

Influence of cell loss after vitrification or slow-freezing on further *in vitro* development and implantation of human Day 3 embryos

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STUDY QUESTION: Is the effect of cell loss on further cleavage and implantation different for vitrified than for slowly frozen Day 3 embryos?

SUMMARY ANSWER: Vitrified embryos develop better overnight than slowly frozen embryos, regardless of the number of cells lost, but have similar implantation potential if further cleavage occurs overnight.

WHAT IS KNOWN ALREADY: After slow-freezing, similar implantation rates have been obtained for intact 4-cell embryos or 4-cell embryos with 1 cell damaged. For slowly frozen Day 3 embryos, lower implantation rates have been observed when at least 25% of cells were lost. Other studies reported similar implantation potential for 7- to 8-cell embryos with 0, 1 or 2 cells damaged. No data are available on further development of vitrified embryos in relation to cell damage.

STUDY DESIGN, SIZE, DURATION: Survival and overnight cleavage were retrospectively assessed for 7664 slowly frozen Day 3 embryos (study period: January 2004–December 2008) and 1827 vitrified embryos (study period: April 2010–September 2011). Overnight cleavage was assessed according to cell stage at cryopreservation and post-thaw cell loss for both protocols. The relationship between cell loss and implantation rate was analysed in a subgroup of single-embryo transfers (SETs) with 780 slowly frozen and 294 vitrified embryos.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Embryos with ≥ 6 blastomeres and $\leq 20\%$ fragmentation were cryopreserved using slow controlled freezing [with dimethyl sulphoxide (DMSO) as cryoprotectant] or closed vitrification [with DMSO-ethylene glycol (EG)-sucrose (S) as cryoprotectants]. Only embryos with $\geq 50\%$ of cells intact after thawing were cultured overnight and were only transferred if further cleaved. For each outcome, logistic regression analysis was performed.

MAIN RESULTS AND ROLE OF CHANCE: Survival was 94 and 64% after vitrification and slow-freezing respectively. Logistic regression analysis showed that overnight cleavage of surviving embryos was higher after vitrification than after slow-freezing ($P < 0.001$) and decreased according to the degree of cell damage ($P < 0.001$). If the embryo continued to cleave after thawing, there was no effect of the number of cells lost or the cryopreservation method on its implantation potential. The implantation rates of embryos with 0, 1 or 2 cells damaged were, respectively, 21% ($n = 114$), 21% ($n = 28$) and 20% ($n = 12$) after slow-freezing and 20% ($n = 50$), 21% ($n = 5$) and 27% ($n = 4$) after vitrification.

LIMITATIONS, REASONS FOR CAUTION: This analysis is retrospective and study periods for vitrification and slow-freezing are different. The number of SETs with vitrified embryos is limited. However, a large number of vitrified embryos were available to analyse the further cleavage of surviving embryos.

WIDER IMPLICATIONS OF THE FINDINGS: Although it is not proved that vitrified embryos are more viable than slowly frozen embryos in terms of pregnancy outcome, vitrification yields higher survival rates, better overnight development and higher transfer rates per embryo warmed. This increases the number of frozen transfer cycles originating from a single treatment and might result in a better cumulative clinical outcome. Based on the present data, the policy to warm an extra embryo before overnight culture depends on the cell stage at cryopreservation and the cell damage after warming. For 8-cell embryos, up to two cells may be damaged compared with only one cell for 6- to 7-cell embryos, before an additional embryo is warmed.

STUDY FUNDING/COMPETING INTEREST(S): none.

Key words: vitrification / slow-freezing / frozen embryo transfer / cell loss / cryopreservation

Introduction

To optimize our cryopreservation program, vitrification was introduced in our centre in 2010 as the standard cryopreservation method for Day 3 embryos instead of slow-freezing. The decision to switch the cryopreservation method was based on the improved survival rate and implantation rate obtained after blastocyst vitrification (Van Landuyt et al., 2011) compared with slow-freezing of blastocysts and on the successful results in a preliminary study on Day 3 vitrification (L. Van Landuyt, unpublished results).

