

Comparison of the Cryopreservation of Human Embryos Obtained After Intracytoplasmic Sperm Injection with a Slow Cooling or an Ultrarapid Cooling Procedure

A. L. MAURI,¹ C. G. PETERSEN,¹ R. L. R. BARUFFI,¹ R. C. FERREIRA,¹ and J. G. FRANCO, Jr.¹⁻³

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Purpose: Our purpose was to compare an ultrarapid method (URM) modified with dimethyl sulfoxide (Me₂SO) to a slow method (SM) with propanediol (PROH) for the cryopreservation of extra human embryos in a program of intracytoplasmic sperm injection (ICSI).

Methods: The extra embryos of 160 patients were cryopreserved in a prospective and randomized manner (drawing lots) by a modified URM (3 M Me₂SO/0.25 M sucrose/thawing in three sucrose gradients) (Group I) or by a SM (1.5 M Propanediol/program 0-Cryologic CL863) (Group II). A total of 103 cycles has been thawed thus far. The number of thawed cycles was 58 for group I and 45 for group II.

Results: The mean age (group I, 31.3 ± 4.5; group II, 31.9 ± 4.3) did not differ between the groups (P = 0.38). The number of frozen embryos (group I, 6.6 ± 3.2; group II, 6.5 ± 3.2) was similar (P = 0.49) for the two groups, as was the number of thawed embryos (P = 0.52) (group I, 6.5 ± 2.9; group II, 6.2 ± 3). The survival rate was higher (P < 0.01) for group II (83.3 ± 23%) than for group I (69.2 ± 28.7%). The cleavage rate was also higher (P < 0.01) for group II (56.8 ± 31%) compared with group I (24.2 ± 22.4%). The number of embryos transferred did not differ (P = 0.14) between the groups (group I, 3.16 ± 1.2; group II, 3.5 ± 1.0). The implantation rate (group I, 6.3%; group II, 13.8%) was significantly different between groups (P = 0.034). Pregnancy rates per thawed and transferred cycle were higher for group II (33.3 and 36.6%, respectively) compared with group I (13.8

and 16%, respectively), and these differences were significant (P = 0.03 and P = 0.03, respectively).

Conclusion: The data obtained suggest that the SM is superior to the URM for the cryopreservation of extra embryos after ICSI.

KEY WORDS: cryopreservation; human embryo; ICSI; ultrarapid method; slow cooling.

INTRODUCTION

The cryopreservation of human embryos has progressed since 1983, when Trounson and Mohr (1) first reported freezing embryos with dimethyl sulfoxide (Me₂SO). Human embryos have usually been cryopreserved in the one-cell, multicellular, or blastocyst stage using different cryoprotectors.

Most of these cryopreservation techniques are based on a slow freezing process that requires expensive equipment and is time-consuming. The ultrarapid method (URM) requires only a short embryo exposure to the cryoprotector at room temperature, followed by immersion in liquid nitrogen (N₂L). In 1987, Trounson *et al.* (2) reported the use of the URM for mouse embryo freezing, which requires rapid embryo exposure (2 to 2.5 min), a high Me₂SO concentration (3 to 4 M), and 0.25 M sucrose, followed by immersion in N₂L and rapid thawing in a 0.25 M sucrose solution. The application of URM in *in vitro* fertilization (IVF) programs has led to variable pregnancy rates (7 to 29%) per embryo transfer (3–6).

Traditionally, the slow method (SM) is used for the cryopreservation of embryos obtained by intracytoplasmic sperm injection (ICSI). In 1994, Van Steirteghem *et al.* (7) studied the efficacy of multicellular extra human embryos obtained after ICSI. The embryos were cryopreserved on day 2 or 3 using

¹ Centre for Human Reproduction, Sinhá Junqueira Maternity Foundation, Ribeirão Preto, SP, Brazil.

² Department of Obstetrics and Gynecology, University of Ribeirão Preto (UNAERP), Ribeirão Preto, SP, Brazil.

³ To whom correspondence should be addressed at Centre for Human Reproduction, Sinhá Junqueira Maternity Foundation, Rua D. Alberto Gonçalves 1500, 14085-100, Ribeirão Preto, SP, Brazil. Fax: 55-16-628-3755. E-mail: franco@highnet.com.br.

the SM with Me₂SO (8). The authors reported a 53% survival rate and an overall pregnancy rate [positive β -human chorionic gonadotropin (hCG)] of 21.8% per transfer. The clinical pregnancy rate was 12% and the delivery rate 5.9%. The preclinical abortion rate was 40.9%.

