Clinical experiences of ICSI-ET thawing cycles with embryos cryopreserved at different developmental stages

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Purpose: The aim of our study was to analyze factors including survival, implantation and pregnancy rate, patients' age and BMI, abortions and extra uterine pregnancies that might influence the outcome of ICSI-ET thawing cycles.

Methods: A total of 147 cycles with embryos cryopreserved at different developmental stages were retrospectively evaluated.

Results: No difference was found in the survival, implantation and pregnancy rates of embryos cryopreserved on Day 2–3 and 5. However, in the pregnant group significantly higher implantation rate was observed with Day 5 blastocysts than with Day 2 or 3 early embryos. We found no difference in the number of abortions and extra uterine pregnancies between fresh and frozen ICSI-ET cycles. Higher BMI was found in the pregnant than in the non-pregnant group. However, the age of patient had no effect on the results.

Conclusions: Developmental stage of embryo and patients' BMI influences the success of ICSI-ET thawing cycles.

KEY WORDS: Embryo cryopreservation; developmental stages; BMI; cleavage stages; blastocyst; glycerol; propanadiol.

INTRODUCTION

The use of controlled ovarian stimulation protocols led to the production of large number of human oocytes and consequently embryos. In order to avoid the risk of multiple pregnancies without discarding the embryos in excess of the number appropriate for safe transfer embryos are cryopreserved. Thus, the importance of embryo cryoconservation procedure has increased and the efforts to study the factors influencing the outcome of freezing have intensified. However, improvements in cryopreservation and cryoprotective agents have made slow progress

since the last 10 years. There is still a large debate on the best stage, protocol and cryoprotectant to freeze. The potential of a frozen stored embryo to become a living child lies in the order of 4%, and babies born from cryopreserved embryos do not represent more than 8% of the total of ART babies born in most programs (1). Until now, the preferred stages for human embryo cryopreservation were the zygote and early cleavage stages. Blastocyst freezing was abandoned for years, since 25% of zygotes only were able to reach the blastocyst stage in vitro in usual culture media and low pregnancy rates were reported (1–3). Zygote and early cleavage stage embryos are most often concerned by cryopreservation and many reports have focused on the main parameters involved in its efficacy (4–10). Recently, new embryo culture systems—such as the co-culture on feeder cells and the sequential media—have been developed allowing obtaining good blastocysts with higher rates of

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50–60% (11–14). However, the efficacy and results of blastocyst culture, cryopreservation and frozen blastocyst transfer reported are still very controversial (2,15,16). Furthermore, human blastocysts obtained using sequential media appear to be half less cryoresistant than the co-cultured ones (17–19). Recently, studies have been published focusing on micromanipulation insemination technique (ICSI) that may affect the success of in vitro blastocyst formation and freezing–thawing cycles. Lower rate of formation and quality and/or higher sensitivity of ICSI blastocysts to freezing need to be considered (16,20,21).

The aim of our retrospective study was to compare the survival, implantation and pregnancy rates (biochemical and clinical) of frozen embryos produced exclusively with ICSI and cryopreserved at different developmental stages. Other factors including woman's age and Body Mass Index (BMI), abortion and extra uterine pregnancy rates (in fresh and frozen ICSI cycles) that might influence the success of ICSI-ET thawing cycles were analyzed as well.

MATERIALS AND METHODS

Patient Characteristics, Ovarian Stimulation Protocol and Embryology Data

The study population consisted of patients who underwent ICSI-ET program between January 2003 and June 2004 and who in addition to having a fresh embryo transfer had supernumerary embryos, which were cryopreserved. Ovarian stimulation protocol, follicular aspiration, and in vitro culture and insemination (ICSI) of oocytes were performed as previously described (22). Midluteal long protocol was used and when down-regulation was confirmed by hormone determination and ultrasound (US), treatment with recombinant human follicle stimulating hormone (rhFSH Puregon, Organon) or human menopausal gonadotrophin (hMG Menogon, Ferring) was initiated. Ovulation was induced with 10,000 IU hCG (Profasi, Serono) when at least three leading follicles attained a mean diameter of 17 mm and serum E2 level increased above 1000 pg/ml. Transvaginal ultrasound-guided oocyte retrieval was performed 35–36 h following hCG administration. The quality/maturation of the obtained oocytes was evaluated than they were inseminated with ICSI. Fertilization was checked 16–18 h after insemination. Cryopreservation of supernumerary zygotes and embryos was routinely offered to all patients. All patients agreeing to embryo storage signed a specific

written consent. After thawing, all viable embryos having at least 50% of the blastomeres survived were transferred (3 embryo/patient). In case of blastocyst, the re-expansion of blastocoel was also required. The rates of embryo survival, implantation per transferred embryo and abortion were recorded for all patients. Between the pregnant and non-pregnant groups of patients the following data were compared: patients' age and BMI, survival and implantation rate of embryos frozen at different developmental stages. The biochemical and clinical pregnancy rates of different patient groups receiving day 2, 3 and 5 frozen embryos were evaluated as well. Furthermore, the number of abortions and extra uterine pregnancies were compared between fresh and frozen ICSI-ET cycles.