The developmental potential of cryopreserved cleavage-stage embryos has been investigated by several authors and was initially based on slow-freezing and thawing results. Higher implantation rates have been obtained for fully intact Day 2 embryos than that for damaged ones (Van den Abbeel et al., 1997; El-Toukhy et al., 2003). Blastocyst formation rate was higher for intact Day 2 embryos than that for damaged ones (Archer et al., 2003). Later on, similar implantation rates were reported for intact 4-cell embryos and 4-cell embryos with 1 cell damaged after thawing (Gabrielson et al., 2006; Edgar et al., 2007). For day 3 embryos, lower implantation rates were observed when at least 25% of cells were damaged (Tang et al., 2006). However, other studies reported a similar implantation potential for 7- to 8-cell embryos with 0, 1 or 2 cells damaged (Zheng et al., 2008).

Several studies showed higher survival rates and a higher number of fully intact embryos after vitrification than after slow-freezing (Kuwayama et al., 2005; Raju et al., 2005; Balaban et al., 2008; Valojerdi et al., 2009; Wang et al., 2012). Balaban et al. (2008) reported that the development to the blastocyst stage was higher after vitrification than after slow-freezing. However, overnight cleavage rate and implantation potential of intact versus damaged embryos of different cell stages after vitrification have not been analysed so far.

In the present study, we investigated the survival, further cleavage and transfer rate of different cell stages on Day 3 of development after cryopreservation using either slow-freezing or vitrification. The impact of cell loss on the implantation potential was analysed in single frozen-embryo transfers (SFETs) for both cryopreservation methods.

Materials and Methods

Study design

In a first retrospective analysis, morphological survival was assessed for 7664 frozen-thawed (slow-freezing) Day 3 embryos of all IVF/ICSI thawing cycles carried out between January 2004 and December 2008. The same analysis was performed for 1827 vitrified-warmed Day 3 embryos of warming cycles between April 2010 and September 2011. Frozen embryo transfer (FET) cycles originating from *in vitro* maturation cycles were excluded from the study.

Morphological survival was analysed in relation to cell stage before cryopreservation. The overnight development was assessed according to the number of cells damaged and this was done for each of the different cell stages.

In a second analysis, the implantation rate was analysed after SFET in patients <37 years old for 780 slow-freezing cycles and 294 vitrification cycles. The implantation rate was correlated with the cell stage before cryopreservation and cell loss after thawing/warming.

Ovarian stimulation and IVF/ICSI treatment

Female patients underwent ovarian stimulation using urinary (Menopur[®], Ferring, Limhamn, Sweden) or recombinant FSH (Puregon[®], MSD, Oss, The Netherlands; Gonal F[®], SA Merck Serono, Geneva, Switzerland) in combination with GnRH antagonist (Orgalutran[®], MSD, Oss, The Netherlands; Cetrotide[®], SA Merck Serono, Geneva, Switzerland) or GnRH agonist (Suprefact, Aventis Pharma, Frankfurt, Germany) co-treatment. Final oocyte maturation was induced by injection of 10 000 IU hCG (Pregnyl[®]; MSD, Oss, The Netherlands), as soon as three follicles of 17 mm were observed on ultrasound scan. Oocyte retrieval was carried out using vaginal ultrasound-guided puncture of ovarian follicles 36 h after hCG administration. IVF and ICSI treatments were carried out as described by Van Landuyt et al. (2005).

Embryo selection for cryopreservation and evaluation after thawing/warming

Supernumerary day 3 embryos with at least six blastomeres and $\leq 20\%$ fragmentation were selected for cryopreservation after fresh Day 3 embryo transfer. Embryos were evaluated for morphological survival immediately after thawing/warming. The morphological survival rate was determined by counting the number of intact blastomeres compared with the number of blastomeres at cryopreservation. Embryos with at least 50% of their cells intact were considered sufficiently intact and were cultured overnight in a blastocyst medium. Further cleavage was evaluated the next morning, after ~ 24 h culture. Only embryos with further cleavage of at least one cell were considered for assessment of implantation after single-embryo transfer (SET). In case of SET, only few patients received a non-cleaving embryo and their results were analysed separately.

Preparation of the FET cycle

In our centre, the most common modality for FET is the natural cycle, either with administration of human chorionic gonadotrophin (hCG) for planning the FET or by detecting the spontaneous LH peak. In patients with amenorrhea or oligomenorrhea, an artificial cycle is proposed for endometrial preparation with exogenous estrogen and progesterone, with or without the addition of a GnRH agonist, as described by Kolibianakis et al. (2003).

