Vitrification of human blastocysts previously cryopreserved by slow controlled-rate freezing at the cleavage stage

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Abstract

Purpose The objective of this study was to evaluate the efficiency of vitrified blastocysts derived from frozen-thawed cleavage stage embryos in terms of morphological survival and re-expansion status of the blastocoelic cavity.

Results After warming 162 blastocysts derived from fresh embryos (= control group) and 90 blastocysts from frozen-thawed cleavage stage embryos (= study group) and after 2–3 h of in vitro culture the percentage of blastocysts with morphological survival was not different between the two groups. After 24 h of in vitro culture, the percentage of fully expanded, hatching or hatched blastocysts was not different between both groups.

Conclusion(s) The results show that blastocysts derived from frozen-thawed cleavage stage embryos can be cryopreserved successfully a second time by vitrification method. Re-cryopreservation by vitrification still needs to be approached with some caution because little data on long term safety of multiple freezing is available.

Keywords Re-cryopreservation · Vitrification · Human blastocyst · Morphological survival

Introduction

The objective of IVF should be to provide couples with a single healthy baby per transfer and if possible more than

one healthy baby per treatment cycle. To accomplish this goal single embryo transfer (SET) is becoming an increasingly accepted procedure [1]. A consequence of SET is an increase in the number of embryos available for cryopreservation and optimized cryopreservation after SET should be part of the strategy to decrease multiple pregnancies [1, 2]. Human embryos have been cryopreserved at the two pronuclear stages, at different multicellular cleavage stages or at the blastocyst stage using different slow-controlled-rate freezing and/or vitrification protocols. Although slow freezing procedures are still an option, vitrification appears to have better results for post-warming survival and for pregnancy rates [3]. In our centre, human embryos were frozen using a slow-controlled rate freezing procedure with 1,2-propanediol as cryoprotectant. Up to two embryos were frozen per straw. Consequently after thawing and overnight culture more than one good-quality embryo might be available for transfer. Therefore, in order to avoid double embryo transfer and the occurrence of multiple pregnancy, re-cryopreservation procedures at the blastocyst stage could be considered. So far, limited data has been published with human embryos that were frozen-thawed twice and the documentation on successful live births derived from re-frozen embryos or blastocysts is mostly limited to case reports [4–11]. Sheehan et al. [12] demonstrated in a mouse model that vitrification methods allowed multiple cryopreservations with minimal damage.

The papers of Kumasako et al., [11], Valle et al. [13] and Stanger et al. [14] described that embryos and blastocysts that had been frozen once either with slow-freezing or vitrification can be re-frozen by vitrification on day 3 or day 5/6.

The aim of our study was to evaluate the efficiency of re-cryopreservation by vitrification of day 6 blastocysts derived from slow-controlled-rate frozen and thawed cleavage stage human day 2/3 embryos.

Capsule Blastocysts derived from cryopreserved cleavage-stage embryos can be re-cryopreserved successfully by vitrification method. This finding opens possibilities to maximize the efficiency of the cryopreservation of cleavage-stage human embryos.

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Materials and methods

Source of embryos

Embryos for research were obtained after informed consent of the patients. The research was approved by the Federal Ethical committee (approval Adv027) and local Ethical Committee (approval 2009/660).

Embryos included in the study were either fresh or frozen–thawed spare embryos obtained in our IVF programme. They were spare embryos because they were of insufficient quality to be used for patient treatment. Insufficient quality was defined as embryos with high fragmentation ($\geq 25\%$) on day 2 or day 3 of development, and (or) multinucleated blastomeres on day 2, and (or) delayed development (< 5 blastomeres on day 3 or ≤ 1 blastomere increase from day 2 to 3). These embryos will be further referred to as poor quality embryos. The embryos were further cultured up to day 6 and used for the study when they reached at least the expanded blastocyst stage. Expanded blastocysts were then vitrified and as a result two groups of expanded vitrified blastocysts could be identified: (1) vitrified expanded blastocysts derived from non-frozen poor quality cleavage-stage embryos (once cryopreserved=control group) and (2) vitrified expanded blastocysts derived from slow controlled-rate frozen and thawed poor-quality cleavage-stage embryos (twice cryopreserved=study group).

