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ORIGINAL ARTICLE ***Embryology***

A prospective randomized controlled trial investigating the effect of artificial shrinkage (collapse) on the implantation potential of vitrified blastocysts

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STUDY QUESTION: What is the effect of artificial shrinkage by laser-induced collapse before vitrification on the implantation potential after transfer of vitrified–warmed blastocysts?

SUMMARY ANSWER: The artificial shrinkage by laser-induced collapse did not significantly increase the implantation rate per transferred collapsed blastocyst (37.6%) compared with non-collapsed blastocysts (28.9%) [odds ratio (OR): 1.48, 95% confidence interval (CI): 0.78–2.83].

WHAT IS KNOWN ALREADY: Retrospective studies have demonstrated that artificial shrinkage of the blastocyst prior to vitrification can have a positive effect on blastocyst survival after warming. A recent study found a similar survival rate but higher implantation rate for collapsed blastocysts. So far, no randomized controlled trial has been conducted to investigate the implantation potential of collapsed blastocysts.

STUDY DESIGN, SIZE, DURATION: Prospective randomized trial. Patients were recruited from December 2011 until April 2014 and warming cycles were included until July 2014. Patients were randomized in the fresh cycle if blastocysts were available for vitrification and were allocated to the study or control arm according to a computer-generated list. In the study group, blastocysts underwent laser-induced collapse before vitrification. In the control group, blastocysts were vitrified without collapsing.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In total, 443 patients signed informed consent and 270 patients had blastocysts vitrified. One-hundred and thirty-five patients were allocated to the study group and 135 to the control group. Sixty-nine patients from the study group and 69 from the control group returned for at least one warming cycle in which 85 and 93 blastocysts were warmed in the first cycle, respectively. Primary outcome was implantation rate per embryo transferred in the first warming cycle. Secondary outcomes were survival and transfer rates, blastocyst quality after warming, clinical pregnancy rate and implantation rate per warmed blastocyst. Blastocysts were vitrified–warmed one by one using closed vitrification and one or two blastocysts were transferred per warming cycle.

MAIN RESULTS AND THE ROLE OF CHANCE: We calculated that the group sample sizes of 80 embryos in the collapse group and 80 embryos in the control group were needed to achieve 80% power to detect a difference between the group proportions of +20% with $P < 0.05$. In the study group, 69 first warming cycles resulted in 69 transfers with 1.2 blastocysts ($n = 85$) transferred. In the control group, an average of 1.3 blastocysts ($n = 83$) were transferred in 67 out of 69 warming cycles. Implantation rates per embryo transferred in the first warming cycle were not different between both groups (38 versus 29%, OR: 1.48; 95% CI: 0.78–2.83), neither was the implantation rate per warmed embryo (38 versus 26%, OR: 1.74; 95% CI: 0.92–3.29). When all warming cycles were considered ($n = 135$ in each group), survival rate after collapse was significantly higher compared with the control group (98.0 versus 92.0%, OR: 4.25; 95% CI: 1.19–15.21). Furthermore, a higher percentage of high-quality blastocysts (36.3 versus 23.5%, OR: 1.86; 95% CI: 1.12–3.08) and hatching blastocysts (19.2 versus 5.4%, OR: 4.18; 95% CI: 1.84–9.52) were found compared with the control group.

LIMITATIONS, REASONS FOR CAUTION: The study lasted more than 2.5 years since fewer patients than expected returned for a warming cycle because of the high ongoing pregnancy rates in the fresh IVF/ICSI cycle.

WIDER IMPLICATIONS OF THE FINDINGS: Although no significant higher implantation rate was found after collapse, the better survival and post-warm embryo quality convinced us to recognize a clinical benefit of artificial shrinkage and to implement it in routine vitrification practice.

STUDY FUNDING/COMPETING INTERESTS: None.

TRIAL REGISTRATION NUMBER: NCT01980225, www.clinicaltrials.gov. The first patient was included November 2011 and the study was registered October 2013.

Key words: vitrification / blastocyst / artificial shrinkage / collapse / implantation