Embryos were thawed/warmed 1 day prior to embryo transfer and replaced as a Day 4 embryo in a Day +3 endometrium.

Cryopreservation protocols

Slow-freezing of Day 3 embryos involved a slow controlled freezing and thawing protocol with dimethylsulphoxide (DMSO) as the cryoprotectant as described previously (Van den Abbeel and Van Steirteghem, 2000). For vitrification of embryos, the closed vitrification procedure was performed using CBS-VIT high-security (HS) straws (Cryo Bio System, L'Aigle, France) in combination with DMSO-ethylene glycol (EG)-sucrose (S) as the cryoprotectants (Irvine Scientific[®] Freeze Kit, Newtownmountkennedy, County Wicklow, Ireland) (Van Landuyt et al., 2011).

Outcome measures

Morphological survival after thawing was expressed as the percentage of thawed embryos that remained fully intact or retained at least 50% of their cells intact. Further cleavage was defined as the formation of at least one extra cell during the overnight incubation and was expressed as the percentage of surviving embryos that exhibited overnight cleavage. A clinical pregnancy was defined as an intrauterine gestational sac observed at

transvaginal ultrasound scan at least 5 weeks after embryo transfer (Zegers-Hochschild *et al.*, 2009).

Statistical analysis

Data are presented as number of events with numerator/denominator and percentages. Groups of interest were compared by using χ^2 or Fisher's exact tests with the significance level set at $P < 0.05$. For each outcome of interest, logistic regression analyses were performed to detect those factors determining outcomes. Computational procedures were performed using Excel 2003 (Microsoft® Office Excel 2003, Redmond, Washington, USA) and IBM® SPSS® Statistics, version 20 (IBM Corporation, 2011, Armonk, New York, USA).

Results

Analysis of immediate morphological survival

Table I presents the number of cells damaged for the different embryo stages for 7664 slowly frozen and 1827 vitrified-warmed day 3 embryos. The overall survival rate after vitrification was 94.3% and the percentage of fully intact embryos was 77.5%. These rates were significantly higher ($P < 0.001$) than that for frozen-thawed embryos (64.4 and 39.2%). The percentage of embryos with $\geq 50\%$ intact cells after slow-freezing was lower for embryos that had >8 blastomeres on day 3 (60.7%) than that for 6, 7 or 8-cell embryos (64.6%, 64.3% and 65.9% respectively) ($P < 0.01$). After vitrification, there was no difference in the total survival rate for the different cell stages.

Analysis of further cleavage and transfer rates

The overnight cleavage rate of all embryos with at least 50% cells intact is higher after vitrification (88.8% and 1530/1723) than that after slow-freezing (80.6%, 3978 of 4935 and $P < 0.001$). Figure 1 presents the overnight cleavage of surviving embryos according to the number of cells lost after both slow-freezing and vitrification. Logistic regression analysis showed a significant and independent effect of the number of cells damaged and the cryopreservation method (both $P < 0.001$, Fig. 1).

The overnight cleavage rate of the surviving embryos after both slow-freezing and vitrification was further stratified according to the cell stage at cryopreservation and the number of cells damaged after thawing/warming (Table II). In the case of slow-freezing, logistic regression analysis showed a highly significant independent effect of the number of cells damaged ($P < 0.001$) and a significant independent effect of the cell stage at freezing ($P = 0.046$) on overnight cleavage was demonstrated. After vitrification, however, embryos with at least eight cells seemed to have higher cleavage rates than 6- and 7-cell embryos ($P < 0.001$), but no independent effect of the cell stage at vitrification on further cleavage was found ($P = 0.367$). This finding might presumably be related to the lower numbers of embryos included in each group.

The overall transfer rate per embryo thawed was 49.1% after slow-freezing compared with 86.3% for vitrified embryos ($P < 0.0001$, Table III). For both methods, embryos with at least eight cells before cryopreservation had a higher transfer rate after thawing/warming than embryos with six or seven cells (Table III).

Analysis of implantation rates in single FETs

In total, two subgroups of 780 and 294 SET cycles of frozen-thawed and vitrified Day 3 embryos, respectively, were analysed. The following analyses include only the transfer of surviving embryos (at least 50% of cells intact) with overnight cleavage. Only a minority of SET was performed with a non-cleaving embryo and the outcome of those embryo transfers was described separately.