Embryo culture and scoring of blastocysts

Before vitrification at the expanded blastocyst stage, poor quality non-frozen or frozen thawed cleavage-stage embryos were cultured in vitro in COOK media formulations (Cook Ireland Ltd., Limerick, Ireland).

Before vitrification, the quality of the trophectoderm (TE) and the inner cell mass (ICM) cells in the expanded blastocysts was evaluated according to the scoring system described by Stephenson [15]. Briefly, ICM grades A and B are given to large and distinct ICMs, with grade A being more compact and B having larger, less compact cells. Grade C is used for small ICMs, grade D for degenerating ICMs, and grade E when no apparent ICM is visible. For the scoring of the trophectoderm, grades A, B, or C correspond to good, moderate and poor quality respectively. Blastocyst expansion was scored from 1 (no expansion of overall size) to 6 (fully hatched from zona pellucida).

Blastocysts with minimal grade C trophectoderm and grade C inner cell mass were vitrified.

Blastocysts with a minimal grade B trophectoderm and grade B inner cell mass were considered as good-quality blastocyst.

Artificial shrinkage of expanded blastocyst

All expanded blastocysts were vitrified after initial artificial shrinkage (AS) of the blastocoele cavity by means of mechanical pipetting. Briefly, the expanded blastocyst was aspirated through a glass pipette which was slightly smaller in diameter than the diameter of the expanded blastocyst. After confirmation of initial shrinkage of the blastocoele, pipetting was performed with a pipette slightly smaller in diameter than the first one. This procedure was repeated two or three times until the blastocoele collapsed completely.

Vitrification of blastocysts

The vitrification procedure was done according to Kuwayama et al. [16] with some modifications. Briefly, all media were pre-warmed at 37°C , and all vitrification and warming steps were done on a warm plate set at 37°C .

Expanded blastocysts collapsed after AS were equilibrated for 2 min in equilibration medium consisting of Hepes-buffered medium supplemented with 20% v/v human serum albumin (HSA, Rode Kruis, Belgium) and also containing 7.5% v/v ethylene glycol (Sigma, Bornem) and 7.5% v/v dimethylsulfoxide (Sigma). The blastocysts were then transferred to the vitrification solution, consisting of Hepes-buffered medium with HSA and also containing 15% v/v ethylene glycol and 15% v/v dimethylsulfoxide and 0.5 M sucrose and after 45 to 60 s loaded onto a CBS-HS vitrification straw (Cryo BioSystem, I.M.V. Technologies) in a very small volume. After sealing, the straws were immediately plunged into liquid nitrogen (LN_2).

The total time required for loading, sealing, and plunging did not exceed the 30 s.

For warming, the CBS-HS straw was first transferred from the LN_2 into a 37°C warm Hepes-buffered medium also containing 1 M sucrose. The warmed blastocysts were then immediately transferred into a new drop of 37°C warm 1 M sucrose in Hepes-buffered medium supplemented with HSA and further incubated for 1 min. After that, the blastocysts were consecutively transferred for 3 min to a dilution solution of 37°C warm 0.5 M sucrose in Hepes-buffered medium supplemented with HSA and for 5 min to 37°C warm Hepes-buffered medium plus HSA. Warmed blastocysts were then incubated for 24 h in Cook Blastocyst medium and assessed for in vitro survival.

Assessment of survival

Main outcome measures were the survival rates of the blastocysts after warming and further in vitro culture for 2–3 h and 24 h. At the two different time points the blastocysts were examined under an inverted microscope at $\times 400$ magnification, and morphological survival rate was assessed based on the morphological integrity of the ICM and TE (fully intact

and moderately damaged). After 24 h of in vitro culture survival rate was also assessed as the capability of warmed blastocysts to expand or re-expand.

