Effectiveness of different protocols for slow freezing of human blastocysts

Učinkovitost različnih postopkov počasnega zamrzovanja človeških blastocist

Borut Kovačič, Nina Hojnik, Veljko Vlaisavljević

Department of Reproductive Medicine and Gynecologic Endocrinology, University Clinical Centre Maribor, Ljubljanska 5, SI-2000 Maribor, Slovenia

Korespondenca/ Correspondence:
Borut Kovačič
Department of Reproductive Medicine and Gynecologic Endocrinology, University Clinical Centre Maribor, Ljubljanska 5, SI-2000 Maribor, Slovenia

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Abstract

Background: Pregnancy rate after the transfer of frozen / thawed human blastocysts has been lower in comparison with the pregnancy rate in *in vitro* fertilization (IVF) cycles with fresh embryos. There is a need for the optimization of freezing protocols.

Methods: In the years between 2000 and 2004, blastocyst culture was performed on 3087 IVF/ICSI cycles. In the cases with more developed blastocysts one or two were transferred into the uterus. In 1031 cycles the surplus blastocysts were cryopreserved using one of four slightly modified two-step freezing, two-step thawing glycerol protocols. In protocol A (n = 41) we used a complex freezing medium (modified Ham F-12) and in the others (B, C, D) a simple one (without amino acids). In protocol B (n = 177) the blastocysts were thawed according to four-step thawing protocol. Protocol C (n = 81) retained two thawing steps. Protocol D (n = 37) was the same as C, but with the addition of hyaluronan into the freezing medium. Eight hundred thirty-four frozen blastocysts were thawed for transfer in 336 thawing cycles. In the retrospective study we compared survival according to blastocyst quality (in Groups B–D) as well as pregnancy and delivery rates between different protocol groups. We also compared the clinical outcome between the transfers of thawed blastocysts in natural (n = 249) or artificial cycles (n = 80).

Results: The blastocyst survival rate does not differ between protocols B, C and D (68.7 % vs. 64.8 % vs. 69.8 %), but it was lower in Group A (53.2 %, P < 0.05). In groups B, C and D, the morphologically optimal blastocysts survived cryopreservation in 82.3 % vs. 81.1 % and 88.9 %, the suboptimal blastocysts survived in 67 % vs. 64.6 % and 64 % and poor blastocysts in 49 % vs. 48.7 % and 66.7 %, respectively. The clinical pregnancy rate was lower in Group A in comparison with Groups B, C and D (9.8 % vs. 21.5 % vs. 18.5 % vs. 16.2 %; P< 0.1). The mean delivery rate per thawing was 15.2 %. When at least one optimal blastocyst was thawed and transferred into the uterus, the clinical pregnancy rate was significantly higher than in cycles with only suboptimal blastocysts (27.4 % vs. 14.7 %, P< 0.05). The implantation rate and take- home-baby rate per one thawed blastocyst were higher when blastocysts were replaced in natural than in artificial cycles (15.4 % vs. 8.2 % and 8.3 % vs. 3.8 %; P < 0.05).

Conclusions: All freezing protocols using a simple freezing medium were more effective than protocol with a complex medium. Their application in IVF patients with frozen surplus blastocysts (these patients delivered in fresh cycles in 51.4 %) increases the cumulative delivery rate by an additional 15.2 % with every thawing cycle. The morphology of blastocysts and cycle preparation for transfer had the strongest influence on the success of freezing programme.

Izvleček

Izhodišče: Metoda zanositve po prenosu zamrznjenih/odmrznjenih blastocist v maternico je še vedno manj uspešna v primerjavi z uspešnostjo postopka oploditve z biomedicinsko pomočjo s svežimi zarodki. Metodo zamrzovanja blastocist je zato potrebno izpopolniti.

Metode: V obdobju od leta 2000 do 2004 smo v 3087 ciklih IVF/ICSI gojili zarodke do stadija blastociste. V ciklih z več blastocistami smo eno ali dve prenesli v maternico. V 1031 ciklih smo nadštevilčne blastociste tudi zamrznili po enem od štirih, delno razlikujočih se dvostopenjskih zamrzovalnih in odmrzovalnih postopkih z glicerolom. V postopku A (n = 41) smo kot osnovo za zamrzovanje uporabili kompleksno gojišče (prilagojeni Ham F-12), v ostalih postopkih (B, C, D) pa enostavno gojišče (brez aminokislin).