Introduction

The method of vitrification to cryopreserve human Day 5 and Day 6 blastocysts has been used in our centre since 2008 (Van Landuyt *et al.*, 2011). Vitrification yields higher survival rates after warming than the standard slow freezing method (Loutradi *et al.*, 2008 and meta-analysis of Kolibianakis *et al.*, 2009). However, the clinical pregnancy rates between the two cryopreservation methods were not significantly different. In the literature, no consensus is obtained regarding the most successful method to cryopreserve blastocysts or embryos in terms of pregnancy or live birth rates. In a recent large population-based cohort study of autologous fresh and thaw cycles (both vitrification and slow freezing), vitrification of blastocysts resulted in higher clinical pregnancy and live delivery rates compared with the slow freezing method (Li *et al.*, 2014). Compared with fresh embryo transfer, vitrified–warmed blastocyst transfer led to 14% lower risk of preterm birth in singleton pregnancies. After introducing vitrification for the cryopreservation of blastocysts, Vanderzwalmen *et al.* (2002) found that survival rates were dependent on the stage of blastocyst development, with expanded blastocysts showing lower survival, transfer and implantation rates than morulae or early cavitating blastocysts. Although the vitrification procedure should avoid intracellular ice-crystal formation compared with slow controlled freezing, the large fluid-filled cavity in expanded blastocysts may inhibit sufficient permeation of cryoprotectant inside the blastocoel, resulting in a little ice formation. Applying artificial shrinkage, i.e. reducing the volume of the blastocoel by puncturing it with a glass pipette, resulted in increased survival rates (from 29% up to 70.6%). In other retrospective studies, a hole was created in the trophectoderm layer, either by puncturing it with a needle (Son *et al.*, 2003), by repeated micropipetting of the blastocyst (Hiraoka *et al.*, 2004) or by laser pulse (Mukaida *et al.*, 2006). These distinct procedures all induce immediate collapse of the blastocoelic cavity just before vitrification, which may have a positive effect on the survival after warming. However, in the later paper of Vanderzwalmen *et al.* (2009), their vitrification protocol was optimized by performing a gradual 3-step exposure to cryoprotectant concentration resulting in increased concentration of cryoprotectant inside the blastocyst and better survival rates. The need to remove the blastocoelic fluid from the blastocyst before vitrification was questioned and thus blastocyst collapse was no longer performed. A successful closed system vitrification without the use of artificial shrinkage was developed by Stachecki *et al.* (2008). The S^3 or large volume vitrification is a dimethylsulphoxide (DMSO) free vitrification procedure using ethylene glycol, glycerol and sucrose, as the cooling/warming cryoprotectants, allowing sufficient time for cryoprotectant exposure in larger volumes. The first clinical application on human blastocysts resulted in survival and implantation rates of 89.2 and 47.5%, respectively. More recently, Reed *et al.* (2015) could optimize the survival rates of trophectoderm biopsied and non-biopsied blastocysts using large volume vitrification (96.9%) instead of microvolume vitrification (84.3%).

In the open Cryotop device, similar survival rate but an increased implantation rate of collapsed blastocysts compared with non-collapsed blastocysts was observed (Iwayama *et al.*, 2011). Moreover, survived blastocysts tended to re-expand faster when they had undergone artificial shrinkage before vitrification, a phenomenon also observed in other studies (Desai *et al.*, 2008; Cao *et al.*, 2014).

To date, no randomized controlled trials (RCTs) have been conducted to compare the survival and/or implantation rate after transfer of vitrified collapsed and non-collapsed human blastocysts. In our IVF centre, the question was raised whether we could further optimize the outcome of our blastocyst vitrification programme by applying collapse, especially in terms of implantation rate per transferred blastocyst. Therefore, a prospective RCT was set up to investigate the effect of artificial shrinkage by laser-induced collapse on the implantation potential of vitrified–warmed Day 5/6 blastocysts. Additionally, survival and transfer rates after warming were compared for collapsed and non-collapsed blastocysts.

Materials and Methods

Study design

The study was a prospective RCT approved by the Ethical Committee of the Universitair Ziekenhuis Brussels. Female IVF/ICSI patients <39 years of age, who were scheduled for Day 5 blastocyst transfer and vitrification of supernumerary blastocysts, were informed about the study and signed the informed consent on the day of oocyte retrieval. Also patients with Day 3 cleavage stage embryo transfer and with supernumerary embryos vitrified at the blastocyst stage were eligible for the study. Oocyte donation cycles, preimplantation genetic diagnosis and *in vitro* maturation cycles were excluded from the study. Besides, couples with testicular or epididymal sperm were excluded. Patient recruitment had started in November 2011 and was ended in April 2014. Warming cycles were included until July 2014.

Patients were allocated to either the control or the study arm (in blocks of six) according to a computer-generated randomization list if at least one blastocyst was available for vitrification on Day 5 or Day 6 of *in vitro* embryo culture. In the study group, all blastocysts selected for vitrification underwent artificial shrinkage by laser-induced collapse of the blastocoelic cavity. In the control group, all blastocysts were vitrified without laser-induced collapse. Patients, clinicians who prescribed the treatment at consultation (IVF/ICSI, day of transfer, number of embryos for transfer in the fresh or frozen cycle) as well as clinicians who performed the fresh or frozen embryo transfer (FET), were blinded for the study since randomization was done in the lab by the embryologist at the time of vitrification. However, the embryologist who performed the collapse and the embryologist who randomized the patient were not blinded. However, the embryologist had to accept the computer-generated code to randomize the patient and could therefore not influence the decision.

As a primary outcome measure, the implantation rate per blastocyst transferred in the first warming cycle was assessed. Patients received one or two blastocysts per transfer according to the clinician's decision at consultation, mainly depending on the patient's age, the number of previous treatment

cycles and the number of embryos replaced in the previous treatment cycles. Secondary outcome parameters were blastocyst survival rate, transfer rate and quality after warming in the first cycle. These secondary end-points were also analysed in all warming cycles performed. The study was registered on the Clinical Trial website (www.clinicaltrials.gov, NCT01980225). The first patient was enrolled November 2011 and the study was registered October 2013.

Embryo selection for cryopreservation and evaluation after warming

Blastocysts were vitrified on Day 5 or Day 6 if they had reached at least the full blastocyst stage with an inner cell mass (ICM) and trophectoderm (TE) score of at least type B (Gardner and Schoolcraft, 1999). High-quality blastocysts had an ICM/TE score type AA or AB, good-quality blastocysts were type BA or BB blastocysts. Blastocysts were vitrified one by one and warmed until one or two (the necessary number of blastocysts for transfer) were obtained. The morphological survival of the blastocyst was assessed immediately after warming. Only blastocysts with >50% of cells intact were eligible for transfer. If the blastocyst was severely (> 50% of the cells damaged) or completely damaged, an extra one was warmed immediately. Blastocysts were transferred only if they showed signs of re-expansion and no further impairment between the time of warming and the moment of transfer.