In 1996, Al-Hasani *et al.* (9), using the SM with propanediol, concluded that the pronuclei obtained after ICSI can be successfully frozen/thawed and that the pregnancy rates obtained (17%) are comparable to those for zygotes obtained by IVF (20%). In 1997, Hoover *et al.* (10), using the SM, observed that the pronuclei resulting from ICSI can be frozen and thawed with rates comparable (14%) to those for standard IVF (17.4%). In 1998, Macas *et al.* (11) reported that zygotes obtained by ICSI survive cryopreservation at rates similar to those for IVF zygotes, but their ability to implant and develop may be affected by the cryopreservation process.

In 1999, our group reported a positive clinical experience with the cryopreservation of embryos obtained by ICSI using the URM (6). In the previous study, embryos from 42 cycles were thawed and a total of 24 transfers was performed, with a 16.6% rate of clinical pregnancy per thawing cycle and a 29.2% rate per transfer, with an implantation rate of 13.2%.

The objective of the present study was to compare the cryopreservation of embryos using the URM with Me₂SO versus the SM with propanediol.

MATERIALS AND METHODS

A total of 160 cycles of embryos obtained by ICSI was cryopreserved in a prospective and randomized manner. A total of 103 thawing cycles was evaluated, with 58 cycles consisting of embryos cryopreserved by the URM (group I) and 45 consisting of embryos cryopreserved by the SM (group II). At present there are no further cycles to thaw since the remaining patients became pregnant or do not wish to receive the cryopreserved embryos.

In the ovarian stimulation cycle preceding ICSI we used blockade of the second phase with leuprolide acetate at a dose of 0.5 mg a day (Lupron; Abbott, São Paulo, Brazil). Fourteen days after the use of the analogue, with blockade established, we used follicle stimulating hormone (FSH) at a fixed dose of 150 or 225 IU for a period of 7 days (12). Follicular development was monitored only by vaginal ultrasonography 8 days after ovarian stimulation began, with the doses of FSH being adapted according to

the ovarian response. When a minimum of two follicles measuring ≥ 17 mm in diameter was observed, hCG was administered at a dose of 10,000 IU. Oocyte retrieval was carried out by transvaginal ultrasound-guided puncture of follicles 34–36 hr after hCG. After identification in the follicular fluid, the oocytes were classified for maturity. The cumulus–corona complex was removed by exposure to a solution of type IV hyaluronidase (H-4272; Sigma Chemical Co., St Louis, MO) at a concentration of 80 IU/ml. The denuded oocytes were incubated in IVF-50 medium (IVF Science Scandinavia, Sweden) until the time of ICSI. Spermatozoa were separated from seminal fluid using discontinuous Sperm-Prep-100 gradients (IVF Science Scandinavia) prepared with 40 and 90% fractions.

ICSI was performed by an established method (13). The embryos were routinely transferred after 48 hr in culture and supernumerary embryos were cryopreserved at the end of the second day.

Ultrarapid Method

Embryo Freezing. In the URM, we used 3 M Me₂SO (Sigma; D-2650) and 0.25 M sucrose (Sigma; S-1888) but we modified the thawing process (6).

Two solutions were first prepared: (i) a culture solution consisting of IVF-50 medium plus 20% inactivated patient serum and (ii) an Me₂SO solution consisting of 3.9 ml culture solution and 14 M Me₂SO in a volume of 1.1 ml. A freezing solution was then prepared, in which 0.43 g sucrose was added to the final 5.0 ml volume of the Me₂SO solution. The freezing solution, containing 3 M Me₂SO and 0.25 M sucrose, was filtered through a 0.22- μ m membrane, exposed to an atmosphere of 90% N₂, 5% CO₂, and 5% O₂, and kept at 4°C for a minimum of 12 hr and a maximum of 72 hr. The embryos were exposed to the freezing solution for exactly 2.5 min in a freezing straw at room temperature and the straw was then plunged directly into liquid N₂.

Embryo Thawing. A thawing solution (TS) was prepared with 0.43 g sucrose and diluted with culture solution to a final volume of 5.0 ml on the thawing day. The TS (0.25 M sucrose) was diluted at three concentrations with culture solution: TS-1, consisting of TS at a final proportion of 0.17 mol/L sucrose solution; TS-2, consisting of TS at a final proportion of 0.13 mol/L sucrose solution; and TS-3, consisting of TS at a final proportion of 0.08 mol/L sucrose solution. The solutions were filtered through a 0.22- μ m membrane, exposed to an atmosphere of 90% N₂, 5% CO₂,

and 5% O₂, placed on Nunc (Copenhagen, Denmark) culture plates, and kept at room temperature.