The overall implantation rate was similar after slow-freezing (20.5% and 160/780) and vitrification (20.7%, 61/294). Live birth rate after slow-freezing was 16.1% (125 of 777, 3 pregnancies with unknown outcome) and was similar to the live birth rate after vitrification (13.0%, 37 of 285, 9 pregnancies with unknown outcome).

Figure 2 presents the implantation rates in relation to the cell stage before cryopreservation for both methods. After logistic regression analysis, no independent effect was found neither for the cell stage at cryopreservation (6, 7, 8 or >8 cells) on the implantation rate, nor for the method of cryopreservation.

Table I Characterization of 7664 frozen-thawed (slow-freezing [SF]) and 1827 vitrified-warmed (VIT) Day 3 embryos according to cell stage at freezing and cell loss after thawing. Data are *n* (%)

Cell stage	Number thawed	Number of cells damaged					Total survived
		0	1	2	3	4	
6-cell SF	1341	555 (41.4)	140 (10.4)	87 (6.5)	84 (6.3)	—	866 (64.6)
6-cell VIT	127	102 (80.3)	9 (7.1)	6 (4.7)	2 (1.6)	—	119 (93.7)
7-cell SF	1493	599 (40.1)	153 (10.2)	113 (7.6)	95 (6.4)	—	960 (64.3)
7-cell VIT	278	212 (76.3)	23 (8.3)	16 (5.8)	12 (4.3)	—	263 (94.6)
8-cell SF	3551	1415 (39.8)	342 (9.6)	240 (6.8)	169 (4.8)	173 (4.9)	2339 (65.9)
8-cell VIT	903	726 (80.4)	63 (4.8)	27 (3.0)	18 (2.0)	20 (2.2)	854 (94.6)
>8 -cell SF	1279	438 (34.3)	125 (9.8)	72 (9.4)	61 (7.9)	74 (5.8)	770 (60.7)
>8 -cell VIT	519	376 (72.4)	44 (8.5)	41 (7.9)	16 (3.1)	10 (1.9)	487 (93.8)
Total SF	7664	3007 (39.2)	760 (9.9)	512 (6.7)	409 (5.3)	247 (3.2)	4935 (64.4)
Total VIT	1827	1416 (77.5)	139 (7.6)	90 (4.9)	48 (2.6)	36 (2.0)	1723 (94.3)

Total survival rate compared between all cell stages after vitrification: Fisher's exact test P value 0.910. Total survival rate compared between all cell stages after slow-freezing: Fisher's exact test P value 0.004. Values (%) in bold are total survival rates for all slowly-frozen and vitrified embryos: χ^2 -test, $P < 0.001$. Percentages in bold are the mean survival rates (all cell stages) for vitrification and slow freezing: P value < 0.001 .

Figure 3 presents the implantation rate in relation to the number of cells damaged after thawing/warming for both methods. The results were analysed irrespective of their developmental stage at cryopreservation or at the moment of embryo transfer. Similar implantation rates were obtained for intact embryos versus damaged embryos. However, only a limited number of transfers were performed for embryos with two or three cells lost. Furthermore, no independent effect was observed for the method of cryopreservation.

Although embryos that cleaved further were preferably selected for embryo transfer, in a few patients embryos without resumption of mitosis were transferred in SFET (not included in Figs 2 and 3). After slow-freezing, 12 embryos that did not cleave further were transferred but no pregnancies were obtained. After vitrification, 23 transfers were performed with non-cleaving embryos resulting in two positive hCGs. However, one pregnancy was biochemical and the second one

resulted in a miscarriage. Thus, none of the post-thaw arrested embryos resulted in an ongoing pregnancy.