V postopku B (n = 177) smo blastociste odtajali v štirih stopnjah. Pri protokolu C smo obdržali samo dve stopnji. Postopek D (n = 37) je bil enak postopku C, le da je gojišče vsebovalo še hialuronan. Odmrznili smo 834 blastocist v 336 ciklih. V retrospektivni raziskavi smo primerjali delež preživelih blastocist glede na njihovo kakovost (med protokoli B – D) ter delež zanositev in porodov med različnimi postopki zamrzovanja. Primerjali smo tudi klinične rezultate med prenosi zarodkov v naravnih ciklih (n = 249) in v umetnih ciklih (n = 80).

Rezultati: Stopnja preživetja blastocist se med postopki B, C in D ni razlikovala (68,7 % oz. 64,8 % oz. 69,8 %). Nižja je bila le v skupini A (53,2 %, P < 0,05). V skupinah B oz. C in D so morfološko optimalne blastociste preživele zamrzovanje v 82,3 % oz. 81,1 % in 88,9 %, suboptimalne v 67 % oz. 64,6 % in 64 % in slabe blastociste v 49 % oz. 48,7 % in 66.7 %. Delež kliničnih nosečnosti je bil v skupini A nižji v primerjavi s skupino B, C in D (9,8 % oz. 21,5 % oz. 18,5 % oz. 16,2 %; P < 0,1). Delež porodov je bil v povprečju 15,2 %. Po odmrznjenju vsaj ene optimalne blastociste in prenosu v maternico je bilo kliničnih nosečnosti več kot v ciklih s samo neoptimalnimi blastocistami (27,4 % oz. 14,7 %, P< 0,05). Stopnja implantacije in delež rojenih otrok na eno odmrznjeno blastocisto sta bila višja ob prenosu odmrznjene blastociste v naravnem kot pa v umetnem ciklu (15,4 % oz. 8,2 % in 8,3 % oz. 3,8 %; P < 0,05).

Zaključki: Vsi zamrzovalni postopki z enostavnim gojiščem so bili v primerjavi s kompleksnim gojiščem bolj učinkoviti. Z njimi lahko pri IVF bolnicah z zamrznjenimi nadštevilčnimi blastocistami (te rodijo v svežih ciklih v 51,4 %) povečamo celokupni delež rojstev za 15,2 % z vsakim dodatnim prenosom odmrznjenih blastocist. Morfologija zamrznjeniht blastocist in način priprave cikla za prenos zarodkov imata najmočnejši učinek na uspešnost zamrzovanja.

Introduction

The aim of the cultivation of human embryos *in vitro* to day 5 is in the selection of the most vital embryos for transfer into the uterus. About half of the embryos reach the blastocyst stage on day 5. The remaining embryos usually stop developing after the second or third division of the zygote due to some developmental irregularities. In comparison with earlier stages, blastocysts have a higher implantation potential. With respect to this, the transfer of one or two blastocysts will result in pregnancy in the same or even in a higher percentage compared to the transfer of three or more early-stage embryos.[1]

Supernumerary blastocysts can be frozen by slow cooling and stored in liquid nitrogen. The cryopreservation of blastocysts is similar to the freezing of early stage embryos. The blastocysts have a fluid-filled blastocoele, thus representing an unfavorable stage for cryopreservation. Besides, the blastocyst is a stage with variously differentiated cells–some form a trophectoderm, others the inner cell mass (ICM) – having different morphology, intercellular junctions and also permeability characteristics. Therefore, for the difference from early stage embryos, the dehydration of blastocysts took longer and was stepwise. During cryopreservation, blastocysts were exposed to cryoprotective solutions with increased concentrations of glycerol. After thawing the blastocysts were rehydrated in as many as 9 solutions with decreased glycerol concentrations.[2]

It is very interesting that there is no generally accepted blastocyst freezing protocol. In the 1990s, the blastocyst cryopreservation protocol had a series of freezing and thawing steps, allowing the blastocysts' slow osmotic equilibration through 10 increasing and decreasing glycerol concentrations.[2] In 1992 and 1993 Menezo reported a better survival rate using a simplified two-step freezing and two-step thawing protocol.[3,4] Today this is the most frequently used protocol in IVF centers using commercial sequential media for blastocyst culture.

