Clinical evaluation of two formulations of slow-freezing solutions for cleavage stage embryos

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Abstract

Purpose The aim was to investigate if improved survival rates could be achieved using a new formulation of solutions for slow freezing of human cleavage stage embryos.

Methods The evaluation was divided into two parts. The first part was a retrospective analysis of results obtained after freezing and thawing of day 3 embryos from 400 women using an old formulation of cryopreservation solutions compared to results from 108 women for which cryopreservation had been performed using new compositions of solutions. The second part was prospective, adding cycles until similar numbers of patients had been included in both groups. In total, 2274 embryos from 897 patients were thawed using the old formulation of solutions while 1273 embryos from 542 patients were frozen and thawed using the new solutions. The primary endpoint was survival rate.

Results With the new solutions, the survival rate increased from 82.1 to 94.4 % and the complete embryo survival rate increased from 54.9 to 81.3 %. The implantation rate, clinical pregnancy rate per embryo transfer, and per cycle were 28.2, 45.2, and 43.7 %, respectively, using the old formulations of cryosolutions. With the new solutions, the results reached 33.7, 54.1, and 54.1 %, respectively. All differences in results were statistically significant. The number of cancelled embryo transfers due to no survived embryos was 18 with the old solutions and 0 using the new solutions.

Capsule With the new composition of solutions for slow freezing and thawing of embryos, significantly improved results were obtained. Additionally, the number of cancelled embryo transfers was reduced.

Conclusion With the new composition of solutions for slow freezing and thawing of embryos, significantly improved results were obtained. Additionally, the number of cancelled embryo transfers was reduced.

Keywords Cryopreservation · Slow-freezing · Human embryo freezing · Survival rates

Introduction

Freezing of human embryos has been a clinical routine in IVF clinics since the 1980s [1] and is still a common procedure, especially for pronuclear and cleavage stage embryos. Even though vitrification has become a very popular cryopreservation method during the last decade, many clinics still prefer freezing [2]. The reasons may be that, for example, the equipment is already in place in many clinics, the method is not very operator-demanding, and the results for the developmental stages mentioned are reasonably good. With vitrification, however, much higher survival rates can be obtained [3, 4].

Slow freezing is the traditional method used for cryopreservation of oocytes, pronuclear zygotes, cleavage stage embryos, and blastocysts. In 1972, Whittingham reported the first successful birth after mouse embryo freezing. Based on the slow-freezing technique derived from mouse embryo cryopreservation, Trounson applied the slow-freezing technique to human cleavage stage embryos resulting in the first pregnancy after human embryo cryopreservation. The first live birth was reported by Zeilmaker et al. 1 year later [5]. The first report of implantation of human embryos was derived from oocytes cryopreserved by slow freezing appeared in 1986 [6], and the survival rate has been improved to 70–80 % with the introduction of high sucrose concentration during pre-freeze dehydration [7]. For blastocyst, the first

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pregnancy after cryopreservation of blastocysts was achieved by slow freezing, using glycerol as permeable cryoprotectant [8].

The original composition of the freezing medium presented by Testart [9] had phosphate buffer saline (PBS) as base medium and sucrose and propanediol as cryoprotectants. This composition has remained more or less unchanged since its introduction. In a study by Edgar et al. [10], a new composition of cryopreservation solutions for cleavage stage embryos was presented. To increase the intracellular dehydration during slow cooling, the new formulation contains a higher concentration of sucrose than used by Testart and it was shown that significantly improved survival rates could be achieved, similar to results obtained with vitrification.

Higher survival rates improve the efficiency of a cryopreservation program and are likely to have a substantial impact on the cumulative success rates of any IVF clinic. The aim of our evaluation was to investigate if survival rates similar to those obtained with vitrification could be obtained in our clinic with the new composition of solutions for freezing and thawing of cleavage stage embryos. We therefore used products and methods from two manufacturers; one method used the original composition and concentrations of cryoprotectants in the solutions while the other manufacturer recently introduced freezing and thawing solutions with the concentrations of sucrose suggested by Edgar et al.