Discussion

The present study aimed to analyse the post-thaw survival and the overnight cleavage rate of Day 3 embryos in relation to their cell stage before cryopreservation and cell loss post-thawing/warming for both slow-freezing and vitrification. Survived vitrified embryos developed better overnight than slowly frozen embryos, irrespective of the number of cells damaged and the cell stage at cryopreservation. Implantation rates were assessed in SFET to determine the impact of cell stage at cryopreservation and cell loss after thawing/warming on the viability of the embryo. In 2010, our centre switched from slow-freezing to vitrification of Day 3 embryos, and thus data on both cryopreservation methods were available and were compared retrospectively. The decision to switch was based on the successful results obtained with blastocyst vitrification (Van Landuyt et al., 2011) and with Day 3 vitrification in a preliminary study (L. Van Landuyt, unpublished results). Because of the retrospective design of the study and the different study periods for slow-freezing and vitrification, we agree that some bias may have influenced the results of this study. However, only because of this retrospective nature, it was possible to include a large number of embryos providing valuable information on survival in relation to cell stage and further cleavage in relation to cell loss. Moreover, embryo selection criteria for cryopreservation and the embryologist staff evaluating the embryos did not change over time. Cryopreservation protocols did not change for slow-freezing or vitrification, nor did or culture system or gas environment. We always used sequential media for embryo culture although from different companies. It is true that using different sequential media can result in different cleavage patterns or developmental speed of embryos in the fresh cycle, but we never experienced an effect on post-thaw survival. Only the presence or absence of post-thaw further cleavage and not the pattern or speed of development was taken into consideration for this analysis.

In the study of Balaban et al. (2008), a cohort of 466 Day 3 embryos donated for research (at least five cells and <20% fragmentation) was randomized to undergo either vitrification or slow-freezing. After post-thaw culture to Day 6, significantly more blastocysts were obtained in the vitrified group (60.3 versus 49.5%). However, no information was

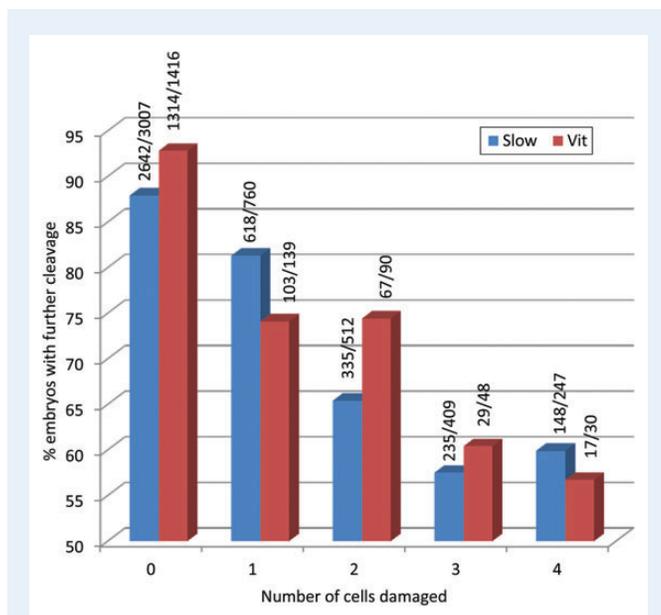


Figure 1 Overnight cleavage in relation to the number of cells lost in frozen-thawed (slow) and vitrified-warmed (Vit) Day 3 embryos for all cell stages. Results of logistic regression analysis: independent effect of numbers of cells damaged: $P < 0.001$; independent effect of cryopreservation technique: $P < 0.001$.

Table II Overnight cleavage in relation to the number of cells lost and cell stage at cryopreservation in slow frozen-thawed (SF) and vitrified-warmed (VIT) Day 3 embryos. Data are embryos with further cleavage/embryos to survive thawing (%)

Cell stage	No. of cells damaged				
	0	1	2	3	4
6- and 7-cell SF	1014/1154 (87.9)	223/293 (76.1)	114/200 (57.0)	77/179 (43.0)	/
8- and >8-cell SF	1628/1853 (87.9)	395/467 (84.6)	221/312 (70.8)	158/230 (68.7)	148/247 (59.9)
6- and 7-cell VIT	280/314 (89.2)	18/32 (56.3)	11/22 (50.0)	6/14 (42.9)	/
8- and >8-cell VIT	1034/1102 (93.8)	85/107 (79.4)	56/68 (82.4)	23/34 (67.6)	17/30 (56.7)

Results of logistic regression analysis after slow-freezing (SF): independent effect of numbers of cells damaged: $P < 0.001$; independent effect of cell stage: $P = 0.046$. Results of logistic regression analysis after vitrification (VIT): independent effect of numbers of cells damaged: $P < 0.001$; independent effect of cell stage: $P = 0.367$.