Menezo et al. used modified Ham F-10 medium as the basic freezing medium, which later proved to be nonoptimal because of its complexity. Our study was started with a complex freezing medium, which was later replaced with a simpler HEPES-buffered medium (without glucose, amino acids and antibiotics). After being in use for some time, this simple freezing medium

was supplemented with hyaluronan – a glycosaminoglycan, which increases implantation if the embryos are exposed to it.[5-7] We analyzed the success of different blastocyst cryopreservation protocols, which varied according to the composition of the basic medium and the number of thawing steps.

In 2000, when we introduced prolonged cultivation of embryos to blastocysts, little was known about the implantation potential of different morphological types of blastocysts. For this reason we froze all supernumerary blastocysts irrespective of their morphology. In most similar studies, a rigorous selection was made among blastocysts for freezing.

Material and methods

Study design

Our statistics include all consecutive frozen-thawed blastocyst cycles from years 2000 to the end of 2004 (n = 336). The study is a retrospective analysis of different blastocyst freezing and thawing protocols. They differed by the composition of basic freezing-thawing medium and by the numbers of thawing steps for rehydration of thawed blastocysts (four protocols). We also compared the cycles differing by the quality of frozen blastocysts (cycles with at least one frozen morphologically optimal blastocyst and cycles with only morphologically suboptimal frozen blastocysts). Finally, we analyzed the success of thawed blastocyst transfers in natural and in artificial cycles.

The success of freezing-thawing protocols was expressed by blastocyst survival rate, pregnancy rate and delivery rate per thawing, and also by implantation rate and take-home-baby rate (babies born per number of thawed blastocysts).

The study was part of a multicentric clinical trial coordinated by Medicult, Jyllinge, Denmark.

Patients and therapy

Between the years 2000 and 2004, the embryos of all patients (n = 3087) were cultured for 5 days according to the protocol described in our previously published work.[8] The transfer of blastocysts was performed in 59.9 % of cycles. The cryopreservation of supernumerary blastocysts was applied in 1031 (33.4 %) of cycles with a transfer.

Patients younger than 40 years, who had their frozen blastocysts thawed (n = 336), were included in the analysis.

In the group of patients with regular menstrual cycle the first ultrasound monitoring was performed on day 7, and the second on day 9 of the menstrual cycle. When the follicles reached 13–14 mm, the patients started to use quick urinary LH tests daily. When the LH test was positive, the patients informed our center, and we decided when to do the embryo transfer of frozen embryos.

The group of patients with irregular menstrual cycles took 4–6 mg daily doses of estradiol valerate (Estrofem, NovoNordisk, Copenhagen, Denmark or Progynova, Schering AG, Berlin, Germany). On day 9 ultrasound monitoring was performed. On day 12 the patients started taking progesterone (Utrogestan, Asta Medica, Wien, Austria) or didrogesteron (Dabroston, Belupo, Koprivnica, Croatia) 20–30 mg daily. Another ultrasound monitoring was performed on day 14 and it was then decided when to carry out the embryo transfer.

In some cases women are downregulated with 0.1 mg of agonist of GnRH (Diphereline, Ipsen Pharma, Beaufour, Switzerland), usually until day 12 of taking Estrofem or Progynova. When the last Diphereline is taken, they start taking Utrogestan or Dabroston. Ultrasound monitoring is performed on days 10, 14 and 16 of the cycle.

Cryopreservation protocols

The supernumerary blastocysts were cryopreserved by one of four cryopreservation protocols, exposing the blastocysts to freezing or thawing solution for 10 minues each. All the procedures were performed at room temperature. During procedure, the petri dishes with cryopreservation media and embryos were put in dark place.

Study Group 1

In the first group (n = 41) we used a two-step freezing, two-step thawing protocol

Table 1: Clinical results of blastocyst cryopreservation using 4 different freezing-thawing protocols.