Collapse and vitrification procedure

Artificial shrinkage of the blastocoel was induced by applying one or two laser pulses (2.0 ms) at the junction between trophectoderm cells using the 1.48-μm diode laser (Octax, MTG, Germany), providing a safe distance from the ICM. Collapse was performed on full, expanded, hatching and completely hatched blastocysts. Full collapse of the trophectoderm layer was not always observed immediately after applying the laser pulse. For some blastocysts reacting slowly, it took up to 5 min to see the complete shrinkage and disappearance of the blastocoel. After collapse of the blastocoelic cavity, the vitrification procedure was immediately started. Blastocysts were vitrified and warmed using closed CBS-VIT High Security (HS) straws (CryoBioSystem, L'Aigle, France) in combination with DMSO-ethylene glycol (EG)–sucrose (S) as cryoprotectants (Irvine Scientific^{R}Freeze kit, Newtownmountkennedy, County Wicklow, Ireland) according to the method previously described by us (Van Landuyt *et al.*, 2011) with minor adaptation using a first droplet of 150 μl instead of 25 μl of thawing solution (TS).

Preparation of the FET cycle

Day 5 or Day 6 blastocysts were warmed in the morning of the day of transfer. They were transferred in the afternoon in a Day 5 endometrium.

The most common modality for FET used was 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 amenorrhoea or oligomenorrhoea, an artificial cycle was 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).

Outcome measures

The primary outcome parameter is the implantation rate in the first warming cycle, defined as the number of intrauterine gestational sacs observed at transvaginal ultrasound scan at least 5 weeks after FET, upon the number of embryos transferred.

Secondary outcomes are the implantation rate per warmed embryo, the survival and the transfer rates after warming, defined as the percentage of blastocysts survived and transferred per warmed blastocyst, respectively. Also the percentage of high- and good-quality blastocysts obtained after warming was assessed.

Sample size calculation

At the start of the study, the implantation rate per vitrified–warmed and transferred full or expanded blastocysts in our centre was 20.3% (Van Landuyt *et al.*, 2011). According to the data from observational studies reported by others, implantation rates (fetal sac per transferred blastocyst) following artificial shrinkage range between 46.7 and 59.7% (Mukaida *et al.*, 2006; Iwayama *et al.*, 2011; Ren *et al.*, 2013). In order to perform a realistic sample size calculation for the current RCT and taking into account the implantation rates reported in the literature, our study was designed based on the less optimistic scenario that implantation rates following collapse may increase to a maximum of 40%. Sample size calculation was performed in PASS 2008 statistical software. We calculated that the group sample sizes of 80 embryos in the collapse group and 80 embryos in the control group without collapse were needed to achieve 80% power to detect a difference between the group proportions of +20% with $P < 0.05$. The proportion in the collapse group was assumed to be 20% under the null hypothesis and 40% under the alternative hypothesis. The proportion in the control group was 20%. The statistical test used is the two-sided *Z*-test with pooled variance. This means that 160 vitrified–warmed blastocysts are needed for the study, used in the first warming cycle.

Although our sample size calculation was performed on the number of embryos, given that our study was designed to detect differences in implantation rates of vitrified–warmed embryos, it was essential to calculate the number of patients needed to be randomized in order to ensure an adequate sample size for the study. To determine this, we estimated that the proportion of patients expected to use their frozen blastocysts within a short-term was ~66%, while 34% of patients was expected not to use their vitrified blastocysts shortly after the fresh (blastocyst) transfer. This was estimated based on a previous study by our group according to which the clinical pregnancy rate (with FHB) for patients with fresh single blastocyst transfer was 34.3% (Papanikolaou *et al.*, 2006). Consequently, in order to compensate for this loss of pregnant patients for the study, more patients needed to be included (factor 1/0.66 = 1.51). Based on the above calculation, we estimated that the inclusion of ~242 patients would be adequate in order to obtain 160 embryos to be transferred in the first frozen cycle.

Statistical analysis

Categorical variables were analysed with use of chi square or Fisher's exact test. Continuous variables were analysed with independent *t*-test or Mann–Whitney *U*-test depending on the normality of the distribution. Normality was assessed by the use of Shapiro–Wilk test. All values were two-tailed with the level of significance set at 0.05. All analyses were performed with the use of SPSS 22 Statistical software.

Finally, taking into account the partially unblinded trial set up and the potential risk of bias in maintaining balance between the groups, regression analysis was performed in order to provide adjusted odds for variables that might have a confounding effect on the final outcome. Therefore, ORs were adjusted for baseline characteristics (age, indication of infertility, cycle rank, BMI) variables related to the preceding fresh cycle (number of oocytes retrieved, number of 2PN embryos, number of top/good-quality embryos and positive pregnancy outcome) and variables related to the current FRET cycle (number of embryos transferred and type of protocol used being a natural cycle or an artificially prepared frozen cycle).