Table III Transfer rate (no. of transferred embryos/no. of thawed embryos (%)) of frozen-thawed/warmed Day 3 embryos in relation to cell stage at cryopreservation

Cell stage	Slow-freezing*	Vitrification**
6-cell	604/1341 (45.0) ^{a,c}	98/127 (77.2) ^{d,e}
7-cell	704/1493 (47.2) ^b	226/278 (81.3) ^{f,g}
8-cell	1831/3551 (51.6) ^{a,b}	792/903 (87.7) ^{d,f}
>8-cell	627/1279 (49.0) ^c	461/519 (88.8) ^{e,g}
Total	3766/7664 (49.1)	1577/1827 (86.3)

Percentages in bold are the mean transfer rates after slow freezing and vitrification.

^a*P* < 0.001.

^b*P* < 0.01.

^c*P* < 0.05.

^d*P* < 0.01.

^e*P* < 0.001.

^f*P* < 0.01.

^g*P* < 0.01.

*For all cell stages in the slow-freezing group: Fisher's exact test *P* value < 0.001.

**For all cell stages in the vitrified group: Fisher's exact test *P* value < 0.001.

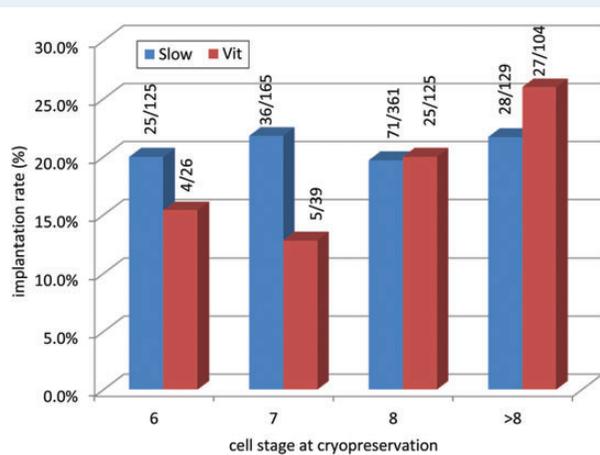


Figure 2 Implantation rates of embryos that showed further cleavage in relation to cell stage at cryopreservation analysed in 780 (slow-freezing) and 294 (vitrification [Vit]) single FETs. Results of logistic regression analysis: independent effect of cryopreservation technique: *P* = 0.965; independent effect of cell stage at cryopreservation: *P* = 0.885.

given on the cell stage at cryopreservation and post-thaw cell damage in both groups.

The present data confirmed that damaged Day 3 embryos have a lower overnight developmental potential than intact embryos, both after vitrification and slow-freezing. Furthermore, overnight cleavage significantly decreases with increasing number of cells lost. In the slow-freezing group, the impact of cell damage on overnight cleavage was more detrimental in 6- and 7-cell embryos than in embryos with at least eight cells. This finding was not observed after vitrification, probably due to the lower number of damaged embryos. Regardless of the cryopreservation method, our data confirmed our expectations that the impact of the number of cells lost is more detrimental in 6 and 7-cell

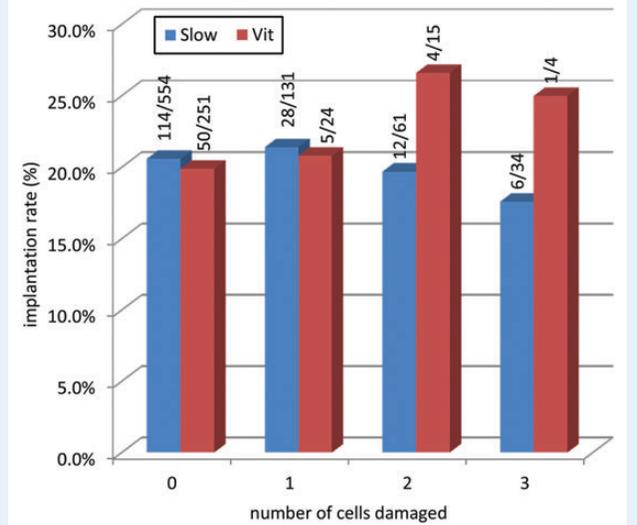


Figure 3 Implantation rates of embryos that showed further cleavage in relation to the number of cells damaged analysed in 780 (slow-freezing) and 294 (vitrification [Vit]) single FETs. Results of logistic regression analysis: independent effect of cryopreservation technique: *P* = 0.921; independent effect of number of cells damaged: *P* = 0.990.