Freezing–thawing protocol:	A	B	C	D
No. of thawing cycles	41	177	81	37
No. of thawed blastocysts	111	441	196	86
Optimal blastocysts (%)	-	124 (28.1)	53 (27)	18 (20.9)
Fair blastocysts (%)	-	264 (59.9)	104 (53.1)	50 (58.2)
Poor blastocysts (%)	-	53 (12.0)	39 (19.9)	18 (20.9)
No. of survived / thawed blastocysts (%)	59/111 (53.2) [a]	305/441 (69.2)	129/196 (65.8)	60/86 (69.8)
Optimal blastocysts (survival rate, %)	-	102/124 (82.3)	43/53 (81.1)	16/18 (88.9)
Fair blastocysts (survival rate, %)	-	177/264 (67)	67/104 (64.4)	32/50 (64)
Poor blastocysts (survival rate, %)	-	26/53 (49)	19/39 (48.7)	12/18 (66.7)
No. of transfers (ET)	35	161	74	36
No. of transferred blastocysts	57	297	123	58
ET 1	16	45	29	16
ET 2	16	96	41	18
ET 3	3	20	4	2
Positive beta hCG / thawing (%)	7/41 (9.8)	49/177 (27.7)	19/81 (23.5)	10/37 (27)
Biochemical pregnancies	3	11	4	4
Clinical pregnancies / thawing (%)	4/41 (9.8)	38/177 (21.5)	15/81 (18.5)	6/37 (16.2)
No. of gestational sacs / transferred bl. (%)	4/57 (7) [b]	46/297 (15.5)	15/123 (12.2)	7/58 (12.1)
Miscarriages, extrauterine gestations	0	11	1	0
Deliveries / thawing (%)	4/41 (9.8)	27/177 (15.3)	14/81 (17.3)	6/37 (16.2)
Deliveries / transfer (%)	4/35 (11.4)	27/161 (16.8)	14/74 (18.9)	6/36 (16.7)
Babies / thawed blastocyst (%)	4/111 (3.6)	33/441 (7.5)	14/196 (7.2)	7/86 (8.1)
Babies / transferred blastocyst (%)	4/57 (7)	33/297 (11.1)	14/123 (11.4)	7/58 (12.1)

A: 2-freezing, 2-thawing steps; complex medium. B: 2-freezing, 4-thawing steps; simple medium. C: 2-freezing, 2-thawing steps; simple medium. D: 2-freezing, 2-thawing steps; hyaluronan in simple freezing medium.
Statistical significance: a $P < 0.05$.

and commercially available Blastocyst freezing and thawing media (Medicult, Jillinge, Denmark), which contained Medicult's complex M3 (modification of Ham F-12) medium. The first cryopreservation solution contained 5 % glycerol, and the second one 9 % glycerol and 0.2 M sucrose. After thawing, the blastocysts were first exposed to M3 medium with 0.5 M sucrose and then to M3 medium with 0.2 M sucrose.

Study Group 2

In the second group (n = 177) M3 medium was replaced with HEPES buffered simple TL-medium (Medicult). Two additional steps were added to the thawing, so that the blastocysts were washed firstly in 4.5 % glycerol, 0.35M sucrose, after that in 0.5 M sucrose, 0.35 M sucrose, 0.2 M sucrose and finally in TL-HEPES.

Study Group 3

In the third group (n = 81) we used TL-HEPES medium, but the thawing was per-

formed in only two steps, exposing the blastocysts to 0.5 M sucrose and after that to 0.2 M sucrose.

Study Group 4

The fourth group (n = 37) had the blastocysts frozen in TL-HEPES medium with added hyaluronan. The blastocysts were thawed in two steps, like in the third group.

Blastocysts were packed by a maximum of two from the same morphologic category in the straw (CryoBio System, L'Aigle, France) and cooled by a computer controlled freezing device (Minicool, Air Liquide, Marne la Vallee, France) using Menezo's standard curve: from 22 °C to -6.5 °C (manual seeding) at 2 °C/min, from -6.5 °C to -37 °C at 0.3 °C/min, and from -37 to -150 °C at 50 °C/min. The straws were then plunged in liquid nitrogen. The thawed blastocysts were cultured for an additional 2–3 hours in BlastAssist Medium 2 (Medicult).

