Analyzing Factors Affecting the Success Rate of Frozen–Thawed Embryos

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Purpose: In recent years the infertile population applying for IVF treatments was changed and so the indications for performing intracellular sperm injection (ICSI). The aim of this study was to analyze predicting factors of our thawing cycles.

Methods: From December 1998 to July 2001, 440 consecutive thawing cycles were performed. Patient characteristics were examined. The number of cryopreserved embryos, number of transferred embryos, the timing of cryopreservation (48 h vs. 72 h), and embryo survival rate were analyzed as a possible predictor for pregnancies achievement.

Results: Conventional IVF patient's characteristic was significantly different from ICSI population and analysis has been performed for every population separately. In the IVF population the women age, the number of transferred embryos, and timing of cryopreservation were factors significantly influencing the pregnancy rate. Interestingly, in the ICSI population only the number of transferred embryos was found to be a predictive factor.

Conclusion: ICSI and IVF cycles should be analyzed separately. Not all the factors influencing the success rate in the conventional IVF population are valid in the ICSI population.

KEY WORDS: Cryopreservation; embryos; in vitro fertilization; predictive factors.

INTRODUCTION

During the past few years, efforts to study the factors influencing embryo cryopreservation outcome have intensified. Ovulation induction produces many matured oocytes and embryos. Most IVF centers aim to reduce the number of transferred embryos in order to avoid multiple pregnancies. This has prompted further investigation and improvement of cryopreservation techniques. The first pregnancy from frozen–thawed human embryo was achieved in 1983 (1). Since then, many IVF laboratories have tried to optimize the process, using diverse protocols and cryoprotec-

tants (2,3). Cryopreservation is performed for embryos of varying quality and at different stages of development (4,5). However, in parallel to this progression, the infertile population applying for treatments by IVF has changed and the indications for performing ICSI as well. ICSI procedure is used now in many cases of conventional IVF failure and not only for male factor infertility. The aims of this study were to characterize the IVF and ICSI population in our IVF program. Accordingly, the predictive factors affecting the success rate of frozen–thawed cycles for every population were examined. The parameters analyzed were women's age, the number of preserved embryos as an index to the success in the original cycle, embryo's stage at cryopreservation, embryo survival rate, number of transferred embryos, and embryos quality after thawing. Outcome measures were pregnancy, implantation rates, and delivery rates.

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MATERIALS AND METHODS

Patients

In this study, we retrospectively performed 440 consecutive thawing cycles in between December 1998 and July 2001.

In their fresh IVF and/or ICSI cycles, patients were treated by gonadotrophin-releasing hormone analogue (GnRHa), and Buseraline (Suprefact®) nasal spray (Hoechst, Frankfurt, Germany) (600 µg/day) continued up to human chorionic gonadotrophin (HCG) administration. When laboratory testing indicated pituitary suppression (oestradiol < 40 pg/mL), 3 ampoules per day of human menopausal gonadotrophin (HMG) were administered, until three or more follicles of >18 mm mean diameter were present on transvaginal ultrasound (TVS). Follicular monitoring by serum oestradiol, luteinizing hormone (LH), progesterone, and serial transvaginal ultrasound (TVS) scan were performed as previously described (6). Patients then received 5000 IU of HCG i.m. 35–36 h before the scheduled TVS-guided follicular aspiration.

In vitro fertilization technique was applied as previously described (7). ICSI procedure was carried out according to Van Steirteghem *et al.* (8) on the Nikon microscope by using Nomarski modulation optics with two three-dimensional manipulators (Narishige, Tokyo, Japan) with modifications as previously described (9). Fertilization was confirmed 16–20 h postinsemination by the presence of two distinct pronuclei under inverted microscope. Cleavage was assessed 24 or 72 h later, and the embryos were classified according to their morphological appearance. In 75% of the cases, up to three thawed embryos were selected for replacement into the uterine cavity. In 110 cases (25%), four embryos were transferred and in 24 selected cases (5.4%), five embryos were transferred. Most of the thawed embryos were replaced following artificial preparation of the endometrium with Estrogen and Luteal supplementation consisted of Gestone in oil (Gestone, Paines & Byrne, Surrey, U.K.), 100 mg/day i.m. Few cycles, either IVF and ICSI cycles, were natural cycles without any endometrial preparation.