Results

In total, 443 patients signed the informed consent on the day of oocyte retrieval. In 270 patients (60.9%, 270/443), at least one supernumerary blastocyst fulfilled the criteria for cryopreservation. Of these, 135

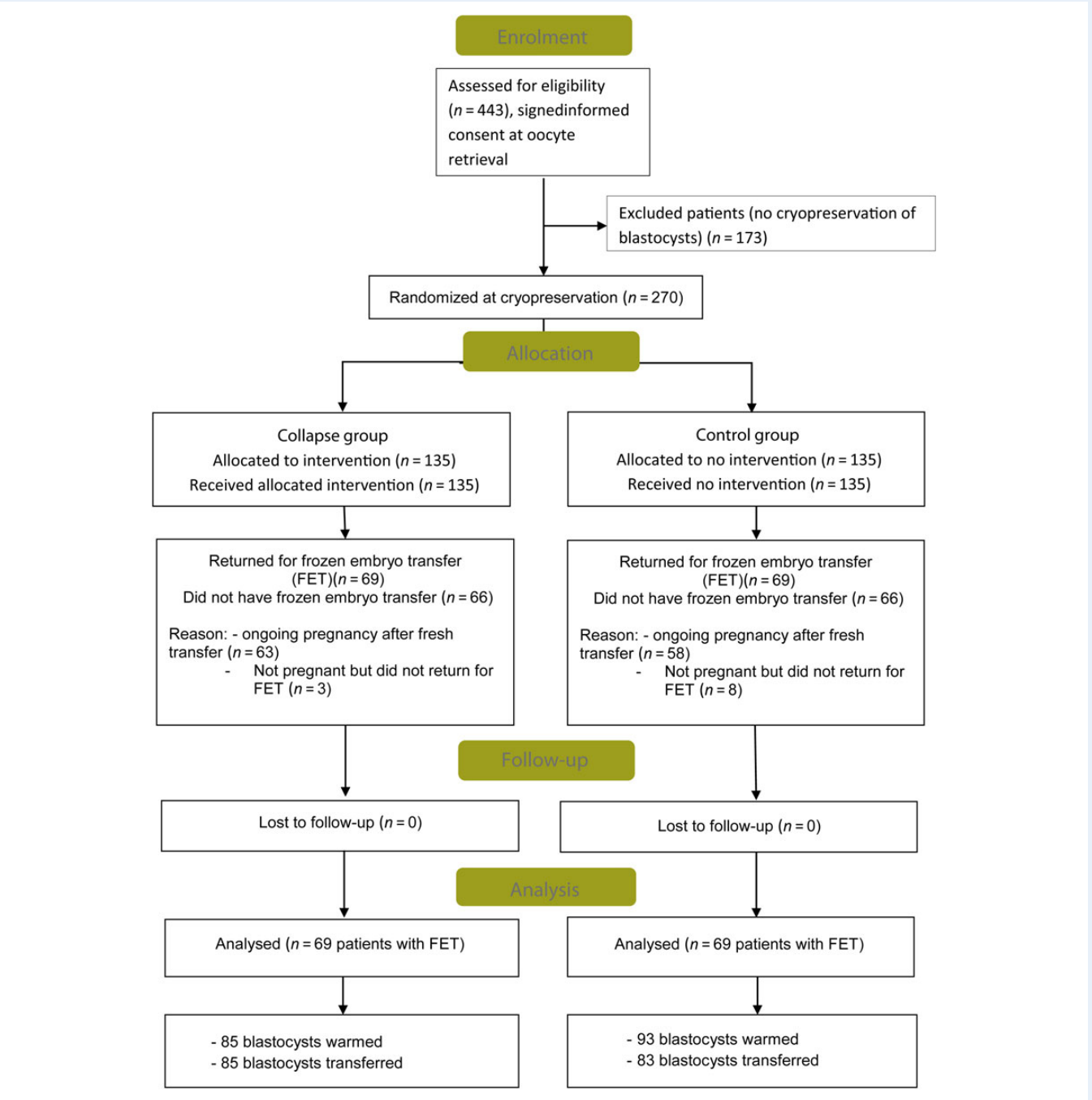

Figure 1 CONSORT flowchart.

patients were randomized to the study group (with collapse) and 135 to the control group (without collapse) (Fig. 1).

Fresh cycle characteristics

Patient characteristics and results of the fresh cycle are presented in Table I. The mean female age, the mean number of cumulus–oocyte complexes (COC), the mean number of 2 pronucleate (PN) oocytes, the mean number of cleavage stage embryos/blastocysts transferred and the mean number of blastocysts vitrified were comparable between the study group and control group. The proportion of Day 3 and Day 5 transfers was similar in the two groups, as well as the number of patients without embryo transfer where all blastocysts were vitrified because of risk of ovarian hyperstimulation (Table I).

The positive hCG rate and clinical pregnancy rate in the fresh cycles was 66.9% (89/133) and 56.4% (75/133), respectively, in the study group and 59.7% (77/129) and 49.6% (64/129), respectively, in the control group (Table I). The implantation rate per transferred embryo was 53.4% (86/161) and 48.7% (77/158) for study group and control group, respectively.

Table I Patient and fresh cycle characteristics of all 270 patients randomized at cryopreservation.

	Study group (collapse)	Control group (no collapse)
Patients randomized (*N*)	135	135
Age (years; Mean ± SD)	31.0 (±3.8)	31.5 (±3.5)
COC (*N*; Mean ± SD)	13.3 (±6.3)	13.7 (±7.6)
2 PN oocytes (*N*; Mean ± SD)	8.7 (±4.2)	9.4 (±5.3)
Embryos transferred (*N*; Mean ± SD)	1.2 (±0.4)	1.2 (±0.5)
N cycles with *n* transferred = 2 (%)	28 (20.7)	29 (21.5)
Transfers	133/135 (98.5%)	129/135 (95.6%)
Day 5	109/135 (80.7%)	108/135 (80%)
Day 3	24/135 (17.8%)	21/135 (15.6%)
No transfer (ovarian hyperstimulation)	2/135 (1.5%)	6/135 (4.4%)
Blastocysts vitrified (*N*; Mean ± SD)	3.4 (±2.7)	3.5 (±2.6)
Positive hCG	89/133 (66.9%)	77/129 (59.7%)
Clinical pregnancies (with FHB)	75/133 (56.4%)	64/129 (49.6%)
Implantation rate (fetal sacs/ embryos transferred)	86/161 (53.4%)	77/158 (48.7%)