Material and methods

The patients were treated with FSH (Gonal-F; Serono, Geneva, Switzerland) after pituitary function was down-regulated with gonadotropin releasing hormone (GnRH-a, Decapeptyl; Ferring A.B., Lausanne, Switzerland). Follicular development was monitored using serial vaginal ultrasound and serum E₂ levels. A dose of 10,000 U of hCG was administered when two or more follicles reached 18 mm in mean diameter. Oocytes were transvaginally retrieved under ultrasound guidance 36 h after triggering ovulation. Retrieved mature oocytes were cultured in supplemented G-IVF medium (Vitrolife, Sweden) at 37 °C in a humidified atmosphere with 6 % CO₂. About 17 h after insemination, the oocytes were checked for pronuclei and polar bodies. Fertilized zygotes were cultured in supplemented G-1 medium (Vitrolife, Sweden). The majority of embryos were transferred or cryopreserved on day 3.

After thawing, the survived embryos were cultured in supplemented G-1 medium for at least 2 h before embryo transfer. One to three embryos were transferred for each patient. For young patients (<35 years) who underwent the first IVF cycle, no more than two embryos were transferred.

The products used for cryopreservation were FreezeKit Cleave™ and ThawKit Cleave™ (new formulation, Vitrolife Sweden AB, Sweden, group A) and Embryo Freeze media and Embryo Thaw media (Irvine Scientific, USA, group B). The new composition of Vitrolife solutions contained not only

the cryoprotectants concentrations by Edgar but also a new base medium composition. The base medium is a modification of a G-Series culture medium (Vitrolife Sweden AB, Sweden) containing amino acids for support of embryo viability and with the addition of MOPS buffer for physiological pH maintenance plus hyaluronan, which has been shown to increase embryo survival after cryopreservation [11, 12].

The first evaluation period started in October 2013 when the new method was introduced (group A, Vitrolife) in the clinic and ended in January 2014. The Irvine freezing method had been used in the clinic during 5 years prior to the evaluation. During the initial evaluation period, the results of 108 freeze-thaw cycles in group A were analyzed. The data was then compared to retrospective data from 400 cycles in group B. After analysis of the data from the initial evaluation period, it was decided to continue the evaluation until similar numbers of cycles in both groups were reached. During the second evaluation period, freezing was performed using the two methods on alternating days. The second evaluation period continued until October 2014 when approximately 1000 freezing cycles and almost 500 thawing cycles had been performed for each group.

Only good-quality day 3 embryos were cryopreserved using a Planer freezer (Planer Ltd, UK). Good quality embryos were defined as having 6–10 cells, equal-sized mononucleated blastomeres with <25 % fragmentation. Embryos were thawed and transferred on the same day, at the 6–10 cell stage.

Survival of embryos was defined as ≥ 50 % of blastomeres still intact after freezing and thawing. Clinical pregnancies were determined using ultrasound verifying the presence of a gestational sac. Statistical difference is $P < 0.05$.

We used the freezing programs following manufactures' instructions. For group A embryos: Starting temperature 20 °C, cooling rate -2 °C/min to -6 °C, seed after 2 min, wait another 8 min, cool at -0.3 °C/min to -30 °C, -50 °C/min to -150 °C, and plunge into liquid nitrogen. For group B embryos: Starting temperature 20 °C, -2 °C/min to -7 °C, 5 min of soaking followed by manual seeding, wait 10 min, cool at -0.3 °C/min to -30 °C, -30 °C/min to -120 °C, and directly into liquid nitrogen.

This study was approved by the Ethics Committees of the Women's Hospital. A formal registration of the study was not performed since the study is partially retrospective.

Statistical analysis was performed with the Statistics Package for Social Sciences for Windows software package version 18.0 (SPSS, Chicago, IL, USA). Comparison of survival rate, complete embryo survival rate, blastomere survival rate, implantation rate, and pregnancy rate between old formulation group and new formulation group was performed using Fisher's exact test considering two groups at a time. Age of female patients, number of retrieved oocytes, fertilization rate, number of good quality embryos, and number of transferred embryos of the two groups were compared with *t* test. Statistical differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

During the first evaluation period, there was no difference between group A and B regarding age of female patients, number of retrieved oocytes, fertilization rate, number of good quality embryos, or the number of embryos per transfer (Table 1). A noticeable difference was that in group B, 18 cycles had to be cancelled due to no surviving embryos while there were no cancelled cycles in group A.