Statistics

Statistic analyses was performed using the Instat V 2.0 software package (Graph Pad Software, San Diego, CA, USA) and SPSS version 19 (SPSS software, Chicago, IL, USA)

Percentages of survival rate, developmental capacity were compared by contingency table analysis followed by chi-square subtests. The Mann–Whitney *U*-test was used to analyse the distribution of the different qualities of blastocysts vitrified in study and control group.

When $P < 0.05$, the difference was considered to be significant.

Results

During the study period 162 blastocysts were vitrified in the once cryopreserved control group and 90 blastocysts were vitrified in the twice cryopreserved study group. The distribution of the different qualities (ICM and TE) of the expanded blastocysts vitrified in both groups, is presented in Table 1. There was no difference in the distribution of the different qualities of expanded blastocysts vitrified between both groups (Mann–Whitney *U*-Test, $P = 0.12$). In both groups expanded blastocysts with at least grade B ICM and grade B TE were considered as good-quality blastocysts.

The percentage of blastocysts with morphological survival after 2–3 h and 24 h was not different between the twice

cryopreserved study group and the once cryopreserved control group [71.1 % (64/90) vs. 78.4 % (127/162), $P = 0.22$] and [66.7 % (60/90) vs. 72.8 % (118/162), $P = 0.32$] Furthermore, the percentage of good-quality blastocysts with morphological survival after 2–3 h and 24 h was not different between the twice cryopreserved study group and the once cryopreserved control group [68.4 % (52/76) vs. 76.3 % (74/97), $P = 0.30$] (Table 2). In the study group of twice cryopreserved blastocysts, the blastocysts were derived from a mixture of frozen cleavage-stage embryos that were either fully intact or had more than 50 % intact blastomeres after thawing. After 24 h culture, vitrified and warmed blastocysts derived from fully intact frozen cleavage stage embryos after thawing had a morphological survival rate of 71.2 % (37/52) which was comparable to 60.5 % (23/38) for vitrified and warmed blastocysts derived from non-fully intact frozen and thawed cleavage-stage embryos (data not show in Table 2).

After 24 h of in vitro culture, the expansion/hatching status of the surviving blastocysts was evaluated. The results are presented in Table 3. The percentage of fully expanded [46.7 % (28/60) vs. 42.4 % (50/118), $P = 0.63$, hatching [30.0 % (18/60) vs. 26.3 % (31/118), $P = 0.59$ or hatched blastocysts [13.3 % (8/60) vs. 16.9 % (20/118), $P = 0.27$] obtained was not different between the twice cryopreserved study group and the once cryopreserved control group respectively.

Furthermore, after 24 h of in vitro culture the expansion/hatching status of the surviving good-quality blastocysts was evaluated and presented in Table 3.

The percentage of fully expanded [50.0 % (26/52) vs. 41.9 % (31/74), $P = 0.47$], hatching [30.8 % (16/52) vs. 35.1 % (26/74), $P = 0.70$] and hatched blastocyst [13.5 % (7/52) vs. 22.9 % (17/74), $P = 0.24$] obtained was not different between the twice cryopreserved study group and the once cryopreserved control group respectively.

Table 1 Distribution of the different qualities of blastocysts vitrified in study and control group

	Study group	Control group
No. of blastocysts	90	162
Blastocyst scoring (ICM – TE)		
No. of A-A blastocysts (*)	23	41
No. of A-B blastocysts (*)	10	12
No. of A-C blastocysts	1	3
No. of B-A blastocysts (*)	16	17
No. of B-B blastocysts (*)	27	27
No. of B-C blastocysts	7	11
No. of C-A blastocysts	1	3
No. of C-B blastocysts	4	17
No. of C-C blastocysts	1	31

(*) These blastocysts were considered as good-quality blastocyst

There was no significant difference in the distribution of the different qualities of expanded blastocysts vitrified between both groups (Mann–Whitney *U*-Test, $P = 0.12$)

