The Outcome of Cryopreserved Human Embryos After Intracytoplasmic Sperm Injection and Traditional IVF

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Objective: Our objective was to analyze the outcome of cryopreserved embryos obtained after intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) in terms of survival rate, implantation rate (IR), total and clinical pregnancy rate (PR) in a retrospective, comparative study.

Methods: Three hundred seventy-five IVF and 463 ICSI surnumerary cleaved embryos, frozen on Day 2 with 1,2-propanediol, were thawed.

Results: Thirty-two percent of the thawed IVF embryos survived and 11 pregnancies (8 clinical) were obtained from 68 transfers (16.1%). Forty-seven percent of the ICSI embryos survived, with 19 pregnancies (18 clinical) from 116 transfers (16.4%). The IR was 8.5% (8/94) in IVF cycles and 10.8% (20/185) in ICSI cycles.

Conclusions: A significantly better survival rate of ICSI embryos was observed but with no difference in PR, preclinical, and clinical abortion rate, or IR.

KEY WORDS: in vitro fertilization; intracytoplasmic sperm injection; cryopreservation; survival rate; pregnancy rate; implantation rate.

INTRODUCTION

Since the first live birth after transfer of embryos obtained with intracytoplasmic sperm injection (ICSI) (1), the technique has been introduced in most in vitro fertilization (IVF) programs (2–4). The higher fertilization rate achieved with this technique with respect to classical IVF (72 vs 54% at our center) produces a

large excess of embryos that should be cryopreserved. Publications concerning the outcome of cryopreserved embryos are still scarce. Several groups analyzed the outcome of cryopreserved embryos obtained after ICSI or IVF and frozen at the pronuclear stage (5,6) and early-cleaved stage (7–10). In the study by Van Steirteghem *et al.* (7), the authors did not find any difference in the survival rate between IVF and ICSI cleaved embryos, but they found a higher incidence of preclinical abortion after transfer of cryopreserved ICSI embryos compared to IVF embryos. Cattoli *et al.* (9) and Kovalik *et al.* (10) did not find any impairment in the survival and developmental potential of frozen-thawed ICSI embryos. Owens *et al.* (8) found a lower survival rate of multicellular IVF embryos with respect to ICSI embryos. This paper reports our own experience and reveals a difference in the outcome between IVF and ICSI cryopreserved embryos. The survival rate, the total and clinical pregnancy rate (PR), the clinical and preclinical abortion rate, and the implantation rate (IR), obtained by thawing 375 IVF embryos and 463 ICSI embryos, frozen and thawed during the same period, are compared.

MATERIALS AND METHODS

Patients

The study analyzed retrospectively frozen/thawed 2-day-old embryos from 118 IVF and 174 ICSI cycles, between the 15th of September 1994 and the 31st of August 1998.

Stimulation Protocol in Retrieval Cycles

Details of the ovarian stimulation protocol using gonadotropin releasing hormone analogue (buserelin

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acetate; Suprefact spray; Hoechst Inc., Frankfurt, Germany), human menopausal gonadotropin [hMG; Humegon (Organon Inc., Oss, The Netherlands) and Pergonal (Serono Inc., Aubonne, Switzerland)], and human chorionic gonadotropin [hCG; Pregnyl (Organon Inc.) and Profasi (Serono Inc.)] oocyte retrieval through vaginal puncture under ultrasound guidance, and transcervical replacement have been described elsewhere (4).

In Vitro Oocyte Culture and Preparation

In IVF cycles, collected oocytes were put in culture in modified Earl's balanced salt solution (mEBSS), supplemented with 0.5% human albumin (Belgian Red Cross), in a 30- μ l drop covered with mineral oil (Sigma, St. Louis, MO) and incubated at 37°C in a 5% CO₂, 5% O₂, 90% N₂ atmosphere before the insemination. After 3 or 4 hr the husband's pretreated sperm was added to a concentration of between 100,000 and 500,000/ml. In ICSI cycles, the cumulus and corona radiata were removed immediately after oocyte retrieval by incubation in Hepes-buffered mEBSS containing 60 to 80 IU/ml hyaluronidase (Type VIII; Sigma) for a time period of between 30 sec and 1 min. The oocytes were then observed under an inverted microscope to assess the presence of the first polar body (metaphase II oocytes), then they were incubated again for 3 to 4 hr and ICSI was carried out on all the metaphase II oocytes. After the ICSI procedure the oocytes were put in culture under the same conditions as the IVF oocytes.

Assessment of Fertilization and Embryo Score

The presence of two pronuclei was controlled 18 hr after insemination. Oocytes were considered to be fertilized if they exhibited two clear pronuclei. Precisely 42 hr after injection the cleavage stage was recorded and a score was given to the embryos. The scoring scale is based on the presence of anucleated cytoplasmic fragments as well as the regularity and the number of blastomeres, with a maximum of 6 points per embryo (11). Up to three embryos were replaced.