Blastocyst quality assessment before and after freezing

The morphology of fresh blastocysts was evaluated after 5 days of cultivation *in vitro*. First evaluation was performed under a stereo microscope (SMZU, Nikon, Japan). The score was checked once again under an invert microscope (IMT-2, Olympus, Japan) at the magnification of 100–300 X. Fresh blastocyst morphology was assessed by taking into consideration four morphological parameters at once.[9] The first was the percentage of embryos reaching the blastocyst or the compact stage with early cavitation (early blastocysts), the second the expansion of the blastocyst (when the blastocoele filled whole embryo, the blastocyst was considered expanded). The third parameter was the shape and compactness of ICM, evaluated only in expanded blastocysts (optimal blastocysts had oval and compact ICM). With the fourth parameter we evaluated the trophectoderm (in optimal blastocysts the trophectoderm consisted of numerous ellipsoid and connected cells without any fragments or necrotic foci among them).

Taking into consideration all four parameters, we distributed the blastocysts into three categories: optimal blastocysts (all four parameters were optimal), fair (at least one parameter was non-optimal) and poor blastocysts (at least two parameters were non-optimal).

The survival of frozen-thawed blastocysts was evaluated two hours after thawing. Blastocysts with more than 50 % of intact blastomeres and signs of re-expansion of blastocoele after collapse caused by hyperosmotic solution during the rehydration process were considered normal. In morulae more than 50 % of blastomeres had to be intact to allow the embryo to be assessed as vital and appropriate for transfer.

The surviving blastocysts and morulae were transferred into the uterus by a soft catheter (Labotect, Goettingen, Germany).

Evaluation of pregnancy

Pregnancy was confirmed 14 days after the transfer of embryos into the uterus by the quantitative evaluation of beta human chorionic gonadotrophin (beta-hCG) in the serum and 14 days later by ultrasonographic evaluation of the presence of the gestational sac and heart beats. Biochemical pregnancies were not considered as clinical pregnancies.

Statistical analysis

The data on patient characteristics, hormone therapy and laboratory procedures were processed with the Statistica (Statsoft, Tulsa, USA) statistical program. The differences in the frequencies of the groups compared were evaluated by the chi-square test. The differences were considered statistically significant if the P value was ≤ 0.05.

Results

The compared groups of thawing cycles did not differ as regards mean patient age, type of insemination procedure (IVF or ICSI), mean number of thawed and transferred blastocysts.

Groups B, C and D were comparable by the quality of frozen-thawed blastocysts. We had no data about blastocyst morphology for Group A, since in the beginning of pro-

longed cultivation we had not yet developed the blastocyst scoring system.

The results were presented in Table 1. The survival rate did not differ between protocols B, C and D (68.7 % vs. 64.8 % vs. 69.8 %). It was statistically significantly lower only in Group A (53.2 %, P < 0.05). Morphologically optimal blastocysts survived freezing and thawing at a higher percentage (more than 80 %) than morphologically fair (64–67.6 %) or poor blastocysts (50–66.7 %). The survival rate of the same morphologic type of blastocysts did not differ between Groups B, C and D.

The pregnancy rate (positive beta hCG) per cycle was comparable in Groups B, C and D (27.7 % vs. 23.5 % vs. 27 %) and was lower in Group A (9.8 %; not statistically significant because of the small number of cases). Biochemical pregnancies were evidenced in 25.9 % of all positive beta hCG.

Also, the clinical pregnancy rate was lower in Group A in comparison with Groups B, C and D (9.8 % vs. 21.5 % vs. 18.5 % vs. 16.2 %, respectively).

Compared to Groups B, C and D, Group A had lower implantation rate (7 % vs. 15.5 % vs. 12.2 % vs. 12.1 %). We also found less deliveries per thawing (9.8 % vs. 15.3 % vs. 17.3 % vs. 16.2 %), deliveries per transfer (11.4 % vs. 16.8 % vs. 18.9 % vs. 16.7 %), and a lower take-home-baby rate per thawed blastocyst (3.6 % vs. 7.5 % vs. 7.2 % vs. 8.1 %) and take-home-baby rate per transferred blastocyst (7 % vs. 11.1 % vs. 11.4 % vs. 12.1 %), but the differences were not statistically significant.

Nineteen percent (12/63) of clinical pregnancies in all groups together terminated in miscarriage.