Embryo Freezing and Thawing Protocols

Embryos were cryopreserved 48 or 72 h after ovum pickup (i.e., two- to eight-cell stage). Embryos were scored before and after freezing, on the basis of blas-

tomere symmetry, the presence of enucleated fragments, the brightness and texture of the blastomere cytoplasm, and number of blastomers. Three types of embryos were considered for freezing: type I embryos had regular blastomers with no fragmentation and 3–4 cells at 48 h/7–8 cells at 72 h; type II embryos had irregularity and symmetry of blastomers or up to 20% fragmentation or 2 cells at 48 h/5–6 cells at 72 h; and type III embryos had up to 50% fragmentation. Embryos with more than 50% fragmentation or with only 2–4 cells at 72-h embryo were not eligible for freezing. The decision to cryopreserved embryos 48 or 72 h post insemination was randomized according to the day of the week.

Cryopreservation was performed using a slow freezing protocol in a programmed biological freezer (Planer Kryo II; Planer Products Ltd., England). Freezing and thawing were performed using the Medi Cult 1,2 Propandiol and Sucrose kit. Embryos were cryopreserved in cryotubes (nunc, Roskilde, Denmark) containing 0.25 mL of freezing medium. Temperature was lowered from room temperature at a rate of $-2^{\circ}\text{C}/\text{min}$ until a temperature of -7.5°C was reached. Manual seeding was performed after 10 min at this temperature. Seeding was performed as previously described (10). After seeding, the tubes were held at -7.5°C for another 15 min before the temperature was lowered to -30°C at a rate of $-0.3^{\circ}\text{C}/\text{min}$. Finally, the temperature was lowered at the rate of $-10^{\circ}\text{C}/\text{min}$, until a temperature of -130°C was reached. At this stage, cryotubes were transferred to a marked canister and into a liquid nitrogen container to be kept until thawing.

Thawing of embryos was performed by removing the tube from the liquid nitrogen, placing it at room temperature for 1 min, and then bringing it to complete thawing in a water bath set at 37°C . Embryos were transferred through a series of decreasing concentration mediums, as instructed by the Medi-Cult kit. Finally, the embryos were placed in a fresh, equilibrated, warmed culture medium, ready for transfer. Embryo survival rate was calculated as the number of transferred embryos per number of embryos that have been thawed.

Statistical Analysis

Since in every cycle, embryos with various qualities were transferred, it was a dilemma how to estimate the embryo quality for every thawing cycle. We had determined weighted mean of embryo quality according to the function: $\text{Quality} = 3 \times \text{number of high embryos}$

+2× intermediate +1× low: number of transferred embryo.

The quality score includes I for high-quality, II for intermediate, and III for low-quality embryos. Laboratory and clinical outcomes of the thawing cycles were compared among the various groups. Results were analyzed using *t* test and chi-square test for univariate analysis. Multiple logistic regression analysis was used for multivariate analysis. The association between embryo quality and the number of transferred embryo was examined using Kendall's τ -*b* test.

RESULTS

Patients' population of the thawed embryos from ICSI cycle was significantly different from patient population treated by conventional IVF (Table I). IVF patients were older; nevertheless, they had more embryos for cryopreservation, more embryos for transfer but lower survival rate of thawed embryos.

During the period evaluated, 440 consecutive thawing cycles were performed. One thousand five hundred and sixty embryos were cryopreserved, and 1339 embryos were transferred (85.8% survival rate for the embryos in the study). Ninety-eight clinical pregnancies (22.3%) were achieved. Forty-five singleton and 12 twins were delivered which is a "Take-home-baby" rate of 12.9%.