Freezing and Thawing Procedure

On the day of transfer (about 45 hr after oocyte retrieval), supernumerary embryos with regular-shaped blastomeres and fragments less than one-third of the total volume (minimum score, 3 for <4-cell

embryos and 4 for $\geq$ 4-cell embryos), were frozen with 1,2-propanediol (PROH), following the protocol described by Testart *et al.* (12). Briefly the embryos were placed in a freezing solution containing 1.5 M PROH (Sigma) in Hepes-buffered mEBSS for 30 min at room temperature, then they were transferred into the same solution with 0.1 M sucrose (Sigma) and loaded into plastic straws (100 μ l; IMV L'Aigle, France) within 10 min.

The freezing process was done in a Planer Kryo 10 machine (Planer Products, Sunbury-on-Thames, UK) programmed for the following curve:

- (a) from 25 to -7°C at a rate of -2°C/min;
- (b) 1 min of soaking after manual seeding at -7°C;
- (c) from -7 to -35°C, at -0.3°C/min;
- (d) from -35 to -150°C, at -45°C/min; and
- (e) 1 hr at -150°C.

Then the straws were plunged into liquid nitrogen.

The thawing procedure was performed by quickly removing the straws from the liquid nitrogen, holding them in the air for 30 sec and then in a water bath at 30°C for 4–5 sec and expelling their contents in the first thawing solution, containing 1.0 M PROH and 0.2 M sucrose. After 10 min the embryos were transferred to 0.5 M PROH and 0.2 M sucrose, to 0.2 M sucrose alone, and then to Hepes mEBSS, for an interval of 10 min in each one. Finally, the embryos were put into culture in mEBSS at 37°C in 5% O₂, 5% CO₂, 90% N₂ atmosphere until the moment of transfer. We considered that an embryo survived the thaw if at least 50% of the blastomeres were intact.

Replacement Cycles

Two days after spontaneous ovulation or ovulation induction [hCG; Pregnyl (Organon Inc.) and Profasi (Serono Inc.)] in stimulated cycles with clomiphene citrate (Clomid; Hoechst Marion Roussel, Brussels, Belgium), the thawed embryos were replaced in the uterus. Pregnancies were diagnosed by at least two positive hCG measurements (>1 mIU/ml) (Behring Inc., Germany) 3 days apart, the first at day 11 after oocyte retrieval. A clinical pregnancy was defined by the presence of a fetal sac at 5 weeks after oocyte retrieval or by villosity being present in the miscarriage material.

Statistical Analysis

Statistical analysis was done using the χ^2 test and Student *t* test. Statistical significance was defined as *P* < 0.05.

Table I. Outcome After Thawing of IVF and ICSI Embryos (Figures in Parentheses Are Percentages)

	IVF	ICSI
No. thawing cycles	118	174
No. survived embryos/No. thawed embryos	120/375 (32)*	220/463 (47)*
No. survived blastomeres/No. thawed blastomeres	381/1325 (29)**	644/1642 (39)**
No. cleaved embryos/No. survived embryos	38/64 (59)	115/160 (72)

* The survival rate of embryos was significantly different in the IVF and ICSI groups ($P < 0.05$).

** The number of intact blastomeres after thawing was significantly different in IVF and ICSI embryos ($P < 0.0001$).

RESULTS

During the 44-month period 3540 IVF and 4212 ICSI embryos were produced. Of these embryos, 35% (1263) and 36% (1529), respectively, were transferred at day 2 and 24% (558) and 30% (809), respectively, were frozen on the same day, approximately 45 hr after insemination. In the IVF group 375 embryos and in the ICSI group 463 embryos were thawed and 120 (32%) and 220 (47%), respectively (Table I), survived ($P < 0.05$). The number of intact blastomeres after thawing was 381 of 1325 (29%) for IVF embryos and 644 of 1642 (39%) for ICSI embryos ($P < 0.0001$). As shown in Table II there was no difference between IVF and ICSI in the average cell number or the average score of embryos thawed. Table III compares the IVF

Table II. Frozen-Thawed Embryo Characteristics in IVF and ICSI Cycles^a

	IVF	ICSI
Average score $\pm$ SD	3.9 $\pm$ 0.5	3.9 $\pm$ 0.5
Average cell number $\pm$ SD	3.4 $\pm$ 1.0	3.2 $\pm$ 1.2

^a No significant differences were observed in the average embryo score or the average number of cells between the IVF and the ICSI frozen-thawed embryos. SD, standard deviation.

Table III. Survival Rate of ICSI and IVF Embryos at Different Cleavage Stages (Figures in Parentheses Are Percentages)

	IVF	ICSI
2-cell stage	34/99 (34)**	91/151 (60)*, **
4-cell stage	73/216 (33.7)***	106/235 (45)*, ***

* The survival rate of ICSI embryos was significantly different between two-cell- and four-cell-stage embryos ($P < 0.005$).

** The survival rate of two-cell-stage embryos was significantly different between IVF and ICSI embryos ($P < 0.0001$).

*** The survival rate of four-cell-stage embryos was statistically different between ICSI and IVF embryos ($P < 0.02$).

and ICSI embryos at the two-cell and four-cell stage. A better survival rate of the two-cell- and four-cell-stage embryos in the ICSI group (60 and 45%, respectively) appeared with respect to the IVF group (34 and 33.7%, respectively), and the difference was statistically significant ($P < 0.05$).