Discussion

Vitrification of reproductive cells is a relative new approach in human IVF but it is clear from literature that vitrification is a

Table 2 Morphological survival of vitrified blastocysts

	Study group	Control group	<i>P</i> -value
Morphological survival of blastocysts after			
2–3 h	64/90 (71.1 %)	127/162 (78.4 %)	0.22
24 h	60/90 (66.7 %)	118/162 (72.8 %)	0.19
Morphological survival of good quality blastocysts after			
2–3 h	56/76 (73.7 %)	80/97 (82.5 %)	0.32
24 h	52/76 (68.4 %)	74/97 (76.3 %)	0.30

Table 3 The expansion status of the survived blastocysts after 24 h in vitro culture

	Study group	Control group	<i>P</i> -value
No. of survived blastocyst after 24 h of culture	60	118	
No. of fully expanded blastocyst (%)	28 (46.7)	50 (42.4)	0.63
No. of hatching blastocysts (%)	18 (30.0)	31 (26.3)	0.59
No. of hatched blastocysts	8 (13.3)	20 (16.9)	0.27
No. of survived good quality blastocyst (%) after 24 h of culture	52	74	
No. of fully expanded blastocyst (%)	26 (50.0)	31 (41.9)	0.47
No. of hatching blastocysts (%)	16 (30.8)	26 (35.1)	0.70
No. of hatched blastocysts (%)	7 (13.5)	17 (22.9)	0.24

better technique for the cryopreservation of reproductive cells as compared to slow-controlled-rate freezing [3, 17]. Therefore in the present study we vitrified expanded blastocysts with a minimal grade C trophectoderm and grade C inner cell mass. For clinical purposes blastocyst morphology before cryopreservation is an important tool, because the quality of the blastocyst will have a significant impact on survival rate. It is known that the efficiency of the vitrification method depends on the expansion status of the blastocyst, with better survival rates at morula or early blastocyst stages compared to full or expanded blastocysts [18, 19]. Therefore as suggested by Vanderzwalmen et al. [18, 19] in the present study we artificially collapsed the blastocyst to minimize the cavity volume. The capacity to vitrify and warm expanded artificially collapsed blastocysts that have already been cryopreserved at the cleavage-stage demonstrate that vitrification causes minimal cryo injury. Minimal morphological damage is observed and in terms of viability by retaining the capacity to expand and re-expand after further culture in vitro. The results of our study demonstrate the feasibility of vitrification of expanded human blastocysts obtained from slow-controlled rate frozen and thawed cleavage-stage embryos. The morphological survival of these vitrified expanded blastocysts, which are cryopreserved twice, and their developmental potential after 24 h of further culture in vitro was not different from vitrified human blastocysts that were derived from cleavage stage embryos that has been cryopreserved once. The results obtained indicate that vitrification at the expanded blastocyst stage may be an efficient method for cleavage-stage embryo freezing and subsequent re-cryopreservation after thawing. In literature, there have been case reports and papers detailing on the re-cryopreservation by either slow-freezing and slow-refreezing, slow freezing and re-vitrification, vitrification and re-vitrification involving several species and at different developmental stages, [12–14, 20–28] which demonstrates that re-cryopreservation of mammalian embryos is possible. However, there are limited reports describing the vitrification of human expanded blastocysts obtained from slow-

controlled-rate frozen and thawed cleavage-stage embryos. Stanger et al. [14] reported on 7 cases only where initial freezing was at the cleavage stage and re-vitrification at the blastocyst stage. He obtained 4 clinical pregnancies. Our results support and extend the observations by Stanger et al. [14]. Of course, we do not know the in vivo implantation potential of these blastocysts but the promising results on the morphological survival and re-expansion of these blastocysts may have an important implication for the practical management of infertility cases where cleavage stage freezing has been or still is considered.