Analyzing the factors that may affect the success rate of thawing cycle derived from conventional IVF revealed that the women's age, number of transferred embryos, and age of embryos at cryopreservation were found to be significant factors affecting the success rate (Table II). The age of the embryos at cryopreservation was found to be in correlation with implantation rate too. These variables remain significant also when we used a logistic regression analysis model. Only the number of transferred embryos was

Table I. Patients' Characteristics

	IVF	ICSI	<i>P</i>
No. of cycles	129	311	
Age of women patients (mean ± SD)	32.6 ± 5.2	30.9 ± 5.2	0.002
No. of frozen embryos (mean ± SD)	4.0 ± 1.5	3.3 ± 1.1	0.001
No. of transferred embryos (mean ± SD)	3.3 ± 1.0	2.9 ± 0.9	0.001
Survival rate (mean ± SD)	0.85 ± 0.18	0.90 ± 0.18	0.01

Table II. Factors Associated with Conventional IVF Treatment Success

	Success	Failure	<i>P</i>
No. of cycles	36 (27.9%)	93	
Age of women	31.0 ± 5.48	33.0 ± 5.07	0.02
No. of frozen embryos (mean ± SD)	4.3 ± 1.19	4.0 ± 1.65	ns
No. of transferred embryos (mean ± SD)	3.7 ± 0.97	3.1 ± 1.02	0.009
Survival rate (mean ± SD)	0.88 ± 0.14	0.84 ± 0.19	ns
Age of embryos at cryopreservation			
PR			
48 h	16/88 (18.2%)		
72 h	20/41 (48.8%)		0.001
IR			
48 h	18/290 (6.2%)		
72 h	22/141 (15.6%)		0.002

Note. ns = not significant; PR = pregnancy rate; IR = implantation rate.

found to be a factor that may influence the success rate of thawed embryos generated from an ICSI cycles (Table III). In ICSI cycles, the women age, the number of cryopreserved embryos, the age of embryos at cryopreservation, and the embryos survival rate were not found to be predictors for pregnancy achievement (Table III). Embryo quality (either from IVF or from ICSI) at 48 h or at 72 h were found to be similar. The number of transferred embryos found to be a factor affecting the success rate of thawing cycles in both populations—IVF and ICSI.

We analyzed this factor also as a categorical parameter. We divided the transferred embryos into groups of 1–2 embryos, 3 embryos, and 4 or more. Table IV

Table III. Factors Associated With ICSI Treatment Success

	Success	Failure	<i>P</i>
No. of cycles	72 (19.9%)	249 (80.1%)	
Age of women	29.9 ± 5.66	31.1 ± 5.58	ns
No. of frozen embryos (mean ± SD)	3.5 ± 0.94	3.3 ± 1.22	ns
No. of transferred embryos (mean ± SD)	3.2 ± 0.89	2.8 ± 0.97	0.009
Survival rate (mean ± SD)	0.92 ± 0.15	0.90 ± 0.19	ns
Age of embryos at cryopreservation			
PR			
48 h	46/221 (20.8%)		
72 h	16/90 (17.8%)		ns
IR			
48 h	48/659 (7.2%)		
72 h	19/249 (7.6%)		ns

Note. ns = not significant; PR = pregnancy rate; IR = implantation rate.

Table IV. Embryo Quality According to Number of Transferred Embryos

Quality	1–2 Embryos	3 Embryos	4+ Embryos	total
IVF				
I	5 (17.9%)	14 (31.1%)	4 (7.1%)	23 (17.8%)
II	14 (50.0%)	28 (62.2%)	43 (76.8%)	85 (65.9%)
III	9 (32.1%)	3 (6.7%)	9 (16.1%)	21 (16.3%)
ICSI				
I	30 (26.1%)	35 (33%)	19 (21.1%)	84 (27.0%)
II	62 (53.9%)	64 (60.4%)	50 (55.6%)	176 (56.6%)
III	23 (20.0%)	7 (6.6%)	21 (23.3%)	51 (16.4%)

Note. Differences between embryo quality according to number of transferred embryo are not significant. Embryo-types I for the best while III is for the poorer.

demonstrates that in every group of transferred embryos, their quality is similar. Embryos quality is similar also between ICSI and IVF either at 48-h embryos or at 72-h embryos (data are not shown). The data in Table V demonstrates the distribution of pregnancies and embryos quality between the different groups of transferred embryos. By transferring three embryos, the chance for pregnancy is significantly higher compared with transfer of 1–2 embryo for both populations (Table V).