In 118 IVF thawing cycles, 68 had replaced embryos (58%), and of 174 ICSI thawing cycles, 116 resulted in a replacement (67%) (Table IV). The total pregnancy rate (PR) per transfer was 16.1% (11/68) in IVF cycles and 16.4% (19/116) in ICSI cycles, with an average number of transferred embryos (1.6 ± 0.6 and 1.8 ± 0.6 , respectively) that was not statistically different (Student's *t*). The clinical PR was 11.7% in the IVF cycles (8/68) and 15.5% (18/116) in the ICSI cycles. We had 3 preclinical losses in IVF (27.3%) and 1 in ICSI (5.0%) and 3 miscarriages of 13 term pregnancies (23%) (5 are still ongoing) in the ICSI group. No clinical miscarriages were observed in the IVF group, where six patients delivered and two pregnancies are still ongoing. The implantation rate (IR) was 8.5% (8/94) in IVF patients and 10.8% (20/185) in ICSI patients.

DISCUSSION

The difference in the survival rate of frozen/thawed embryos observed here could probably be the result of several factors, like the age of the patients, the embryo quality, and the type of fertilization technique employed. Our ICSI patients are younger (34 ± 0.4 in the IVF group and 31 ± 0.4 in the ICSI group) and

Table IV. Thawing Activity After IVF and ICSI Cycles (Figures in Parentheses Are Percentages)^a

	IVF	ICSI
No. thawing cycles	118	174
No. cycles with replaced embryos	68 (58)	116 (67)
Average No. embryos transferred $\pm$ SD	1.6 $\pm$ 0.6	1.8 $\pm$ 0.6
No. pregnancies	11	19
No. clinical pregnancies	8/68 (11.7)	18/116 (15.5)
Pregnancy rate per embryo transfer	11/68 (16.1)	19/116 (16.4)
Pregnancy rate per cycle	11/118 (9.3)	19/174 (10.9)
No. sacs	8	20
Implantation rate	8/94 (8.5)	20/185 (10.8)

^a No statistical differences were observed in implantation or total and clinical pregnancy rates after transfer of IVF and ICSI frozen-thawed embryos. No statistical differences were observed in the average number of embryos transferred in IVF and ICSI cycles.

the difference is statistically significant (Student's *t*, $P < 0.001$). An impairment of PR and IR in frozen cycles of >40-year-old women was described previously but the same impairment was not observed in the survival rate of frozen/thawed embryos of older women (13,14). An influence of the cleavage stage and embryo grade of cryopreserved embryos on the survival rate after thawing has been described (15,16), but as shown in Table II we did not observe such a difference in the average cell number and the embryo quality that otherwise could explain our data. Biochemical and physical changes in cells during the fertilization process may explain our results. Zona pellucida (ZP) hardening, a process linked to the mechanism of blockage of polyspermy, probably mediated by an oocyte-type plasminogen activator (17–21); the development of free oxygen radicals, especially at a high concentration of spermatozoa (22,23), which can peroxidate polyunsaturated fatty acids in the cell membrane, causing a reduction in flexibility and fluidity; and all the molecular changes in the ultrastructure of the oocyte following fertilization can be involved in influencing the reaction to the cryoprotectant. A comparison of the survival rate of frozen–thawed embryos obtained after SUZI and classical IVF was made by Obasaju *et al.* (24), and a slightly, but not significantly, lower survival rate was observed in cryopreserved embryos after classical IVF. Unfortunately a small series (38 SUZI and 104 IVF embryos) was analyzed. Other authors compared the survival rate after thawing embryos frozen after IVF or ICSI (7–10). In one case (8) the survival rate of IVF embryos was lower; in the other three (7,9,10) no differences were observed. The observed discrepancies from our results could be the consequences of different selection criteria for the embryos to be frozen or differences in the freezing–thawing protocols used. In the Van Steirteghem *et al.* report (2), for example, DMSO was the cryoprotectant used.

The total PR per transfer and the IR for the IVF and ICSI embryos were not different. A higher clinical PR was observed in the ICSI group with respect to the IVF group, but the difference was not significant. Three preclinical miscarriages of 11 positive hCG in IVF patients and 1 of 19 in ICSI patients were observed, in contradiction with the Van Steirteghem *et al.* (2), where a higher preclinical abortion rate was observed in cryopreserved ICSI embryos with respect to IVF embryos. No clinical miscarriages were observed in the IVF patients and 3 preclinical abortions of 13 were observed in the ICSI group, corresponding to 23%. This percentage of clinical abortions does not

exceed the pregnancy loss rate of 42% reported in the study by Miller *et al.* (25) for fertile women after normal conception; this confirms the observations recently presented by Kovalik *et al.* (10).

In our hands, the cryopreservation of embryos achieved after fertilization by the ICSI technique does not impair the developmental potential of the embryos with respect to the IVF embryos. Despite the significantly higher survival rate of the frozen–thawed ICSI embryos, the pregnancy rate per cycle in IVF and ICSI groups is not different.

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