The straws containing the frozen embryos were then removed from liquid nitrogen, left to stand at room temperature for 30 sec, and immersed in a water bath at 30°C for 40 sec. The embryos were then transferred consecutively to the three sucrose dilutions (TS-1, TS-2, and TS-3) for 10 min each time at room temperature. The embryos were placed in culture solution at room temperature for 5 min and then in culture solution microdrops covered with oil at a temperature of 37°C and maintained in a 5% CO₂ atmosphere for 24 hr.

Slow Freezing Method

Embryo Freezing. For the freezing process we used Freeze-kit 1 (IVF Science Scandinavia), which contained the following solutions: PBS, 1.5 M propanediol (PROH), and 1.5 M PROH + 0.1 M sucrose. The solutions were added to Nunc culture plates and stabilized at room temperature for 15 min. Cryopreservation was performed using a CL-863 cryologic apparatus. The extra embryos were first washed in PBS and then transferred to 1.5 M PROH for 10 min. The embryos were then transferred to the 1.5 M PROH solution with sucrose and immediately transferred to the straws. The straws were placed in a cryochamber stabilized at a temperature of 24°C and cooling was started at 2°C per min until a temperature of -6°C was reached, when manual seeding was performed. The temperature then fell at 0.3°C per min to -35°C, followed by a free fall to -150°C, after which the straws were transferred to liquid nitrogen.

Embryo Thawing. In the thawing process Thaw-kit 1 (IVF Science Scandinavia) was used, consisting of four solutions: 1.0 M PROH plus 0.2 M sucrose, 0.5 M PROH plus 0.2 M sucrose, 0.2 M sucrose, and PBS. The solutions were stabilized at room temperature for at least 15 min. The straws were stabilized at room temperature for 30 sec and then transferred to a water bath (30°C) for 40 sec. The embryos were successively added to the four solutions (1.0 M PROH/0.2 M sucrose, 5 min; 0.5 M PROH/0.2 M sucrose, 5 min; 0.2 M sucrose, 10 min; PBS, 5 min). The embryos were then transferred to PBS stabilized at 37°C and 5% CO₂ for 5 min and, finally, incubated in G1.2 culture medium (IVF Science Scandinavia) for 24 hr.

Evaluation of Embryo Survival and Cleavage After 24 hr. Survival was defined by the presence of only one intact blastomere after the thawing process (5). However, the embryos were inspected separately in terms of

embryo development, and only those that presented a continuation of the embryo cleavage process were transferred to the patient (14). Embryo cleavage was considered to be present when the division of at least one of the blastomeres was observed after 24 hr.

Replacement Cycle and Pregnancy

Two hormonal schemes were used for the transfer of thawed embryos, i.e., a natural cycle and a substitutive cycle. In the natural cycle, follicular development was monitored by serial vaginal ultrasonography starting on the 10th day of the cycle. A 5000-IU dose of hCG was administered when the follicle presented a diameter ≥ 17 mm, and thawing was routinely performed on the fourth day after hCG (day of hCG injection = day 1) and embryo transfer on the fifth day after hCG.

In the substitutive cycle, estradiol valerate (Postoval; Wyeth, São Paulo, Brazil) was administered from the 1st to the 13th day of the cycle at a daily dose of 4–6 mg. Progesterone was introduced on the 14th day at a dose of 400 mg/day by the vaginal route, as long as the endometrial thickness was ≥ 6 mm (15). Thawing was performed on the fourth day of progesterone treatment, and transfer on the fifth day of progesterone treatment.

The pregnancy test was performed on the 14th day after transfer, and clinical pregnancy was confirmed during the sixth week by the presence of a gestational sac and an embryo with a heartbeat. Data were analyzed statistically by the Whitney and Fisher tests.

RESULTS

Patient age (group I, 31.3 ± 4.5 ; group II, 31.9 ± 4.3) did not differ between the groups ($P = 0.38$) (Table I). The number of frozen embryos (group I, 6.6 ± 3.2 ; group II, 6.5 ± 3.2) was similar ($P = 0.49$). In addition, the number of thawed embryos was similar ($P = 0.52$) (group I, 6.5 ± 2.9 ; group II, 6.2 ± 3) for the two groups.

The embryo survival rate was higher ($P < 0.01$) in group II ($83.3 \pm 23.2\%$) than in group I ($69.2 \pm 28.7\%$). The rate of embryo cleavage was also higher ($P < 0.01$) in group II ($56.8 \pm 31\%$) than in group I ($24.2 \pm 22.4\%$).