Post-warming survival and transfer rates

Out of the 60 patients in the study group and 71 patients in the control group who did not obtain a clinical pregnancy with FHB after fresh embryo transfer, 57 and 63 returned to use their frozen embryos, respectively. Additionally, 12 patients from the study group and six from the control group who had been pregnant and had delivered from the fresh cycle came back to use their vitrified embryos for a new attempt. Thus, in total 69 patients from the study group and 69 patients from the control group performed at least one cycle with vitrified embryos in which 85 and 93 blastocysts were warmed, respectively. The results are presented in Table II. The pre-vitrification quality of the blastocysts that were selected for warming was not different between the groups. The proportions of high-quality and good-quality blastocysts were 41.2% (35/85) and 58.8% (50/85) in the study group, respectively, versus 46.2% (43/93) and 53.8% (50/93) in the control group, respectively [odds ratio (OR): 0.84; 95% confidence interval (CI): 0.45–1.47; and OR: 1.23; 95% CI: 0.68–2.23; $P = 0.497$, respectively]. The total survival rate was significantly higher in the collapse group (100%, 85/85) than in the control group (91.4%, 85/93; OR 17.00; 95% CI: 0.97–299.19; $P = 0.007$) with 55.2% (47/85) of fully intact blastocysts after collapse and 45.2% (42/93) without collapse (OR: 1.50; 95% CI: 0.83–2.71; $P = 0.177$). After warming, more high-quality blastocysts per surviving blastocyst were observed when collapse was performed [38.8% (33/85) versus 22.4% (19/85); OR 2.21; 95% CI: 1.13–4.31; $P = 0.020$]. The percentage of blastocysts that were hatching after warming was 16.5% (14/85) in the study group compared with 7.1% (6/85) in the control group, which almost reached statistical significance (OR 2.60; 95% CI: 0.95–7.12; $P = 0.056$). The percentage of blastocysts surviving but not fully re-expanding to their original blastocyst stage (remained collapsed or showed a small cavity) was not different between the two groups (11.8%, 10/85 versus 15.3%, 13/85; OR: 0.74; 95% CI: 0.31–1.79, $P = 0.660$). The percentage of blastocysts that were finally transferred per warmed blastocyst was significantly higher in the collapse group (100%, 85/85 versus 89.2%, 83/93, OR: 21.50; 95% CI: 1.24–372.86; $P = 0.002$).

Some patients underwent more than one warming cycle. Table III presents the results of all warming cycles performed including 138 patients: 116 and 115 warming cycles were performed in the study and control group, respectively, in which 149 (1.3 per warming cycle) and 162 (1.4 per warming cycle) blastocysts in total were warmed, respectively. The total survival rate after warming was significantly higher for collapsed blastocysts (98.0%, 146/149 versus 92.0%, 149/162, OR: 4.25; 95% CI: 1.19–15.21; $P = 0.016$). The percentage of fully intact blastocysts was 53.0% (79/149) after collapse compared with 43.2% (70/162) without collapse (OR 1.48; 95% CI: 0.95–2.32; $P = 0.084$). Again, more high-quality blastocysts per surviving blastocyst (36.3%, 53/146 versus 23.5%, 35/149, OR: 1.86; 95% CI: 1.12–3.08; $P = 0.016$) and also less moderate quality blastocysts (15.1%, 22/146 versus 25.5%, 38/149, OR: 0.52; 95% CI: 0.29–0.94; $P = 0.026$) were found in the study group. The percentage of surviving blastocysts that were hatching after warming was higher in the collapse group (19.2%, 28/146) than in the control group (5.4%, 8/149, OR: 4.18; 95% CI: 1.84–9.52; $P < 0.001$). The percentage of blastocysts that did not re-expand after warming was similar in both groups (9.6%, 14/146 versus 14.1%, 21/149; OR: 0.65; 95% CI: 0.32–1.33; $P = 0.232$).

Clinical outcome of the first warming cycle

The clinical outcome of the first warming cycle is presented in Table IV. In the control group, 2 out of 69 patients did not get a transfer because the single vitrified embryo did not survive after warming. A similar number of embryos ($n = 1.2$) were transferred in both groups. The positive hCG rates (43.5%, 30/69 and 40.3%, 27/67; OR: 1.14; 95% CI: 0.58–2.25) and clinical pregnancy rates (37.7%, 26/69 and 31.3%, 21/67; OR: 1.32; 95% CI: 0.65–2.69) were not different for collapsed and non-collapsed blastocysts, respectively. Also implantation rates per transferred embryo (37.6%, 32/85 versus 28.9%, 24/83; OR: 1.48; 95% CI: 0.78–2.83, $P = 0.230$) and per warmed embryo (37.6%, 32/85 versus 25.8%, 24/93; OR: 1.74; 95% CI: 0.92–3.29) were comparable in both groups. The percentage of multiple gestations per clinical pregnancy was 19.2% (5/26) in the collapse group versus 9.5% (2/21) in the control group (OR: 2.26; 95% CI: 0.39–13.06; $P = 0.352$). The multiple gestations also included one monozygotic (MZ) triplet and two MZ twins in the collapse group and one MZ twin in the control group.