embryos than in embryos with ≥ 8 cells, as they lose a higher percentage of cell volume compared with an 8-cell embryo. However, even when the data were analysed with respect to the percentage of cell damage (and not according to the number of cells lost), higher cleavage rates were found for 8-cell embryos (data not included). This was also reflected in the significantly higher transfer rates per thawed/warmed embryo obtained with embryos with at least eight cells. This implies a better intrinsic quality of normal developing embryos. In this view, the decision to warm an extra embryo before overnight culture at our centre now depends on the cell stage at cryopreservation and the post-warming cell damage. For 8-cell embryos, up to two cells can be damaged compared with only one cell for 6- to 7-cell embryos, to be considered sufficient for overnight culture without warming an additional embryo.

This study also showed that once a survived embryo shows resumption of mitosis after thawing, there was neither an impact of the cell stage at cryopreservation, nor of the cell damage on its implantation capacity. Edgar *et al.* (2007) also observed for Day 2 slowly frozen 4-cell embryos that resumption of mitosis of at least two blastomeres, independent of blastomere survival, was indicative for significantly higher implantation rates. Guérif *et al.* (2002) concluded that the most predictive factor for the implantation potential of a Day 2 thawed embryo is the final number of blastomeres present at transfer, irrespective whether these cells resulted from blastomere survival or from resumption of mitosis.

The question whether the higher cleavage rate of vitrified embryos also results in better implantation rates and whether a vitrified embryo per se is a more viable embryo than a slowly frozen embryo can only be answered by performing a randomized controlled trial. The systematic review and meta-analysis of Loutradi *et al.* (2008) and Kolibianakis *et al.* (2009) concluded that further properly designed trials are needed to evaluate the two cryopreservation techniques in terms of pregnancy rates. The meta-analysis of Abdelhafez *et al.* (2010) suggested superiority

of vitrification to slow-freezing regarding clinical outcomes. However, only one randomized trial was included for cleavage stage embryos. Taking into account the large difference in post-thawing/warming survival between slow-freezing and vitrification, it would now be unethical to start a randomized controlled trial. Since vitrified Day 3 embryos survive significantly better, more frozen embryo cycles originating from one oocyte collection cycle can be offered to the patient. Thus, it can be hypothesized that the clinical advantage of vitrification will be reflected in elevated cumulative pregnancy rates. Expressed per embryo transferred, our retrospective data did not reveal superior implantation rates of vitrified embryos upon slowly frozen ones, taking into account that only survived embryos with overnight cleavage were transferred. Recent retrospective data of Wang et al. (2012) showed higher implantation rates after vitrification using Cryoleaf compared with slow-freezing (19.8 versus 14.4% and $P = 0.01$). However, warming and transfer of embryos were planned on the same day, and thus results were analysed irrespective of overnight cleavage. Therefore, comparing those implantation rates with the results of the present study might not be justified.

In the randomized study of Wilding et al. (2010), implantation rates were not significantly different between vitrification and slow-freezing (14.3 versus 13.5%), even though all embryos that contained at least one surviving blastomere were transferred and more intact embryos were transferred in the vitrified embryo group.

Conclusion

Vitrified embryos showed a higher post-warming overnight cleavage rate than slowly-frozen embryos. If a survived ($\geq 50\%$ cells intact) Day 3 vitrified or slowly frozen embryo cleaved further after thawing, the number of cells lost has no impact on its implantation potential. However, damaged embryos have a lower overnight cleavage rate than fully intact embryos in both methods. Therefore, obtaining fully intact embryos is a prerequisite for an optimal cryopreservation programme, which is more likely to occur after vitrification.

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Authors' roles

L.V.L.: conception and design, acquisition of data, analysis and interpretation of data, writing of the article and critical review of the article. H.V.D.V.: critical review of the article. A.D.V.: critical review of the article. P.H.: statistical analysis of the data, interpretation of data and critical review of the article. C.B.: critical review of the article. H.T.: critical review of the article. G.V.: study design, interpretation of data and critical review of the article.

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Conflict of interest

None declared.

