.*, 2001). Oocyte collection was performed transvaginally, under ultrasound guidance, 36 h after HCG injection.

After retrieval, oocytes were cultured in fertilization medium (Cook IVF, Brisbane, Australia) for about 2 h. In compliance with the above-mentioned IVF law, a maximum of three oocytes were inseminated in fresh cycles, while the remaining mature oocytes were frozen and subsequently used, all or in part, in successive frozen cycles. In a very small cohort of patients, the obtained fresh embryos had to be frozen, and embryo transfer was not performed in order to reduce the risk of ovarian hyperstimulation syndrome (OHSS). In preparation for freezing, complete removal of cumulus mass and corona cells was performed enzymatically using hyaluronidase (80 IU/ml; Sigma Aldrich Srl, Milan, Italy), and mechanically by using fine-bore glass pipettes. Only oocytes showing an extruded polar body I and presumably at the metaphase II stage were frozen after culture for a total period of time of about 4 h following retrieval.

Oocyte freezing solutions

Two sets of dehydration solutions were employed for this study: (i) 1.5 mol/l propane-1,2-diol (PrOH) + 20% Plasma Protein Supplement (PPS; Baxter AG, Vienna, Austria) in Dulbecco's phosphate-buffered solution (PBS; Gibco Life Technologies Ltd, Paisley, Scotland) (equilibration solution) and 1.5 mol/l PrOH + 0.3 mol/l sucrose + 20% PPS in PBS (loading solution) previously described by Fabbri *et al.* (2001); and (ii) 0.75 mol/l PrOH and 1.5 mol/l PrOH (equilibration solutions) and 1.5 mol/l PrOH + 0.2 mol/l sucrose (loading solution) (Cook IVF).

Freezing procedure

Oocytes were frozen using one of the two procedures described as follows:

(i) Oocytes were equilibrated in the equilibration solution (1.5 mol/l PrOH) for 10 min at room temperature and then transferred to the loading solution (1.5 mol/l PrOH + 0.3 mol/l sucrose) for 5 min; (ii) Oocytes were equilibrated sequentially in the solutions containing 0.75 and 1.5 mol/l PrOH (7.5 min for each step) and then transferred for 5 min in the loading solution (1.5 mol/l PrOH + 0.2 mol/l sucrose). Oocytes were finally loaded in plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France), individually or in small groups (maximum three oocytes per straw). Straw temperature was lowered through an automated Kryo 10 series III biological freezer (Planer Kryo 10/1,7; Planer Plc, UK) from 20°C to –8°C at a rate of –2°C/min. Manual seeding was performed at –8°C. This temperature was maintained in a hold interval of 10 min in order to allow uniform ice propagation. Temperature was then decreased to –30°C at a rate of –0.3°C/min and finally rapidly to –150°C at a rate of –50°C/min. Finally, straws were directly plunged into liquid nitrogen and stored for later use.

Thawing procedure

Thawing was carried out at room temperature. Straws were removed from liquid nitrogen, warmed in air for 30 s and then plunged into a water bath at 30°C for 40 s. Cryoprotectant step-wise dilution was performed using one of the following procedures: (i) Thawed oocytes were released in 1.0 mol/l PrOH, 0.3 mol/l sucrose and 20% PPS, and incubated for 5 min. Afterwards they were transferred in 0.5 mol/l PrOH, 0.3 mol/l sucrose and 20% PPS for an additional 5 min. Finally oocytes were placed in 0.3 mol/l sucrose and 20% PPS for 10 min before final dilution in buffer including 20% PPS for 20 min (10 min at room temperature and 10 min at 37°C). All these solutions were prepared in PBS buffer; (ii) Thawing was performed as described in (i) employing solutions obtained from Cook IVF containing the same proportions of cryoprotectants.

Embryo freezing

A total of 64 embryos from 23 patients were frozen and thawed using the standard protocol described by Lassalle *et al.* (1985).