Logistic regression analysis

Regarding the results of the regression analysis, none of the considered variables was significantly associated with positive hCG or clinical pregnancy rates. The adjusted OR (95% CI) for the comparison between the study group (collapse) and control group (non-collapse) was 1.06 (0.480–2.320) for positive hCG rate and 1.21 (0.55–2.70) for clinical pregnancy rates.

Discussion

Based on the results of this RCT, the artificial shrinkage by laser-induced collapse did not significantly increase implantation rates per transferred

Table II Morphological survival and quality of blastocysts in the first warming cycle (n = 138).

	Study group (collapse)	Control group (no collapse)	Odds ratio (95% CI)	*P*-value
Warming cycles (*N*)	69	69		
Embryos warmed				
Total (*N*)	85	93		
Per patient (*N*; Mean ± SD)	1.2 ± 0.13	1.3 ± 0.51	−0.1 (−0.24–0.04)	0.174[a]
N cycles with *n* warmed = 1 (%)	53 (76.8)	46 (66.7)	1.66 (0.78–3.51)	
N cycles with *n* warmed = 2 (%)	16 (23.2)	22 (31.9)	0.65 (0.30–1.37)	
N cycles with *n* warmed = 3 (%)	0 (0)	1 (1.4)	0.33 (0.01–8.21)[b]	
Before vitrification				
Blastocyst quality (*N*(%))				
High	35 (41.2)	43 (46.2)	0.84 (0.45–1.47)	0.497
Good	50 (58.8)	50 (53.8)	1.23 (0.68–2.23)	
After warming				
Survived (*N*(%))	85 (100)	85 (91)	17.00 (0.97–299.19)[b]	0.007[c]
100% intact (*N*(%))	47 (55)	42 (45)	1.50 (0.83–2.71)	0.177
≥50% intact (*N*(%))	38 (45)	43 (46)	0.94 (0.52–1.70)	0.838
Blastocyst quality (*N* (*N*/survived %))				
High	33 (39)	19 (22)	2.21 (1.13–4.31)	0.020
Good	35 (41)	43 (51)	0.68 (0.37–1.25)	0.411
Moderate	17 (20)	22 (26)	0.72 (0.35–1.47)	0.556
Hatching	14 (17)	6 (7)	2.60 (0.95–7.12)	0.056
Not re-expanded blastocysts	10 (12)	13 (15)	0.74 (0.31–1.79)	0.660
N transferred (%)	85 (100%)	83 (89.2%)	21.50 (1.24–372.86)[b]	0.002[c]

With the exception of blastocyst quality after warming, percentages are calculated by dividing by the total number of embryos warmed per group.
CI, confidence interval.
[a]Mann–Whitney *U*-test.
[b]Odd ratios and 95% CI were computed by using a continuity correction of 0.5 (addition of 0.5 in all the cells of 2 × 2 tables) in order to overcome problems with zero cell counts.
[c]Fisher's exact test.

or per warmed collapsed blastocyst compared with the control group. Although a difference of ~9% in implantation rate failed to reach statistical significance, our study was not designed to detect such a small difference. In this regard, it is unclear whether inclusion of more patients and cycles would have been able to demonstrate such a difference. Consequently, future multicentre RCTs with a larger sample size or a cumulative meta-analysis of small RCTS are welcome in order to further examine whether artificial blastocyst shrinkage has a beneficial effect on implantation rates. Of interest, although our study failed to identify any difference in the primary end-point (implantation rates), a significantly higher blastocyst survival rate and post-warming blastocyst quality in favour of the collapse group has been demonstrated. Although that we cannot provide solid guidance regarding this finding, owing to the fact that the study was not designed for this purpose, it is interesting to highlight that blastocyst survival rates and quality post-warming were consistently higher in the collapse group either when examining the first or cumulatively all the warming cycles.

Furthermore, blastocysts that were collapsed before vitrification developed more often into hatching blastocysts after warming than the non-collapsed group. In addition, evidence was provided that collapsing the blastocyst before vitrification resulted in better embryo quality preservation, leading to a higher proportion of high-quality blastocysts after warming per survived blastocyst. Similar findings were described by Desai *et al.* (2008), who studied the effect of artificial reduction of the blastocoel on blastocyst morphology, cell number and DNA damage in early and expanded human and mouse blastocysts. In mouse expanded blastocysts without artificial shrinkage, apoptotic cell death was found in 13% of the cells compared with 3% after laser-induced or 5% after mechanically induced collapse. The authors mentioned that this cell death, assessed by counting the number of TUNEL positive cells, was detected though little indication of cell damage was initially visible after evaluating blastocyst morphology through the microscope. Additionally, collapsed mouse blastocysts presented a higher cell number after warming. Also in the bovine, Min *et al.* (2013) observed a higher total cell number and lower number of apoptotic-positive cells after artificial shrinkage of hatching blastocysts obtained after somatic cell nuclear transfer. In the study of Desai *et al.* (2013), the cell death was minimal in both untreated and collapsed early blastocysts, and those blastocysts did not benefit from fluid reduction. In the same study, the survival was similar with or without collapsing of human left-over blastocysts, but the post-warming re-expansion was better and faster in collapsed blastocysts. Moreover, there was a trend of a faster and higher re-expansion rate after laser-assisted collapse compared with the use of a needle. An incomplete collapse was more often seen after mechanical shrinkage with a needle and

Table III Morphological survival and quality of blastocysts in all warming cycles ($n = 231$).

