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ARTICLE

Blastocyst transfer after aseptic vitrification of zygotes: an approach to overcome an impaired uterine environment

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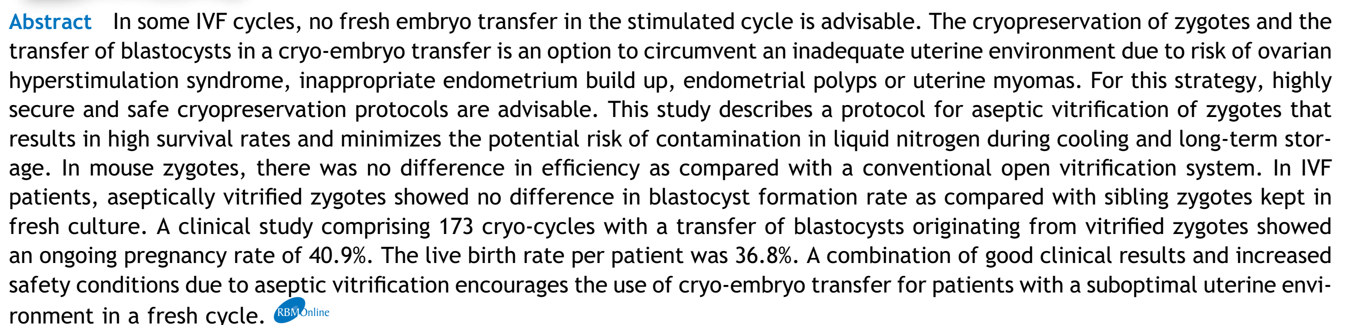

Abstract In some IVF cycles, no fresh embryo transfer in the stimulated cycle is advisable. The cryopreservation of zygotes and the transfer of blastocysts in a cryo-embryo transfer is an option to circumvent an inadequate uterine environment due to risk of ovarian hyperstimulation syndrome, inappropriate endometrium build up, endometrial polyps or uterine myomas. For this strategy, highly secure and safe cryopreservation protocols are advisable. This study describes a protocol for aseptic vitrification of zygotes that results in high survival rates and minimizes the potential risk of contamination in liquid nitrogen during cooling and long-term storage. In mouse zygotes, there was no difference in efficiency as compared with a conventional open vitrification system. In IVF patients, aseptically vitrified zygotes showed no difference in blastocyst formation rate as compared with sibling zygotes kept in fresh culture. A clinical study comprising 173 cryo-cycles with a transfer of blastocysts originating from vitrified zygotes showed an ongoing pregnancy rate of 40.9%. The live birth rate per patient was 36.8%. A combination of good clinical results and increased safety conditions due to aseptic vitrification encourages the use of cryo-embryo transfer for patients with a suboptimal uterine environment in a fresh cycle. RBMOnline

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KEYWORDS: aseptic vitrification, blastocyst transfer, cryopreservation, 2PN, uterine receptivity, zygotes

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Introduction

In a certain percentage of IVF cycles, no fresh embryo transfer is advisable due to health risks for the patients and/or an impaired uterine environment not providing an optimal environment for implantation. One indication to vitrify embryos is ovarian hyperstimulation syndrome (OHSS), which implies – next to the medical dangers for the patient – a potentially decreased endometrial receptivity due to histologically advanced and genomically dysregulated endometrial tissue as suggested by Check et al., 1999 and Shapiro et al., 2011. Other medical situations to cancel fresh embryo transfers are inappropriate endometrium build up, the occurrence of endometrial polyps or uterine myomas. Using highly efficient cryopreservation protocols is a reliable strategy to maximize the chances of pregnancy, substituting fresh with cryo-embryo transfer and thereby transferring the embryo in a well-prepared luteal phase. The cryopreservation of zygotes and the transfer of blastocysts in a cryo-embryo transfer after extended culture to day 5 has already been reported as a reliable strategy (Shapiro et al., 2009; Cohen et al., 2012). Apart from these reasons, in some countries, including, for example, Germany and Switzerland, legal restrictions make it necessary to cryopreserve the fertilized oocytes at the zygote stage. This is also an option for couples with ethical concerns because it circumvents the culturing and cryopreservation of late-stage embryos. Furthermore, cryopreservation at the 2-pronuclei (2PN) stage can be considered a valuable tool to overcome problems of synchronization between donor and recipient in egg donation cycles.

Due to high success rates, vitrification has become the preferred method for the cryopreservation of human oocytes and blastocysts in IVF programmes worldwide. Surprisingly, this method has not reached the same level of popularity when it comes to the cryopreservation of human 2PN embryos. Only a handful of cases and findings have been published (Isachenko et al., 2004, 2005; Liebermann and Tucker, 2002; Naether et al., 2008; Park et al., 2000; Selman and El-Danasouri, 2002) and the number of manuscripts describing the clinical application of 2PN vitrification remains extremely limited (Al-Hasani et al., 2007; Kuwayama et al., 2005), even though cryopreservation is justified for a wide range of different reasons as previously stated.

One drawback of the vitrification protocols employed is the direct contact of the embryos with liquid nitrogen (LN_2) during cooling and the persistent presence of LN_2 in the vitrification device. The study group has, therefore, established a protocol for aseptic hermetically closed straws minimizing the potential risk of contact with pathogens and reactive chemical compounds present in LN_2.