Insemination and culture of cryopreserved oocytes

After freezing and thawing, oocytes showing swelling or shrinkage, vacuoles, membrane blebbing or other anomalies were not considered viable and suitable for treatment. Surviving oocytes were transferred to a 20 μl drop of cleavage medium (Cook IVF) under warm mineral oil (Cook IVF) at 37°C in an atmosphere of 5% CO_2. At 90–120 min after thawing, ICSI was performed as described elsewhere (Borini *et al.*, 2006a).

At 18–20 h post-insemination, two pronucleate eggs were transferred to fresh cleavage medium and cultured until transfer. On day 2 (44–46 h post-insemination), embryos were transferred.

Endometrial preparation and embryo transfer

Endometrial growth was supported with a steadily increasing dosage, 100–300 µg, of micronized 17β in oestradiol in patches (Climara, 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 the vaginal route, was started on the day of oocyte thawing (day 0). Embryo transfers were performed on the third day of progesterone administration (day 2). Endometrium thickness was checked and the cycle suspended if the line was thinner than 8 mm or thicker than 12 mm. In cases of pregnancy, endometrial support treatment was continued for 60 days after transfer. Clinical pregnancy was defined as the presence of a gestational sac at ultrasound examination.

Cumulative pregnancy rates were calculated by dividing the sum of fresh and frozen pregnancies by the number of patients (Jones *et al.*, 1995).

Results

This study included patients whose thawing cycles involved the use of all frozen oocytes as well as those whose frozen oocytes were thawed only in part, having achieved a pregnancy. A total of 749 patients (mean age ± SD, 35.2 ± 4.1) were considered. A mean of 2.88 ± 0.4 oocytes were inseminated in the fresh cycles. Fertilization and cleavage rates were 79.1% and 97.9%, respectively. A total of 1638 fresh embryos were transferred in 680 embryo transfers, with a mean number of 2.2 ± 0.9 embryos transferred. The number of pregnancies obtained was 257. At ultrasound examination, 345 gestational sacs were observed. Singleton, twin and triplet pregnancies were 176 (68.5%), 65 (25.3%) and 16 (6.2%), respectively. The pregnancy rates per patient and per transfer were 34.3% and 37.8%, respectively. An implantation rate of 21.1% was calculated as the ratio between gestational sacs and transferred embryos. In 37 patients, pregnancies terminated spontaneously, with a rate of 14.4%.

After insemination, 23 patients were considered at high risk of OHSS, a condition that suggested the cryopreservation of their embryos as a cautionary measure. Sixty-four embryos were frozen and subsequently thawed at least 1 month after ovarian stimulation. Forty-one embryos that survived fully intact or retained at least 50% of their original cell mass were transferred in 22 transfers. Ten pregnancies, eight singleton and two twin, were achieved, with a rate per transfer of 45.5%.

Considering together the transfers in which either fresh or frozen–thawed embryos were replaced, a total of 267 pregnancies were obtained, generating overall pregnancy rates per patient and per transfer of 35.6% and 38.0%, respectively. The total number of gestational sacs was 366, leading to an implantation rate of 21.8% (**Table 1**).

The total number of frozen oocytes was 5448 (**Table 2**). A total of 510 patients (68.1%) underwent at least one thawing cycle. A total of 3238 oocytes were thawed, with a survival rate of 68.1% (n = 2205). A total of 1815 of the survived oocytes were microinjected, as a result of the restrictions imposed on the number of embryos, which may be generated in each treatment cycle. The number of surviving oocytes exceeded the number of those destined for microinjection in order to respond to the need of excluding some thawed oocytes showing minor intrinsic or extrinsic abnormalities (large or degenerate polar body I, abnormal or fractured zona, etc). The proportion of normally fertilized oocytes was 76.1% (n = 1381). Cleavage rate on day 2 was 91.8%, giving rise to 1268 transferable embryos which were replaced in 590 embryo transfers, with a mean of 1.9 ± 1.0 transferred embryos per transfer. A total of 70 thawing cycles failed to progress to embryo transfer due to post-thaw oocyte degeneration (n = 14), failed fertilization (n = 48) or failed cleavage (n = 8). Ultrasound assessment revealed 88 pregnancies and 101 gestational sacs. Seventy-five pregnancies were singleton, while 11 were twin and one was quadruplet. The pregnancy rates per transfer and per patient were respectively 14.9% and 17.2%. The implantation rate was 8.1%, as calculated by the proportion of implanted embryos with respect to the total number of embryos transferred. Nineteen patients aborted with a rate of 21.6%. Thirteen pregnancies are still ongoing, while 52 single and four twin pregnancies progressed to full term. Two of the 60 babies born (31 females and 29 males) were affected by developmental abnormalities, consisting of choanae atresia and Rubinstein–Taydi Syndrome.