	Study group (collapse)	Control group (no collapse)	Odds ratio (95% CI)	*P*-value
Warming cycles (*N*)	116	115		
Embryos warmed				
Total (*N*)	149	162		
Per patient (Mean ± SD)	1.3 ± 0.47	1.4 ± 0.52	−0.1 (0.21–0.01)	0.285[a]
Before vitrification				
Blastocyst quality (*N*(%))				
High	52 (34.9)	67 (41.4)	0.76 (0.48–1.20)	0.242
Good	97 (65.1)	95 (58.6)	1.32 (0.83–2.08)	
After warming				
Survived (*N*(%))	146 (98.0)	149 (92.0)	4.25 (1.19–15.21)	0.007
100% intact	79 (53.0)	70 (43.2)	1.48 (0.95–2.32)	0.084
≥50% intact	67 (45.0)	79 (48.8	0.86 (0.55–1.34)	0.502
Blastocyst quality (*N* (*N*/survived%))				
N high	53 (36.3)	359 (23.5)	1.86 (1.12–3.08)	0.016
N good	71 (48.6	76 (51.0	0.91 (0.58–1.44)	0.683
N moderate	22 (15.1	38 (25.5)	0.52 (0.29–0.94)	0.026
N hatching	28 (19.2)	8 (5.4)	4.18 (1.84–9.52)	<0.001
N not re-expanded	14 (9.6)	21 (14.1)	0.65 (0.32–1.33)	0.232
N transferred	144 (96.6)	147 (90.7)	2.94 (1.04–8.30)	0.038

With the exception of blastocyst quality after warming, all percentages were calculated by dividing by the total number of embryos warmed per group.
CI, confidence interval.
[a]Mann–Whitney *U*-test.

this could be an explanation for the slower development/re-expansion after warming. The use of the laser to perform artificial shrinkage was also preferred in our lab, since it is a very simple technique requiring minimal training.

The effect of the method used for artificial shrinkage on clinical outcome was recently investigated retrospectively by Cao *et al.* (2014).They compared laser-pulse collapse with the use of a 29 gauge needle in order to shrink the blastocyst. No difference was found in survival rates between both groups (93%), but the blastocysts showed a significantly lower hatching rate (83.6 versus 91.2%) after the use of a needle. Regarding the clinical outcome, similar implantation rates were found (41.8 versus 44.6%) but a significantly higher premature birth rate (40.0 versus 21.15%) was observed after shrinkage with a needle. The authors hypothesized that the larger tip of the needle compared with the small hole made by the laser pulse could cause more damage to the trophectoderm cells and may have detrimental effects in later development and function of the placenta and umbilical cord.

Compared with the implantation rate of 20.3% for BL3 (full blastocysts) and BL4 (expanded)blastocysts in our previous study (Van Landuyt *et al.*, 2011) on blastocyst vitrification, both the implantation rates with and without collapse seemed increased in the present study. In the previous study, the clinical outcome of the first 2 years of blastocyst vitrification at our centre was analysed. Afterwards, our warming protocol was slightly modified by increasing the first warming droplet from 25 to 150 μl, which may have attributed to a better survival and thus better post-warming blastocyst quality. However, also the pregnancy rates of the fresh IVF/ICSI cycles have increased the last 3 years at our centre. Therefore, not only the higher expertise and protocol modification after 2 years of vitrification but other factors including blastocyst culture medium and rebuilding the lab into a cleanroom could have resulted in better intrinsic embryo quality.

In our study, assisted hatching after warming was never applied. It was preferred to investigate the pure effect of shrinking the blastocoel before vitrification and not to include other variables, which strengthens the study design. In literature, several centres reporting successful implantation rates after blastocyst vitrification ranging from 37.0% up till 59.7% (Hiraoka *et al.*, 2004; Zhu *et al.*, 2010; Wikland *et al.*, 2010; Iwayama *et al.*, 2011; Ren *et al.*, 2013; Desai *et al.*, 2013) combined both artificial shrinkage and assisted hatching, thus making it impossible to distinguish the specific effect of each individual treatment on their clinical results. It would be interesting to perform the golden study investigating the benefit of each individual technique (assisted hatching or collapse) and of both together, compared with a control group that did not receive any extra treatment. Unfortunately, this four-group randomized control trial would require many patients as well as an extended study period, and would therefore be unrealistic. We should not forget, however, that successful implantation rates ranging from 30 to 53.6% (fetal sacs) have been obtained also without the use of artificial shrinkage (Kuwayama *et al.*, 2005; Ebner *et al.*, 2009; Liebermann, 2009; Vanderzwalmen *et al.*, 2009; Hashimoto *et al.*, 2013) or with occasional artificial shrinkage only performed in hatching blastocysts (42.3%, Cobo *et al.*, 2012).

Table IV Clinical outcome in the first warming cycle.