Groups B, C and D do not differ in any parameter.

In Table 2 we compared the clinical results of thawing cycles with respect to differences in female hormone treatment before transfer. A significantly higher implantation rate was achieved in the group of transfers in natural cycles in comparison with the group of artificial–hormonally treated cycles (15.4 % vs. 8.2 %; $P < 0.05$). The transfer in the natural cycle also resulted in more pregnancies per cycle (17.3 % vs. 8.8 %), pregnancies per transfer (19 % vs. 9.6 %), babies born per thawed blastocyst (8.3 % vs. 3.8 %; P < 0.05) and babies born per transferred blastocyst (12.6 % vs. 6 %; P < 0.05).

In Table 3 we analyzed the results of freezing-thawing cycles according to the quality of frozen blastocysts. In thawing cycles with at least one morphologically optimal frozen blastocyst we obtained significantly more clinical pregnancies in comparison with cycles in which only suboptimal blastocysts have been frozen (27.4 % vs. 14.7 %; P < 0.05). Such a trend was also noticed in the comparison of deliveries in both groups of cycles (19.7 % vs. 12.7 %, respectively).

Between 2000 and 2004 blastocyst cultivation was performed in 3087 cycles at our center. Supernumerary blastocysts were frozen in 1031 (33.4 %) cycles. In as many as 602 of 1031 (58.4 %) cycles, the patients became pregnant with fresh embryos. The delivery rate in this group was 51.4 % (530/1031). The remaining 501 (48.6 %) patients were potential candidates for thawing cycles. They had the possibility to conceive and deliver in one thawing cycle in 15.2 % (our results: mean delivery rate from all tested groups together (51 deliveries / 336 thawing cycles)). The hypothetical cumulative delivery rate per cycle in patients having more than 2 blastocysts for transfer was thus 51.4 % from a fresh cycle plus an additional 15.2 % from every thawing cycle.

Discussion

The availability of sequential media has led to an increase in the practice of blastocyst freezing. Several groups have reported freezing of blastocysts quite successfully, reaching clinical pregnancy in the range of 10–30 % per transfer,[10-12] results that were not significantly improved when compared with the thawing of earlier stages. Similar success rates with thawed blastocysts and early embryos are indicative of the imperfection of blastocyst freezing procedure when taking into account that fresh blastocysts have higher developmental capacity than early stage embryos. Later there were some more optimistic reports about pregnancy rates in the range of 40–60 % for thawed blastocysts.[13-17]

The increase in the above mentioned pregnancy rates can be the result of some freezing protocol improvements, such as changing the start temperature and cooling rate,[14] adding more thawing steps,[17] increasing the temperature of blastocysts exposed to glycerol before cooling,[12,18] simplifying the base medium or adding extra proteins.[14,17] But there is a lack of prospective randomized studies, which could confirm that the slight protocol changes really mean a better freezing protocol. There are also many controversial reports. For example, some authors report a better protocol with a simple medium used as the basic cryopreservation medium.[14] On the other hand, others obtained the same results using more complex medium.[17] Two-step thawing showed to be a more optimal protocol than the stepwise protocol.[16] Others report superior results when using the stepwise thawing protocol.[14] Some authors found that exposing blastocysts to glycerol at a higher temperature before freezing is necessary for good results,[12] others report a poorer survival rate when blastocysts were exposed to glycerol for the same period of time because of its toxic effect even at room temperature. Therefore they recommend the shortening of this phase of cryopreservation.[17]

In our study, four different study protocols yielded nearly the same results. Only cycles, in which complex M3 medium was used as a basic cryopreservation medium, showed poorer survival and pregnancy rates. Because poor results were also obtained by other centers, this type of cryopreservation medium is no longer available. G1 medium and G2 medium, which showed the best results when used as basic cryopreservation medium,[14,17] are not as complex as M3 medium. G1 and G2 media are simple media, modified especially for blastocyst development. The difference in complexity between G1 and G2 is only in amino acids and energy substrates.[19] The more complex the medium, the more complex the freezing curve, because every component in the medium has its own freezing point.[20] This led us to use a simpler medium (without amino acids, glucose and antibiotics), buffered with HEPES, which allows embryo exposure to the cryoprotectant at room temperature without additional CO_2 gassing. The use of simpler media resulted in increased survival (53.2 % vs. more than 64 %) and delivery rates (9.8 % vs. more than 15 %).