Significantly improved results were obtained for group A regarding survival rate and pregnancy rate per cycle.

During the second evaluation period, age of female patients, the number of retrieved oocytes, fertilization rate, average number of good quality embryos, and the number of embryos per transfer were statistically the same (Table 2) between groups A and B. As during the first evaluation period, there was a big difference in the number of cancelled cycles between the groups.

When the results for the total evaluation period were compiled, it was evident that the new formulation resulted in less cancelled cycles and significantly higher embryo survival rates. Furthermore, the clinical results showed higher implantation rate and higher clinical pregnancy rates per embryo transfer and per cycle (Table 3).

Discussion

Freezing of cleavage stage embryos has been an important part of the IVF treatment for decades, and results were considered acceptable until vitrification was introduced as a more efficient cryopreservation method. With vitrification, considerably higher survival rates can be obtained compared to

standard slow-freezing procedures, commonly around 90 %. Vitrification was initially introduced as a quick and easy cryopreservation method. Still, many clinics find vitrification time-consuming and operator-demanding and prefer to cryopreserve pronuclear-stage and cleavage stage embryos using the conventional slow-freezing method.

The slow-freezing protocols suggested by Testart is a so-called non-equilibrium cryopreservation protocol resulting in intracellular vitrification during the fast temperature decrease of the freezing procedure. In 2009, Edgar et al. presented improved results after slow freezing. Compared to the standard method used for many years, they increased the level of sucrose in the freezing medium as well as in the thawing medium. This increased dehydration before freezing had a substantial effect on survival. During vitrification, cells are exposed to increased levels of sucrose resulting in increased dehydration and increasing levels of penetrating cryoprotectants allowing intracellular vitrification. The improved survival as presented by Edgar seems to result from a similar response of cells as in vitrification.

Seki and Mazur [13, 14] have shown that warming rates are more important than cooling rates for successful cryopreservation. In the slow-freezing program suggested by Testart, fast warming is required. It seems that the improved level of dehydration suggested by Edgar combined with the cooling and warming rates typically applied in this protocol result in a combination providing excellent survival.

In our clinic, we consider slow freezing more practical but wanted to improve the results, especially the survival rate after thawing of the embryos. It was therefore of interest to evaluate the new composition of traditional slow-freezing solutions using a modified protocol. The new composition is based on MOPS-buffered media containing amino acids and

Table 1 Results of the first evaluation period

	Group A New formulation	Group B Old formulation	<i>P</i> value
Age of female patients	30.42 ± 4.29	30.14 ± 4.24	0.943
Thawing cycles	108	400	
No. of retrieved oocytes (mean ± SD)	17.33 ± 6.36	18.04 ± 7.64	0.375
Fertilization rate (mean ± SD)	10.56 ± 5.64	10.51 ± 5.53	0.934
No. of good quality embryos (mean ± SD)	6.16 ± 3.42	6.50 ± 3.99	0.412
No. of frozen embryos (mean ± SD)	5.54 ± 3.53	5.87 ± 4.27	0.473
No. of transferred embryos (mean ± SD)	2.04 ± 0.39	2.08 ± 0.49	0.503
Survival rate of thawed embryos	92.31 % (240/260)	78.21 % (822/1051)	<0.001**
Complete embryo survival rate	78.08 (203/260)	50.24 % (528/1051)	<0.001**
Cycle cancellation rate	0 % (0/108)	4.50 % (18/400)	0.018*
Pregnancy rate per cycle	55.56 % (60/108)	43.50 % (174/400)	0.030*
Pregnancy rate per embryo transfer	55.56 % (60/108)	45.55 % (174/382)	0.081
Implantation rate	31.80 % (69/217)	26.00 % (195/750)	0.100