	Study group (collapse)	Control group (no collapse)	Odds ratio (95% CI)	*P*-value
Warming cycles (*N*)	69	69		
Embryos warmed (mean)				
Total (*N*)	85	93		
Per patient (Mean ± SD)	1.2 ± 0.43	1.3 ± 0.51	−0.1 (−0.24 to 0.04)	0.174[a]
Embryos transferred (*n*/*N* (%))	85/85 (100)	83/93 (89.2)	21.50 (1.24 to 372.86)[b]	0.002[c]
Embryos/transfer (Mean ± SD)	1.2 (±0.43)	1.2 (±0.47)	0.00 (−0.14 to 0.14)	0.763[a]
N cycles with *n* transferred = 0 (%)	0 (0)	2 (2.9)	0.19 (0.01–4.12)[b]	
N cycles with *n* transferred = 1 (%)	53 (76.8)	51 (73.9)	1.17 (0.54–2.54)	
N cycles with *n* transferred = 2 (%)	16 (23.2)	16 (23.2)	1.00 (0.45–2.21)	
N transfers	69	67		
Positive hCG (*n*/*N* transfers (%))	30 (44)	27 (40)	1.14 (0.58–2.25)	0.707
Biochemical pregnancies (*n*/*N* positive hCG (%))	3 (10)	6 (22)	0.39 (0.09–1.74)	0.206
Extra uterine gestation (*n*/*N* positive hCG (%))	1 (3)	0 (0)	2.79 (0.11–71.59)[b]	0.339
Clinical pregnancies (*n*/*N* transfers (%))	26 (38)	21 (31)	1.32 (0.65–2.69)	0.437
Multiple gestations (*n* (*n*/*N* clinical pregnancies %))	5 (19)	2 (10)	2.26 (0.39–13.06)	0.352
Triplets (*n* (monozygotic *n*))	1 (1)	0		
Twins (*n* (monozygotic *n*))	4 (2)	2 (1)		
N sacs	32	24		
Implantation rate per embryo transferred (%)	38	29	1.48 (0.78–2.83)	0.230
Implantation rate per embryo warmed (%)	38	26	1.74 (0.92–3.29)	0.089

CI, confidence interval.
[a]Mann–Whitney *U*-test.
[b]Odd ratios and 95% CI were computed by using a continuity correction of 0.5 (addition of 0.5 in all the cells of 2 × 2 tables) in order to overcome problems with zero cell counts.
[c]Fisher's exact test.

Recently, the effect of artificial shrinkage was investigated in fresh blastocyst transfer cycles (Hur *et al.*, 2011), considering the artificial shrinkage as a type of assisted hatching since a little hole is created in the zona pellucida when applying a laser pulse between the trophectoderm cells or when using a needle to puncture the trophectoderm layer. After shrinking the fresh blastocyst, the blastocyst was evaluated until re-expansion occurred and then transferred in the fresh cycle. Significantly higher clinical pregnancy rates were obtained after artificial shrinkage (58.8%) compared with the control group (39.0%). However, these findings are based on one single study and are to be confirmed by other, preferably randomized controlled studies.

In the present study, despite the fact that the mean number of embryos transferred in the first warming cycle was 1.2, the multiple pregnancy rate was 19.2% in the collapse group and 9.5% in the control group, including three and one MZ pregnancy, respectively. When considering all warming cycles (data not shown), one extra MZ twin was found in the control group, resulting in three MZ pregnancies in the collapse group and two in the control group (8.6 and 6.5% per clinical pregnancy). These MZ twinning rates are high compared with the overall MZ twin rates in IVF/ICSI treatment cycles reported in recent literature (2.3% by Osianlis *et al.*, 2014; 2.1% by Knopman *et al.*, 2014; 1.4% by Nakasuji *et al.*, 2014). Nakasuji *et al.* (2014) and Knopman *et al.* (2014) found an increase in MZ twins in both fresh and frozen–thawed blastocyst transfers compared with cleavage stage embryo transfers on the one hand. On the other hand, frozen–thawed transfer per se did not affect the MZ twinning incidence compared with fresh transfer. In our present study, however, the number of clinical pregnancies is too limited in order to draw strong conclusions regarding MZ twinning. Nevertheless, this phenomenon and its possible relationship with artificial shrinkage should be carefully monitored further.

Although the study was designed to detect differences in implantation rates of vitrified–warmed blastocysts, we were obliged to randomize patients in the fresh cycles making the recruitment process difficult and time-consuming. Recruiting patients and signing informed consents was performed on the day of oocyte retrieval in the fresh cycle since collapse is performed just before vitrification. Only a proportion of these patients (60.9%), those who had blastocysts available for cryopreservation, were finally included in the study and randomized on the day of vitrification. Furthermore, study results could only be obtained when patients came to use their blastocysts in a warming cycle. In order to avoid the introduction of bias in the study, only the first warming cycle of each patient was included for the analysis of pregnancy outcome and implantation rate. Moreover, only less than half of the randomized patients returned for a warming cycle because of the high clinical pregnancy rates [with fetal heart beat (FHB)] of more than 50% in the fresh cycle. This rate was higher than the expected ongoing clinical pregnancy rate of 34.3% after fresh blastocyst transfer in the study by Papanikolaou *et al.* (2006), previously performed in our centre. Hence, more patients (270) than initially had been estimated (242) needed to be included and reaching this adequate number of patients took a period of 2.5 years.

Conclusion

In this study, no significant difference was found between collapsed and non-collapsed blastocysts at the level of implantation but higher survival rates and better post-warm embryo quality were observed when full to expanded blastocysts were collapsed using laser-induced artificial shrinkage before vitrification. However, it should be emphasized that this beneficial effect of collapse is valid in our closed vitrification system with CBS-HS-VIT straws. It is unclear whether these findings can be extrapolated to other vitrification protocols/devices. In this study, the high survival rate of 98% and the implantation rate of 37.6% per collapsed blastocyst transferred, suggest a benefit of artificial blastocyst shrinkage before vitrification. Although verification of a benefit in implantation rates needs validation by future and larger studies, the higher survival rates and the higher embryo quality justify implementation in routine vitrification practice in our centre.

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Authors' roles

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

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Conflict of interest

None declared.

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