In general, further in vitro culture to the expanded blastocyst stage allows us to select those embryos with the highest implantation potential. When patients return for a cryopreserved embryo transfer, they may elect to have individual cleavage stage embryos frozen, thawed and transferred over multiple cycles or to thaw multiple embryos. When thawing multiple embryos as is inevitably the case when more than one embryo is frozen per straw, several surviving (fully intact and partially intact) cleavage stage embryos may co-exist. From literature we know that partially intact thawed cleavage stage embryo's can result in pregnancies, but that the implantation potential drops in parallel with increasing degrees of blastomere lysis [29–31]. Our study supports the idea of extending culture of surviving cleavage-stage embryos up to the blastocyst stage, in order to help to identify the best embryo for transfer. Since our study indicates that vitrification of expanded blastocysts obtained from slow-controlled-rate frozen and thawed cleavage stage embryos is a validated and robust procedure, the advantage in thawing many embryos and extending their culture is that it allows the patient to maximize their chance of pregnancy in a shorter time period.

In conclusion, our results show that blastocysts derived from frozen-thawed cleaved-stage embryos can successfully be cryopreserved a second time by vitrification. The cryopreservation by vitrification could be a valuable option to increase the cumulative pregnancy rate while decreasing the risk of multiple gestations, but still needs to be approached with some

caution because little data is available on the long term safety of multiple cryopreservations.