The number of transferred embryos did not differ ($P = 0.14$) between groups (group I, 3.16 ± 1.2 ; group II, 3.5 ± 1.0). The rate of embryo implantation was significantly higher in group II (13.8%) than in group I (6.3%) ($P = 0.034$).

Table I. Comparison of the Cryopreservation of Embryos Using the Ultrarapid Method with DMSO (Group I) Versus the Slow Method with Propanediol (Group II)

	Group I	Group II	P
Number of thawed cycles	58	45	
Mean age (yr)	31.3 ± 4.5	31.9 ± 4.3	0.38
Number of frozen embryos	6.6 ± 3.2	6.5 ± 3.2	0.49
Number of thawed embryos	6.5 ± 2.9	6.2 ± 3	0.52
Embryo survival rate	69.2 ± 28.7%	83.3 ± 23%	<0.01
Embryo cleavage rate	24.2 ± 22.4%	56.8 ± 31	<0.01
Number of embryos transferred	3.16 ± 1.2	3.5 ± 1.0	0.14
Implantation rate	6.3%	13.8%	0.034
Pregnancy rate per thawed cycle	13.8%	33.3%	0.030
Pregnancy rate per transferred cycle	16%	36.6%	0.030

The pregnancy rate per thawed embryo cycle and per transferred embryo cycle was significantly higher in group II (33.3 and 36.6%, respectively) than in group I (13.8 and 16%, respectively) ($P = 0.03$ and 0.03 , respectively). The abortion rate was 12.5% in group I and 13.3% in group II.

The natural cycle was used in 73.3% of group I patients and in 68.9% of group II patients ($P = 0.66$), and the substitutive cycle was used in 26.6% of group I patients and in 31% of group II patients ($P = 0.67$), with no significant difference in distribution between groups. The incidence of multiple pregnancies was 25% in group I and 21.4% in group II.

DISCUSSION

In 1994, Van Steirteghem *et al.* (7) evaluated for the first time the prognosis of embryos submitted to freezing and thawing after ICSI. Type A or B embryos (type A embryos had regular or irregular blastomeres and showed no anucleate fragments; type B embryos had $\leq 20\%$ of their surface filled with anucleate fragments) were cryopreserved with DMSO by the SM on day 2 or 3 (2–16 cells). The pregnancy rate per transfer was 21.8%, but with a high incidence of abortion during the first trimester.

In 1998, Kowalik *et al.* (16) compared the results for embryos obtained by IVF and ICSI after cryopreservation using the SM. Survival rates were similar for the two groups (70.5 and 73.2% for IVF and ICSI, respectively) and were not affected by cryopreservation stage. In addition, there were no significant differences in pregnancy rates per transfer (31.8 and 32.3%) or in preclinical abortion rates (16.7 and 23.8%) between the IVF and the ICSI groups. The authors concluded that ICSI does not have an adverse impact on the survival and successful implantation of cryopreserved and thawed embryos.

However, Macas *et al.* (11) observed that zygotes arising from ICSI cycles survived cryopreservation at a rate similar to that of IVF zygotes, but their ability to implant and develop further was probably affected by the cryopreservation procedure. These data suggested the continued need for reevaluation of the methods used for embryo cryopreservation after ICSI.

In 1999, Mauri *et al.* (6) reported encouraging initial results with the URM, a fact that motivated the present comparative study of the URM and SM.

However, the results obtained here in a prospective and randomized manner showed that, thus far, the rate of embryo survival ($83.3 \pm 23\%$) and cleavage after 24 hr ($56.8 \pm 31\%$) was significantly higher ($P < 0.01$ and $P < 0.01$, respectively) in the group in which the SM was used compared to the URM group (69.2 ± 28.7 and $24.2 \pm 22.4\%$, respectively).

Furthermore, the pregnancy rate per thawed cycle and transferred embryo cycle was significantly higher in the group with embryos cryopreserved by the SM (33.3 and 36.6%, respectively) than in the group with embryos cryopreserved by the URM (13.8 and 16%, respectively) ($P = 0.03$ and $P = 0.03$, respectively). These data led our group to discontinue the use of URM in our unit, at least with the modifications developed thus far.

In conclusion, compared to the SM, the URM is a less effective method for embryo cryopreservation. However, new technical modifications should be made for the URM to produce the same results as obtained with the SM since it is a rapid and easy method with a lower cost, due mainly to the reduction of laboratory work.

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