Embryo Freezing and Thawing Protocols

Embryos were frozen on day 2, 3 and 5 after in vitro fertilization with ICSI. Embryos on day 2 and 3 were frozen in phosphate buffered solution (PBS) supplemented with 1.5 M 1,2 propanediol (PrOH), 25 mg/mL Human Serum Albumin (HSA) and 0.1 M sucrose (S) (Freeze-Kit 1TM/Vitrolife, Sweden/). Embryos were frozen with conventional slow freezing protocol using PLANER III Kryo 10 cell freezer (Planer Products Ltd., Sunbury-on-Thames, UK). After seeding at -7°C , the embryos were slowly cooled ($0.3^{\circ}\text{C}/\text{min}$) to -30°C , than they were cooled with higher speed ($-10^{\circ}\text{C}/\text{min}$) to -80°C before plunging into liquid nitrogen (LN_2). After thawing, PrOH was removed from the cells in 3 steps with solutions containing PrOH in decreasing concentrations and 0.2 M S (Thaw-Kit 1TM/Vitrolife, Sweden/).

Blastocysts were frozen on day 5 in a PBS based solution containing 8% glycerol (G) and 25 mg/mL HAS (G-Freeze-Kit BlastTM/Vitrolife, Sweden/). Glycerol was added to the embryos in 5 steps. After seeding at -7°C , blastocysts were slowly cooled ($0.3^{\circ}\text{C}/\text{min}$) to -30°C , than they were rapidly frozen ($-15^{\circ}\text{C}/\text{min}$) to -150°C before plunging into LN_2 . Glycerol was diluted out of the cells in 8 steps in solutions containing the G in decreasing concentrations (8%, 6%, 5%, 4%, 2% and 1%) (G-Thaw-Kit BlastTM/Vitrolife, Sweden/). Finally, embryos were evaluated and transferred at different developmental stages on day 2, 3 and 5 into patients ($n = 126$) (max 3 embryos were transferred per patient).

Embryos frozen at early cleavage or blastocyst stages were thawed with the same procedure. First

air thaw was applied for 20 s followed by immersion into 30°C water bath for 20 s. After dilution out of the cryoprotectant, embryos were in vitro cultured for 1 to 1.5 h prior to quality control and embryo transfer. Only those embryos were transferred in which minimum 50% of the blastomeres survived freezing and/or the blastocoelic cavity re-expanded.

Embryo Transfer (ET)

Embryos were transferred to patients in either a natural cycle or a programmed/prepared cycle. In a natural cycle, patients were monitored with serum E2 level determination and US. When follicle bigger than 20–24 mm in diameter together with 10 mm wide endometrium was detected with US ovulation was induced with 1000 IU hCG (Profasi, Serono). Embryo transfer (max 3 embryo/patient) was performed on day 16, 17 or 19 depending on the developmental stage of embryo. In a programmed cycle, patients were prepared for ET with down-regulation starting in the mid-luteal phase of the preceding menstrual cycle. Subsequently endometrial development was achieved/supported by administration of 17 β oestradiol in increasing dose to mimic the natural cycle. Once endometrial development was confirmed by US, progesterone was administered (50 mg/day) to support the luteal phase. Pregnancy was defined as a spontaneous rise in a β hCG concentration at least 10 days posttransfer. Clinical pregnancy implied the presence of an intrauterine gestational sac and fetal heart beat on an ultrasound performed at 7 weeks of gestation.

Statistical Analyses

The efficiency of ICSI-ET thawing cycles was evaluated by survival, implantation and pregnancy rates. During the statistical analysis patient population was re-grouped according to embryo developmental stages (1:2 days, 2:3 days and 3:5 days) and gravidity (1:gravid and 2:non-gravid). In order to find differences between patient groups non-parametric statistics were applied by using comparing multiple independent samples (in case of developmental stages; Kruskal-Wallis median test) and by using two independent samples (in case of gravidity; Kolgomorov-Smirnov test). To clarify the role of BMI and patients' age, one-way analysis of variance was used. BMI and patients' age were the dependent variables and developmental stage and/or gravidity were the effects.