To evaluate the cumulative clinical outcome, pregnancies derived from fresh and frozen–thawed embryos or oocytes were cumulated (355 in total) and then divided by the number of patients (n = 749), giving rise to a rate of 47.4% (**Table 3**).

Considering that 2947 embryos (1638 fresh, 41 thawed and 1268 from thawed oocytes) were transferred, and 458 implanted (345, 12 and 101 respectively), a cumulative implantation rate of 15.5% was obtained.

A total of 2047 oocytes from 295 patients remain frozen and available for future thawing cycles.

Table 1. Cumulative pregnancy outcome from transfers with fresh and frozen–thawed embryos.

No. of patients	749
No. of transfers with fresh or frozen–thawed embryos	702
No. of embryos transferred (mean ± SD)	1679 (2.2 ± 0.9)
No. of pregnancies	267
Pregnancy rate (%) per embryo transfer	38.0 (267/702)
Pregnancy rate (%) per patient	35.6 (267/749)
Multiple pregnancy rate (%)	31.1 (83/267)
Gestational sacs	366
Implantation rate (%)	21.8 (366/1679)
Abortions (*n*)	37
Abortion rate (%)	13.9

Table 2. Pregnancy outcome using frozen–thawed oocytes.

No. patients with at least one thawing cycle	510
No. of thawing cycles	660
No. of oocytes	
Frozen	5448
Thawed	3238
Survived (%)	2205 (68.1)
Microinjected	1815
Two-pronucleate (%)	1381 (76.1)
No. of embryos cleaved (%)	1268 (91.8)
No. of embryos transferred (%)	1268 (100)
No. of transfers	590
No. of pregnancies	88
Gestational sacs	103
Implantation rate (%)	8.1
Pregnancy rate (%) per patient	17.2 (88/510)
Pregnancy rate (%) per transfer	14.9 (88/590)
Abortions (*n*)	19
Abortion rate (%)	21.6

Table 3. Cumulative pregnancy rate derived from fresh and frozen–thawed oocytes.

No. of patients	749
No. of pregnancies	355
No. of gestational sacs	458
Cumulative pregnancy rate (%) per transfer	47.4 (355/749)
Cumulative implantation rate (%)	15.5 (458/2947)