Other modifications, such as four-step or two-step thawing, seem to have no influence on the survival (68.7 % vs. 64.8 %) or delivery rates (15.3 % vs. 17.3 %). Even when the blastocysts were exposed to a cryoprotectant at a higher temperature (30 or 37 degrees C), the survival rate did not differ from survival of blastocysts treated with glycerol at room temperature (personal experience).

The addition of different proteins to cryoprotectants can make the cryopreservation protocol more successful because of their protective properties for membrane systems.[21] Veeck et al. added plasmanate – additional albumins.[14] Many others report on improved results when hyaluronan was added to the cryoprotectant.[5-7] In a small number of thawing cycles with hyaluronan in the cryopreservation medium (n = 37) we could not confirm the superior effect of hyaluronan.

In many centers, blastocyst culture is only applied in patients having a large number of embryos, so that a strong selection between blastocysts for transfer and also for freezing is possible. Many authors reported on the impact of blastocyst selection for freezing on the cryopreservation results.[22] According to Behr et al. (2002), the desire to keep clinicians and patients happy in the early days of sequential blastocyst culture leads many embryologists to cryopreserve late stage embryos / blastocysts that would never have been frozen with the wealth of experience embryologists now have.[22]

In our center blastocyst cryopreservation started at the same time as blastocyst culture in sequential media. From 2000 to 2004 blastocyst culture was applied in all patients, and all supernumerary blastocysts, irrespective of their morphology, were frozen. Because of these two facts the comparison of our cryopreservation results with those of others is not possible.

Regarding our survival rate, which seems to be poorer than in other studies (less than 70 % vs. 90 %),[14,17] we note a significant dif-

Table 2: Clinical results (survival rates, implantation rates, pregnancy rates and take-home-baby rates) of frozen–thawed blastocyst transfers in natural or artificial cycles.

	Natural cycles	Artificial cycles
No. of thawing cycles	249	80
No. of survived / thawed blastocysts (%)	397/590 (67.3)	139/212 (65.6)
No. of transfers (ET)	226	73
No. of transferred blastocysts	389	134
Mean no. of transferred blastocysts	1.6 +/- 0.8	1.7 +/- 0.7
ET 1	85	18
ET 2	119	49
ET 3	22	6
Positive beta hCG / thawing (%)	65/249 (26.1)	18/80 (22.5)
Biochemical pregnancies	13	8
Clinical pregnancies / thawing (%)	52/249 (20.9)	10/80 (12.5)
No. of gestational sacs / transferred bl. (%)	60/389 (15.4) [a]	11/134 (8.2)
Abortions, extrauterine gestations	9	3
Deliveries / thawing (%)	43/249 (17.3)	7/80 (8.8)
Deliveries / transfer (%)	43/226 (19)	7/73 (9.6)
Babies / thawed blastocyst (%)	49/590 (8.3) [a]	8/212 (3.8)
Babies / transferred blastocyst (%)	49/389 (12.6)	8/134 (6)

Statistical significance, Statistična značilnost: a P < 0.05.

ference between different morphological groups of blastocysts. The optimal blastocysts survived cryopreservation in 81.1 % to 88.9 %, the suboptimal in 64 % to 67 % and poor blastocysts in 48.7 to 66.7 %. Consequently the pregnancy rate was expectedly lower in comparison with other studies. But when at least one optimal blastocyst was thawed together with suboptimal blastocysts, the clinical pregnancy rate was significantly higher than in cycles with only suboptimal thawed blastocysts (27.4 % vs. 14.7 %, respectively) (Table 3).