Table 1. Survival Rate (%) of Cryopreserved ICSI Embryos According to Developmental Stages and Gravidity

Groups	<i>N</i>	Mean	<i>SD</i>	Geometric Mean	Median
By developmental stages					
2 days	19	89.95	14.61	88.67	100
3 days	106	95.46	10.97	94.68	100
5 days	22	81.45	21.93	78.30	100
Total	147	92.68	14.39	91.28	100
Median test	$\chi^2 = 0.00$		P = 1.00		
Gravidity					
Pregnant	42	95.40	11.50	94.56	100
Nonpregnant	105	92.18	15.13	90.62	100
K-S test ^a	<i>P</i> > 0.100				

^aKolgomorov-Smirnov test.

The analyses were carried out using Statistica for Windows (23).

RESULTS

During the last 18 months, 1328 embryos were cryopreserved. Until now, 30% of the frozen embryos have been thawed. Cryopreserved embryos were transferred in a total of 147 frozen/thawed ICSI-ET cycles. No differences were found in the survival rates of embryos frozen at different developmental stages on day 2, 3 and 5 after ICSI (ICSI: day 0.) (Table I.).

A total of 66 day 2 early embryos in 2–4 cells stage were transferred into 19 patients. Clinical pregnancy was detected in five patients out of 19 (5/19; 26.3%). Five out of 66 transferred embryos implanted (implantation rate /IR/: 7.6%; 5/66). day 3 embryos frozen at 6 to 8 cell stage were transferred in 106 ICSI-ET cycles and 11 biochemical (11/106; 10.4%) and 29 clinical pregnancies (29/106; 27.3%) were achieved (Total: 40/106; 37.7%). A total of 327 day 3 cryopreserved embryos were transferred and 36 implanted out of them (IR: 11.0%; 36/327). Fifty-five day 5 frozen blastocysts were transferred in 22 ICSI-ET cycles. Out of the 22 cycles, two biochemical (2/22; 9.0%) and 8 clinical pregnancies (8/22; 36.4%) were observed. Out of the 55 frozen blastocyst 10 implanted (IR: 18.1%; 10/55).

A total of 37.4% pregnancy rate (55/147) was observed after 147 ICSI-ET thawing cycles (biochemical pregnancy: 8.8%, 13/147; "ongoing" pregnancy and delivery: 28.6%; 42/147). Comparing the clinical pregnancy results of embryos cryopreserved at different developmental stages, no difference was found (day 2: 26.3%, day 3: 27.3%, day 5: 36.4%). A total IR

Table II. Results of Frozen/Thawed ICSI-ET Cycles with Embryos Cryopreserved at Different Developmental Stages

Developmental stage (day)	ET cycles (n)	Pregnancy			
		BPR (%)	CPR (%)	Total (%)	IR (%)
2	19	—	5/19 (26.3)	5/19 (26.3)	5/66 (7.6)
3	106	11/106 (10.4)	29/106 (27.3)	40/106 (37.7)	36/327 (11.0)
5	22	2/22 (9.0)	8/22 (36.4)	10/22 (45.4)	10/55 (18.1)
Total	147	13/147 (8.8)	42/147 (28.6)	55/147 (37.4)	51/448 (11.4)

Note. ET: embryo transfer; BPR: biochemical pregnancy; CPR: clinical pregnancy (egg sac and fetal heart beat is detected with ultrasound by 7th week of pregnancy); IR: implantation rate (implanted embryos/transferred embryos.)

of 11.4% (51/448) was achieved with embryos cryopreserved on day 2, 3 and 5 (Table II).

We observed significant differences in the IR of embryos cryopreserved at different developmental stages on day 2, 3 and 5 in the group of pregnant patients ($n = 42$) ($p < 0.039$). Higher IR was found with day 5 blastocysts (49.67%) then with day 2 (28.80%) and day 3 (38.89%) embryos. The lowest IR was found after day 2 frozen early embryo transfers.

No difference was found in the number of abortion and extra uterine pregnancies (9.2% vs. 6.8%; 1.5% vs. 1.7%) between fresh and frozen/thawed ICSI-ET cycles carried out in the same period. We found no effect of patients' age to the outcome of frozen/thawed ICSI-ET cycles (Table III). However, comparing the BMI of pregnant and nonpregnant patients, we found significantly higher average BMI value in the pregnant than in the non-pregnant group ($p < 0.029$) (Table IV).