Discussion

Oocyte cryopreservation has long been considered unattainable, principally because of the inability to recover the stored material with high rates of survival (Gook *et al*., 1994; Tucker *et al*., 1996) or preserve unaltered the developmental potential of the ensuing embryos (Tucker *et al*., 1996). Over the last 10 years, though, novel protocols have increased the survival and overall viability of frozen oocytes. Improved slow cooling protocols ensure high yields of frozen-thawed oocytes that can fertilize and cleave with high rates (Porcu *et al*., 2000; Fabbri *et al*., 2001; Boldt *et al*., 2006; Borini *et al*., 2006a; Levi Setti *et al*., 2006; De Santis *et al*., 2007). This has stimulated a wider application of oocyte cryopreservation in response to the necessity to find alternatives to embryo freezing or make oocyte donation easier and safer. Vitrification is the alternative methodology for low temperature preservation. Births from vitrified oocytes were described initially by Kuleshova *et al*. (1999) and Katayama *et al*. (2003). Yoon *et al*. (2003) reported other births from vitrified oocytes, but the overall implantation rate per thawed oocyte (1.7%) was not superior to the one achieved with slow cooling protocols. Recently, this technology has been reported to achieve results equivalent or perhaps superior to those obtained with slow cooling (Kuwayama *et al*., 2005), raising further interest in the potential of oocyte storage. However, clinical evidence on oocyte cryopreservation remains insufficient, mainly because, with very few exceptions (Borini *et al*., 2006b; Levi Setti *et al*., 2006), the studies conducted so far have been small and based on selected cases, e.g. oocyte donation (Fosas *et al*., 2003), whose outcome may be not applicable to more wide-ranging patient populations. In general, the interpretation of the clinical outcome of IVF is not an easy task. A number of factors, such as type of infertility, female age, ovarian response to gonadotrophins, ovarian stimulation and laboratory strategies, are recognized to be critical in determining the efficiency of IVF therapy. In addition, success rates are not reported consistently and vary depending on the definition used. While there is little doubt that the ultimate therapeutic goal of IVF consists in the delivery of a healthy baby, the criteria that should be adopted to measure this standard of success remains a matter of debate (Griesinger *et al*., 2004; Messinis and Domali, 2004; Min *et al*., 2004). In view of the relevant drawbacks in terms of pharmacological (OHSS) and surgical risks, discomfort, psychological stress, cost and time, ovarian stimulation cycles should be minimized and the reproductive potential of a whole cohort of oocytes retrieved from a single recovery capitalized.

Until recently, cryopreservation at either the pronucleate or the cleavage stage has been the option that could translate into practice such principles. Embryo freezing has been available since 1983 (Trounson and Mohr, 1983). However, its contribution to IVF success has been a matter of controversy for many years. In 1995, Jones *et al*. (1995) proposed the definition of cumulative pregnancy rate as the proportion of pregnancies from fresh and frozen embryos per started cycle, discussing its value as a criterion to assess IVF success. In a rather large study, de Jong *et al*. (2002) described that the added value of embryo cryopreservation contributes only marginally (less than 2%) to the cumulative pregnancy rate (43.8%) following three successive fresh IVF cycles. In reality, this conclusion has remained rather isolated. Several other studies emphasized that embryo cryopreservation contributes greatly to the cumulative pregnancy rate. In a large cohort of patients, Ubaldi *et al*. (2004) reported a fresh pregnancy rate of 47% in patients younger than 38 years. Remarkably, after transfer of frozen–thawed embryos the cumulative pregnancy rate was 74%. A benefit from the storage and transfer of frozen embryos was also observed in patients older than 38 years, in which case these rates were 35% and 46%, respectively. It should be said, though, that in this study thawed embryos were subjected to micromanipulation to remove lysed blastomeres before transfer. It has been suggested that embryo freezing also contributes significantly to increase the overall clinical outcome per started cycle in single embryo transfer treatments. In 127 elective single embryo transfers, Tiitinen *et al*. (2001) achieved a pregnancy rate of 38.6%. Replacement of one frozen embryo in 46 transfers and two frozen embryos in 83 transfers led to pregnancy rates of 17.4% and 37.3% and a cumulative pregnancy rate of 52.8%. The goal of maximizing the chances of pregnancy per started cycle can be pursued also by cryopreserving surplus material at the pronucleate stage. In 261 stimulation cycles, Damario *et al*. (2000) adopted the policy of culturing beyond day 1 only the number of fertilized oocytes supposed to be appropriate for a fresh embryo transfer, while cryopreserving and using the others in successive thawing cycles. The rate of liveborn delivery occurring from oocytes derived from a single recovery was 59.7% in patients younger than 39 years, while it dropped to 18.5% in older patients. Overall, these experiences indicate that embryo/zygote cryopreservation is a very successful strategy for improving IVF efficiency.