The positive beta hCG rate in our study is sufficiently high (26.7 %), but there is a high rate of spontaneous embryo reductions expressed early as only biochemical pregnancies (25.9 %) or later as miscarriages (16.7 %). There are also reports from other centers about high abortion rates in thawing cycles[16]. A high miscarriage rate was to be expected, because the best blastocysts from fresh cycles were used for embryo transfer, but the remaining ones, which were cryopreserved, were mostly (in around 70 %) suboptimal. From our previous study we know that the miscarriage rate after the transfer of fresh blastocysts can vary from 9.1 % to as many as 83.3 % depending on the quality of transferred blastocysts.[9] Cryopreservation can cause different celullar and also intracellular damage, such as disruption of mitotic spindles in dividing blastomeres or fusion of two cells, which affects chromosomal normality. There is also some speculation that ICM cells survive freezing differently than trophectoderm cells, because of their different permeability of ICM and trophectoderm for water and cryoprotectants.[22]

The results do not depend only on the freezing procedure, but to a great extent also on hormonal treatment, patient age and indications. Table 2 shows that there is a big difference when thawed blastocysts are transferred in natural cycles compared to artificial–hormonally prepared cycles (de-

Table 3: Clinical results (survival rates, implantation rates, pregnancy rates and take-home-baby rates) of transfers differing by quality of frozen–thawed blastocysts.

	At least 1 optimal blastocyst	Only nonoptimal blastocysts
No. of thawing cycles	117	150
No. of transfers (ET)	115	135
No. of transferred blastocysts	215	226
Mean no. of transferred blastocysts	1.8 +/- 0.7	1.5 +/- 0.8
ET 1	30	52
ET 2	70	75
ET 3	15	8
Positive beta hCG / thawing (%)	39/117 (33.3)	33/150 (22)
Biochemical pregnancies	7	11
Clinical pregnancies / thawing (%)	32/117 (27.4) [a]	22/150 (14.7)
No. of gestational sacs / transferred bl. (%)	38/215 (17.7)	25/226 (11.1)
Abortions, extrauterine gestations	9	3
Deliveries / thawing (%)	23/117 (19.7)	19/150 (12.7)
Deliveries / transfer (%)	23/115 (20)	19/135 (14.1)
Babies / thawed blastocyst (%)	28/332 (8.4)	21/328 (6.4)
Babies / transferred blastocyst (%)	28/215 (13)	21/226 (9.3)

Statistical significance, Statistična značilnost: a P< 0.05.

livery rate 17.3 % and 8.8 %, respectively), but for anovulatory patients natural cycles are not appropriate.

Nevertheless, cryopreservation of blastocysts using all these protocols is not as successful as we expected. The take-home-baby rate calculated per one thawed blastocyst is only between 7 and 8 %. This means that for one baby born at least 12 to 14 blastocysts have to be thawed. But of course this ratio can improve immediately if calculated only from morphologically optimal frozen-thawed blastocysts.

Because of rare reports about good blastocyst freezing programs, which should reach a clinical pregnancy rate of at least 40 %, many scientists around the world are trying to apply new techniques. The most promising one is vitrification, because it prevents the formation of ice crystals in the blastocyst-surrounding medium between cooling and storage in liquid nitrogen.[23-26] Despite a higher concentration and possibly more toxic effect of cryoprotectants used in vitrification, the vitrification has already been proved as more successful cryopreservation method than slow freezing for early embryos and blastocysts.[27,28]

It is very difficult to follow the success of blastocyst freezing programs because in good centers the majority of patients with prolonged cultivation and blastocyst freezing are pregnant in a fresh cycle (58.4 % in our center). In 5 years only 429 patients from the total of 3087 (14 %) having blastocyst cultivation are potential candidates for thawing cycles. Any change in the freezing or thawing procedure thus requires a long time to show its positive or negative effect on the survival or pregnancy rate.

Based on the results from the cryopreservation program, we can also calculate the cumulative delivery rate from fresh and frozen cycles together. In patients having more than 2 blastocysts for transfer it is 51.4 %

from a fresh cycle plus an additional 15.2 % from every thawing cycle.

Summary

Due to the cryopreservation of all supernumerary–also morphologically poor blastocysts, the survival, pregnancy and delivery rates are not comparable with the results of other published studies in which strong selection between patients for prolonged cultivation and between blastocysts for freezing was performed. We show that blastocyst quality is the most important factor affecting the results of the cryopreservation program.

Regardless of which of the freezing protocols with a simple medium was used, equal results were obtained. Using cryopreservation of blastocysts in IVF patients with more than two blastocysts for transfer – who delivered in fresh cycles in 51.4 % – the cumulative delivery rate increased by an additional 15.2 %.

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