DISCUSSION

Some retrospective studies were published comparing factors that may affect freezing–thawing IVF cycles including the influence of micromanipulation procedure on the success rate (10,11). However, the proportion of patients undergoing ICSI procedure has grown and patient's characteristics have changed. ICSI is often performed not only for male factor but

also for conventional IVF failure. The above differences in patients' population led us to analyze IVF cycles separately from ICSI cycles. According to that finding we are not comparing success rate of frozen–thawed embryos generated of ICSI vs. IVF, rather examines every population separately. We found that the number of transferred embryos significantly influenced pregnancy rates in IVF-thawing cycles as well as in ICSI-thawing cycles. The transfer of three embryos resulted in significantly higher pregnancy rates than did the transfer of one or two embryos. In the ICSI population the number of transferred embryos significantly influenced also the implantation rate. This finding is in agreement of some other works (12,13) but it is in contradiction with the policy of many IVF units that limits the number of transferred embryos even in frozen–thawed embryo's cycles to reduce the number of multiple pregnancies. The decision of how many embryos to transfer in a thawing cycle may differ from the fresh cycle and depends on the results of cryopreservation in each individual center and on clinical and social considerations. Several studies were conducted to predict the thawing cycle outcome on the basis of the successful implantation of the original IVF cycle (14,15). The number of frozen embryos and the embryos' survival rate can serve as an index for a successful cycle. These however were not found to be significant factors influencing pregnancy rates in our both populations.

In IVF cycles the age of the patients and the age of the embryos at cryopreservation were found to be predictors of success. The influence of women age in this population is in agreement with other works (16,17). The age of the embryos at transfer has been reported to influence success rate in fresh cycles, given that embryonic genome is activated between the four- and eight-cell stages (18). Several studies reported that delayed selection of embryos, i.e., choosing from embryos that survived cryopreservation and continued to cleave, significantly improved the thawing cycle outcome (4,19). It is not clear yet whether delaying freezing to 72 h after insemination or letting thawed 48-h embryos to develop in culture up to 72 h provides a beneficial test of the viability of the embryos or enables further selection for embryo quality. Surprisingly, in the ICSI-thawing neither the age of the patients nor the age of embryos at cryopreservation has been found to affect the pregnancy rate or the implantation rate. The implantation rate was found to be similar in 48- and 72-h embryos. The pregnancy rate of the 72-h IVF embryos is higher than the ICSI–72 h embryos and higher than the 48-h IVF embryos

Table V. Number of Transferred Embryos in Pregnancy Cycles

	1–2 Embryos	3 Embryos	4+ Embryos	<i>P</i>
No. of pregnancies	3	13	20	
IVF cycles (%)	10.7	27.3	36.8	0.04
IR	5/54	15/132	22/245	ns
Grade I	1	6	0	
Grade II	1	7	17	
Grade III	1	0	3	
No. of pregnancies	12	26	24	
ICSI cycles (%)	10.4	24.5	26.7	0.005
IR	7/216	27/318	33/374	0.03
Grade I	5	14	7	
Grade II	6	11	15	
Grade III	1	1	2	

Note. Numbers in percentage are of all successful cycles, (36 in conventional IVF and 62 in ICSI). Embryo-types I for the best while III is the poorer. IR = implantation rate.

(48.8% vs. 17.8% and 48.8% vs. 18.2% respectively). This may be due to changes in embryo sensitivity following ICSI procedure which we cannot recognize by morphological indications.

CONCLUSION

Other parameters, such as sperm quality, may serve as predictor for the success rate of IVF-ICSI frozen-thawed embryos.

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