Unfortunately, these forms of preservation are not applicable in Italy, because of the veto of inseminating more than three oocytes per treatment cycle and freezing surplus embryos, as decreed by the IVF law introduced in 2004. The authors have adopted oocyte cryopreservation as a measure which could surrogate embryo cryopreservation in the endeavour to offer our patients the best chances of pregnancy per started cycle. In fact, last year the authors published a set of data concerning the cumulative pregnancy rate in cycles in which only three or four fresh oocytes were inseminated and the others were frozen for later use (Borini *et al*., 2006a). The cumulative success rate was 47%. However, having been conducted on a limited number of cycles ($n = 80$), this study could not be considered conclusive. Here, the authors adopted the same policy on a much larger group of patients ($n = 749$) whose selection criterion depended essentially on the possibility of freezing at least three surplus oocytes. Such a size sample clearly makes the present data more reliable. The fresh pregnancy rate was 35.6%, which appears a more than acceptable result, considering that no more than three oocytes were inseminated. Frozen cycles were conducted in order to obtain three viable oocytes after thawing, although this was not possible in all cases because some of the oocytes had been frozen in groups rather than individually. After thawing more than 3200 oocytes, the survival rate approached 70%, confirming that recent slow cooling protocols (including those adopted in this study) have improved the yield of thawed oocytes suitable for treatment. Fertilization and cleavage rates were over 76% and 91%, respectively, i.e. indistinguishable from those normally obtained in our unit with fresh oocytes. The number of embryo transfers corresponded to about 90% of the thawing cycles because of a limited number of failures at the fertilization or cleavage stage. This can be accounted for, at least in part, by the fact that also in frozen cycles no more than three oocytes were inseminated. The implantation rate remained suboptimal (8%). This percentage is rather

lower than the result achieved with fresh oocytes (21%), a fact which may well reflect the possibility that the cryopreservation protocols used may to some extent affect the overall oocyte developmental ability. However, it should be considered that the more morphologically normal oocytes were assigned to the fresh treatments. Irrespective of the possible imperfection of the selection criterion applied, this may have discriminated fresh and frozen oocytes in populations not exactly equivalent. Also, prior to ICSI the overall pre- and post-freezing culture time of the cohort of stored oocytes was 6–7 h. As a consequence, insemination was carried out at 42–43 h after HCG. This time is somewhat later than the optimal time for ICSI (Dozortsev *et al*., 2004) and may have reduced the developmental potential of frozen–thawed oocytes. Pregnancy rate per transfer was over 17%, with an abortion rate of 21%. The rate of cumulative pregnancy (over 47%) confirms the outcome of our previous study (Borini *et al*., 2006a) and above all indicates that oocyte cryopreservation increases significantly the chances of clinical success per started cycle. It should be considered that these data do not include the oocytes that remained frozen (about 40% of the original amount of the stored material), whose reproductive potential in principle could raise the final clinical success rate to well over 50%.

The authors are aware that further efforts need to be made in order to improve the developmental ability of cryopreserved oocytes. Our most recent data appear to confirm that the viability of frozen oocytes may be preserved with improved efficiency (Bianchi *et al*., 2007). Irrespective of future advances in the field of basic cryobiology that are needed, the authors believe that the storage of unfertilized oocytes already offers an alternative to more traditional forms of cryopreservation. In fact, our cumulative success rate is not very dissimilar from the one reported for elective single embryo transfer treatments (Tiitinen *et al*., 2001). In addition, despite the high success rate reported by some authors, cumulative pregnancy rates may not reach 40% in cycles where supernumerary pronucleate eggs were frozen (Schroder *et al*., 2003). This is less than the rate obtained in our experience with frozen oocytes. IVF specialists should feel encouraged to pursue oocyte cryopreservation as a form of IVF treatment, as this can contribute considerably to the overall clinical success, circumventing the ethical and legal complications that may occur when frozen embryos remain unused after the achievement of a fresh pregnancy. In particular ,Italian patients can benefit from oocyte cryopreservation, without facing the financial, practical and psychological burdens derived from seeking treatment in countries where embryo freezing is permitted.

Declaration

Tecnobios Procreazione holds a commercial relationship with Cook Australia for the oocyte freezing and thawing culture media used, in part, in this work.

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