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References

- Gerris J, De Neubourg D, De Sutter P, Van Royen E, Mangelschots K, Vercruyssen M. Cryopreservation as a tool to reduce multiple birth. *Reprod Biomed Online*. 2003;7:286–94.
- Neubourg DD, Mangelschots K, Van Royen E, Vercruyssen M, Ryckaert G, Valkenburg M, et al. Impact of patients' choice for single embryo transfer of a top quality embryo versus double embryo transfer in the first IVF/ICSI cycle. *Hum Reprod*. 2002;17:2621–5.
- Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, et al. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertil Steril*. 2008;90:186–93.
- Baker A, Check J, Lurie D, Hourani C, Hoover L. Pregnancy achieved with pronuclear-stage embryos that were cryopreserved and thawed twice: a case report. *J Assist Reprod Genet*. 1996;13:713–5.
- Yokota Y, Yokota H, Yokota M, Sato S, Araki Y. Birth of healthy twins from in vitro development of human refrozen embryos. *Fertil Steril*. 2001;76:1063–5.
- Check JH, Brittingham D, Swenson K, Wilson C, Lurie D. Transfer of refrozen twice-thawed embryos do not decrease the implantation rate. *Clin Exp Obstet Gynecol*. 2001;28:14–6.
- Farhat M, Zentner B, Lossos F, Bdolah Y, Holtzer H, Hurwitz A. Successful pregnancy following replacement of embryos previously refrozen at blastocyst stage: case report. *Hum Reprod*. 2001;16:337–9.
- Takahashi T, Araki Y. Successfully healthy baby delivery from human refrozen blastocyst embryos by vitrification. *J Mammal Ova Res*. 2007;21:162–5.
- Estes S, Laky D, Hoover L, Smith S, Schinfeld J, Somkuti S. Successful pregnancy resulting from cryopreserved pronuclear and cleaved embryos thawed and cultured to blastocysts, refrozen and transferred. A case report. *J Reprod Med*. 2003;48:46–8.
- Hiraoka K, Funchiaki M, Hiroaka K, Horiuchi T, Murakami T, Kinutani M, et al. Re-cryopreservation by vitrification of human blastocysts developed from frozen-cleaved embryos: a report of 15 cycles. *J Mammal Ova Res*. 2007;24:23–8.
- Kumasako Y, Otsu E, Utsunomiya T, Araki Y. The efficacy of the transfer of twice frozen-thawed embryos with the vitrification method. *Fertil Steril*. 2009;91:383–6.
- Sheehan CB, Lane M, Gardner DK. The CryoLoop facilitates re-vitrification of embryos at four successive stages of development without impairing embryo growth. *Hum Reprod*. 2006;21:2978–84.
- Valle M, Guimarães F, Cavagnoli M, Sampaio M, Geber S. Birth of normal infants after transfer of embryos that were twice vitrified/warmed at cleavage stages: report of two cases. *Cryobiology*. 2012;65:332–4.
- Stanger J, Wong J, Conceicao J, Yovich J. Vitrification of human embryos previously cryostored by either slow freezing or vitrification results in high pregnancy rates. *RBM Online*. 2012;24:314–20.
- Stephenson E, Braude P, Mason C. International community consensus standard for reporting derivation of human embryonic stem cell lines. *Regen Med*. 2007;2:349–62.
- Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *RBM Online*. 2005;11:608–14.
- Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertil Steril*. 2006;86:70–80.
- Vanderzwalmen P, Zech N, Prapas Y, Prapas N, Nijs M, Vandamme B, et al. Pregnancy and implantation rates after transfer of fresh and vitrified embryos on day 4 or 5. *J Assist Reprod Genet*. 1999;16:147.
- Vanderzwalmen P, Bertin G, Debauche C, Standaert V, van Roosendaal E, Vandervorst M, et al. Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum Reprod*. 2002;17:744–51.
- Vitale NJ, Myers MW, Denniston RS, Leibo SP, Godke RA. In-vitro development of refrozen mouse embryos. *Hum Reprod*. 1997;12:310–6.
- Fathi R, Valojerdi MR, Yazdi PE, Ebrahimi B, Alipour H, Hassani F. Development of 4-cell mouse embryos after re-vitrification. *Cryobiology*. 2012;64:23–6.
- Smith LK, Roots EH, Dorsett MJ. Live birth of a normal healthy baby after a frozen embryo transfer with blastocysts that were frozen and thawed twice. *Fertil Steril*. 2005;83:198–200.
- Sills E, Murray G, Genton M, Walsh DJ, Coull G, Walsh A. Clinical features and reproductive outcomes for embryos undergoing dual freeze-thaw sequences followed by blastocyst transfer: critique of 14 consecutive cases in IVF. *Fertil Steril*. 2009;91:1568–70.
- Reed ML, Hamic A, Caperton CL, Thompson DJ. Live birth after anonymous donation of twice-cryopreserved embryos that had been stored in liquid nitrogen for a cumulative storage time of approximately 13.5 years. *Fertil Steril*. 2010;94:2771.e1–e3.
- Murakami M, Egashira A, Murakami K, Araki Y, Kuramoto T. Perinatal outcome of twice-frozen-thawed embryo transfers: a clinical follow-up study. *Fertil Steril*. 2011;95:2648–50.
- Asimakopoulos B, Kotanidis L, Nikolettos N. In vitro maturation and fertilization of vitrified immature human oocytes, subsequent vitrification of produced embryos, and embryo transfer after thawing. *Fertil Steril*. 2011;95:2123.e1–2.
- Chang C, Sharipo D, Bernal D, Wright G, Kort H, Nagy Z. Two successful pregnancies obtained following oocyte vitrification and embryo re-vitrification. *RBM online*. 2008;16:346–9.
- Cobo A, Castellò D, Vallejo B, Albert C, de los Santos JM, Remohí J. Outcome of cryotransfer of embryos developed from vitrified oocytes: double vitrification has no impact on delivery rates. *Fertil Steril*. 2013;99:1623–30.
- Van den Abbeel E, Camus M, Van Waesberghe L, Devroey P, Van Steirteghem A. Viability of partially damaged human embryos after cryopreservation. *Hum Reprod*. 1997;12:2006–10.
- Edgar DH, Bourne H, Speirs A, McBain J. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod*. 2000;15:175–9.
- Guerif F, Bidault R, Cadoret V, Couet M, Lansac J, Royere D. Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal. *Hum Reprod*. 2002;17:1321–6.