References

- Abdelhazef F, Desai N, Abou-Setta A, Falcone T, Goldfarb J. Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis. *Reprod Biomed Online* 2010;**20**:209–222.
- Archer J, Gook D, Edgar H. Blastocyst formation and cell numbers in human frozen-thawed embryos following extended culture. *Hum Reprod* 2003;**18**:1669–1673.
- Balaban B, Urman B, Ata B, Isiklar A, Larman MG, Hamilton R, Gardner DK. A randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Hum Reprod* 2008;**23**:1976–1982.
- Edgar D, Archer J, McBain J, Bourne H. Embryonic factors affecting outcome from single cryopreserved embryo transfer. *Reprod Biomed Online* 2007;**14**:718–723.
- El-Toukhy T, Khalaf Y, Al-Darazi K, Andritsos V, Taylor A, Braude P. Effect of blastomere loss on the outcome of frozen embryo replacement cycles. *Fertil Steril* 2003;**79**:1106–1111.
- Gabrielson A, Fedder J, Agerholm I. Parameters predicting the implantation rate of thawed IVF/ICSI embryos: a retrospective study. *Reprod Biomed Online* 2006;**12**:70–76.
- Guérif F, Bidault R, Cadoret V, Couet ML, Lansac J, Royere D. Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal. *Hum Reprod* 2002;**17**:1321–1326.
- Kolibianakis E, Zikopoulos K, Devroey P. Implantation potential and clinical impact of cryopreservation—a review. *Placenta* 2003;**24**:S27–S33.
- Kolibianakis EM, Venetis CA, Tarlatzis BC. Cryopreservation of human embryos by vitrification or slow freezing: which one is better? *Curr Opin Obst Gyn* 2009;**21**:270–274.
- 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. *Reprod Biomed Online* 2005;**11**:608–614.
- Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, Tarlatzis BC. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertil Steril* 2008;**90**:186–193.
- Raju R, Haranath GB, Krishna KM, Prakash GJ, Madan K. Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates. *Reprod Biomed Online* 2005;**11**:434–437.
- Tang R, Catt J, Howlett D. Towards defining parameters for a successful single embryo transfer in frozen cycles. *Hum Reprod* 2006;**21**:1179–1183.
- Valojerdi MJ, Eftekhari-Yazdi P, Karmian L, Hassani F, Movaghar B. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. *J Assist Reprod Genet* 2009;**26**:347–354.
- Van den Abbeel E, Camus M, Van Waesberghe L, Devroey P, Van Steirteghem A. Viability of partially damaged embryos after cryopreservation. *Hum Reprod* 1997;**12**:2006–2010.
- Van den Abbeel E, Van Steirteghem. A Zona pellucida damage to human embryos after cryopreservation and the consequences for their blastomere survival and in-vitro viability. *Hum Reprod* 2000;**15**:373–378.
- Van Landuyt L, De Vos A, Joris H, Verheyen G, Devroey P, Van Steirteghem A. Blastocyst formation in in vitro fertilization versus intracytoplasmic sperm injection cycles: influence of the fertilization procedure. *Fertil Steril* 2005;**83**:1397–1403.
- Van Landuyt L, Stoop D, Verheyen G, Verpoest W, Camus M, Van de Velde H, Devroey P, Van den Abbeel E. Outcome of closed blastocyst

- vitrification in relation to blastocyst quality: evaluation of 759 warming cycles in a single-embryo transfer policy. *Hum Reprod* 2011;**26**:527–534.
- Wang X, Zhang X, Qin Y, Hao D, Shi H. Outcomes of day 3 embryo transfer with vitrification using Cryoleaf: a 3-year follow-up study. *J Assist Reprod Genet* 2012;**29**:883–889.
- Wilding M, Capobianco C, Montanaro N, Kabili G, Di matteo L, Fusco E, Dale B. Human cleavage-stage embryo vitrification is comparable to slow-rate cryopreservation in cycles of assisted reproduction. *J Assist Reprod Genet* 2010;**27**:549–554.
- Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, Sullivan E, Vanderpoel S, for ICMART and WHO (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology. *Fertil Steril* 2009;**92**: 1520–1524.
- Zheng X, Liu P, Chen G, Qiao J, Wu Y, Fan M. Viability of frozen-thawed human embryos with one-two blastomere lysis. *J Assist Reprod Genet* 2008;**25**:281–285.