DISCUSSION

The ability to cryopreserve the supernumerary embryos and obtain viable pregnancies after thawing and ET serves as an excellent solution to the problem of extra/surplus embryos and increases the over-

all pregnancy rate from one oocyte retrieval attempt. The increasing importance of embryo cryopreservation in the clinical use of IVF-ET intensified the research connected with different factors that may influence the results of embryo cryopreservation.

Several comparative reports have been published in recent years regarding the freezeability of embryos generated by conventional IVF or ICSI (19–21). The issue is very important since 1) the infertile population applying for a treatment by IVF and the indications for performing ICSI has been changed, and 2) the number of patients undergoing ICSI procedure and ICSI-ET cycles increasing rapidly. Nowadays, ICSI is performed not only for male factor infertility but also in many cases of conventional IVF failure. Lahav-Baratz *et al.* (2003) reported that the frozen/thawed ICSI-ET and IVF-ET cycles should be evaluated separately because not all the factors influencing the success rate in traditional IVF-ET cycles are valid in the ICSI-ET cycles (26). The retrospective analyses of conventional IVF-ET cycles indicated that age of patients, number and age (developmental stage) of transferred embryos affected to the pregnancy results. However, in case of ICSI-ET cycles only the number of transferred embryos had effect on pregnancy rate (21,24,25). Our results are partly in contrast with the previously reported

Table III. Body Mass Index (BMI) According to Developmental Stage and Pregnancy

Effect	N	Mean	SD
Developmental stage			
Day 2	19	26.1	8.89
Day 3	106	24.9	5.64
Day 5	22	25.3	5.98
Anova		$f = 0.301$	$p = 0.741$
Gravid	42	26.9	7.23
Nongravid	105	24.4	5.60
Anova		$f = 4.874$	$p = 0.029$

Table IV. Age of Patients According to Developmental Stage and Pregnancy

Effect	N	Mean	SD
Developmental stage			
Day 2	19	31.2	4.22
Day 3	106	33.1	5.69
Day 5	22	31.0	3.32
Anova		$f = 2.118$	$p = 0.124$
Gravid	42	31.4	4.08
Nongravid	105	32.9	5.62
Anova		$f = 2.320$	$p = 0.130$

data. In the present study, we retrospectively analyzed the results of 147 ICSI-ET thawing cycles. We found no difference in the survival and clinical pregnancy rate achieved with embryos frozen/thawed and transferred on day 2, 3 and 5. However, we obtained significant difference in the implantation rates of embryos with different developmental stages in the pregnant group of patients. We found that more day 5 blastocyst implanted than day 2 or day 3 embryo. Our data indicate that developmental stage and age of embryos at freezing affect to the outcome of ICSI-ET thawing cycles. Our ET and IR results are in agreement of others' observations who reported 27–34% clinical pregnancy and 12–16% implantation rate with blastocysts (13,14,19, 25,26).

Simon *et al.* (1998), comparing the pregnancy rates of frozen embryos originating from traditional IVF and ICSI, found higher pregnancy rate after transferring frozen/thawed traditional IVF embryos (32.5% vs. 20%)(24). They explained the difference with the better quality of IVF embryos. Their conclusion was that embryo quality at cryopreservation is one of the most important factor determining survival and pregnancy rate. In our study, working exclusively with frozen/thawed ICSI embryos we obtained similarly high clinical pregnancy rates in all developmental stages indicating that the quality of ICSI embryos are as good as embryos generated by conventional IVF. No difference was found in the number of abortions and extra uterine pregnancies between fresh and frozen ICSI-ET cycles (22,27,25)

We know very little how extremes of weight are associated with low rate of pregnancy in women receiving assisted reproductive treatment and there are discrepancies in the results published in the literature (28–31). Wang *et al.* (2000) examined whether BMI is associated with reduced fecundity in women receiving assisted reproductive treatment (33). They observed that the fecundity of the moderate group was almost 60% higher than that of the very obese group, and the fecundity of the underweight group (BMI: <20) was also significantly lower than that of the moderate group ($p < 0.05$), indicating an “inverted U” relation between BMI and fecundity. However, in a recently published study, Wattanakumtornkul *et al.* (2003) reported no relationship between BMI and success of assisted reproductive treatments (36). Our results support the observation of Wang *et al.*, (2000) indicating that both the high (BMI: >25.0) and low

(BMI: <20.0) BMI has negative effect to the success of assisted reproductive procedures. We found that BMI of non-pregnant group is significantly lower than that of the pregnant group showing the tendency that in case of low BMI the chance to achieve success with assisted reproductive treatment decreases.

In summary, our results indicate that developmental stage of embryos at cryopreservation and BMI of patients influence the outcome of ICSI-ET thawing cycles and cryopreserved ICSI embryos can be used with good success in ET cycles.

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