**Significant ($P < 0.01$) difference between group A and B; *Significant ($P < 0.05$) difference between group A and B

Table 2 Result of the second evaluation period

	Group A New formulation	Group B Old formulation	<i>P</i> value
Age of female patients	30.92 ± 4.25	30.93 ± 4.34	0.448
Freezing cycles	957	1190	
No. of retrieved oocytes (mean ± SD)	16.82 ± 6.67	17.59 ± 7.69	0.109
Fertilization rate (mean ± SD)	10.04 ± 4.98	10.38 ± 5.51	0.335
No. of good quality embryos (mean ± SD)	5.99 ± 3.36	6.43 ± 3.89	0.073
Frozen embryos	2309	2880	
No. of frozen embryos (mean ± SD)	5.32 ± 3.38	5.79 ± 4.17	0.062
Thawing cycles	434	497	
Thawed embryos	1013	1223	
Number of transfers	434	485	
Cycle cancellation rate	0 % (0/434)	2.41 % (12/497)	0.001**
Survival rate	94.47 % (957/1013)	85.36 % (1044/1223)	<0.001**
Complete embryo survival rate	82.13 % (832/1013)	58.95 % (721/1223)	<0.001**
Blastomere survival rate	91.88 % (5994/6524)	79.12 % (6577/8313)	<0.001**
No. of transferred embryos (mean ± SD)	2.09 ± 0.52	2.04 ± 0.51	0.114
Clinical pregnancy rate/embryo transfer	53.69 % (233/434)	44.95 % (218/485)	0.008**
Implantation rate	34.10 % (310/909)	29.94 % (297/992)	0.055
Clinical pregnancy rate/ cycle	53.69 % (233/434)	43.86 % (218/497)	0.003**
Ratio of gestational sacs/thawed embryo	30.60 % (310/1013)	24.28 % (297/1223)	0.001**

**Significant ($P < 0.01$) difference between group A and B

hyaluronan to maintain embryo viability during the handling and cryopreservation procedures and to optimize embryo survival. The cryoprotectants in the new formulation were the same as in the old one, propanediol and sucrose, but the concentration of sucrose in the freezing solution as well as in the first thawing solution was higher. The modification of the

protocol for the new formulation excludes one step which makes the procedure more time-efficient.

The impact of blastomere loss on success rates after slow freezing have been well established [15]. However, with loss of a single blastomere, viability is not significantly affected. Implantation of thawed or warmed embryos is similar when

Table 3 Compiled results of the total evaluation

	Group A New formulation	Group B Old formulation	<i>P</i> value
Age of female patients	30.82 ± 4.26	30.58 ± 4.31	0.464
Number of freezing cycles	1065 (108 + 957)	1590 (400 + 1190)	
Number of thawing cycles	542 (108 + 434)	897	
Number of transfers	542 (108 + 434)	867 (485 + 382)	
Cycle cancellation rate	0 % (0/42)	3.34 % (30/897)	<0.001**
Number of embryos thawed	1273 (260 + 1013)	2274 (1051 + 1223)	
Survival rate	94.02 % 1197 (240 + 957)/1273	82.06 % 1866 (822 + 1044)/2274	<0.001**
Complete embryo survival rate	81.30 % 1035 (203 + 832)/1273	54.93 % 1249 (528 + 721)/2274	<0.001**
Implantation rate	33.66 % 379 (69 + 310)/1126 (217 + 909)	28.24 % 492 (195 + 297)/1742 (750 + 992)	0.002**
Clinical pregnancy rate/embryo transfer	54.06 % 293 (60 + 233)/542 (108 + 434)	45.21 % 392 (174 + 218)/867 (382 + 485)	0.001**
Clinical pregnancy rate/cycle	54.06 % 293 (60 + 233)/542 (108 + 434)	43.70 % 392 (174 + 218)/897 (400 + 497)	<0.001**

**Significant ($P < 0.01$) difference between group A and B

they are fully intact or when a single blastomere is damaged [16]. With the survival rates obtained in group A, few embryos result in loss of more than one blastomere after slow freezing (Table 2). This supports our observation of increasing pregnancy and implantation rates in group A compared to group B.

With the new formulation of the solutions for freezing and thawing of cleavage stage embryos and the modified protocol, we have found that we can achieve the same high survival rates with the traditional slow-freezing method as with vitrification. We can continue using our freezing machine and can spend time on other things in the lab while the machine is running. For the time being, we find slow freezing with this modified method very effective and allowing for optimal time management during busy working days and at the same time obtaining results comparable to those obtained after vitrification of cleavage stage embryos.

Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Conflicts of interest The authors declare that they have no conflicts of interest.

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