Vitrification is the conversion of a liquid into a glass-like state without the formation of crystals. It was postulated that ultra-rapid cooling and warming rates were mandatory during the vitrification process to reduce the risk of intracellular crystal formation and the concomitant damage of cell structures. To achieve ultra-rapid cooling rates, embryos are placed in a very small amount of cryoprotectant of less than 1 μl on open carrier devices, which are directly plunged into LN_2. Thereby, the embryos come into direct contact with LN_2, which is generally non-sterile and contains reactive compounds. The carriers are loaded into straws in the LN_2 bath, which means that the embryos remain in direct contact with LN_2 in the straws during the whole storage time.

Although the question of contamination by bacteria, viruses or fungi during cooling or storage in LN_2 is being debated, the mere probability of contamination raises safety concerns (Bielanski and Vajta, 2009; Bielanski, 2012; Cobo et al., 2012). For this reason, great effort is taken to at least minimize the risk of contamination for the embryos during the vitrification process and when they are directly plunged into LN_2. Various methods for sterilizing LN_2, including ceramic filters (Cobo et al., 2011) or UV-light simultaneously with hermetical cryostorage (Parmegiani et al., 2010, 2011), are under development and are both technically complex and expensive.

However, even avoiding potential contact with infectious material at the point of vitrification, a possible cross-contamination or other detrimental effects of LN_2, i.e. the constant exposure to toxic low-molecular-weight compounds, still remain if the devices are not hermetically closed (Yan et al., 2011). Although the probability of contamination in LN_2 is still being discussed, this risk is important and indicates that the storage system, especially for long-term storage conditions, should be revised. Contact with LN_2 in the cryovial can be avoided through either the storage of the samples in LN_2 vapour (Cobo et al., 2010) or the use of closed embryo carrier systems. Closed loading systems that can achieve suitable cooling and warming rates have successfully been applied for human blastocysts, for example (Vanderzwalmen et al., 2009, 2010).

As far as is known, no clinical data on the aseptic vitrification of human 2PN-embryos vitrified in hermetically closed straws on a large scale are available to date. Before considering aseptic vitrification of human zygotes as a standard for cryopreservation our IVF centre, an aseptic vitrification protocol in closed vitrisafe devices was first validated on mice at the 2PN stage. To achieve this objective, the developmental potential to form blastocysts after vitrification was compared for closed and open devices.

In patients who required the vitrification of embryos for medical reasons, for example, OHSS or insufficient endometrium build up, aseptic vitrification was applied on zygotes. The successful clinical application of the aseptic vitrification protocol was verified by analysing survival rates, blastocyst formation, ongoing pregnancy rates (OPR) and live birth rates (LBR) in 173 vitrification–warming cycles. Furthermore, during the first year of applying the vitrification of zygotes, the efficiency of the vitrification protocol was evaluated in a sibling analysis comparing the developmental capacity of vitrified and non-vitrified 2PN to form blastocysts.

Materials and methods

Mice zygotes: retrieval, culture and study design

FVB/N mouse inbred strains (Charles River, France), age 5–7 weeks, were stimulated with 3.5 IU of pregnant mare's serum gonadotrophin (Folligon, Intervet, The Netherlands) injected intraperitoneally, followed by an injection of 5 IU human chorionic gonadotrophin (HCG; Chorulon; Intervet,

The Netherlands) 36 h later. Soon after, each female was mated with a male of the same strain. The mice presenting a vaginal plug were killed on day 0.5 post coitum, enabling the collection of the zygotes after surgical opening of the ampulla.

Retrieved zygotes were randomly allocated to either vitrification in aseptic straws or in open devices. Vitrification was performed as described below. After warming, zygotes were kept in 50 μl drops of homemade M16 medium, covered with oil (Fertipro-Bernem-Belgium), in a humidified 5% CO2 incubator at 37.5°C. The survival rate after warming and the development of the embryos to expanded (day 5) and fully hatched blastocysts (day 6) were evaluated.

Patients: ovarian stimulation and embryo culture

This retrospective study comprises a total of 159 patients (aged 34.5 ± 4.2 years; range 23–42) with embryos vitrified at the 2PN stage in aseptic vitrisafe devices between July 2007 and March 2011 (**Figure 1**). Embryo transfers took place between January 2009 and September 2011. Fresh embryo transfers were cancelled due to OHSS (117 cycles), insufficient endometrium build up (20 cycles) and other medical reasons (22 cycles), such as the occurrence of endometrial polyps, uterine myomas or fever. Ovarian stimulation was performed using the long protocol (Zech et al., 2007). Oocyte retrieval was carried out 36 h post HCG administration followed by culture of the oocyte–cumulus–complexes in human tubal fluid medium (LifeGlobal, Ontario, Canada) for 2–4 h. Oocytes were then fertilized using either standard insemination, intracytoplasmic sperm injection or intracytoplasmic morphologically selected injection (Vanderzwalmen et al., 2008). Subsequently, the injected oocytes were cultured in Global medium (LifeGlobal) supplemented with 7.5% human serum albumin (HSA; LifeGlobal). Fertilization was assessed 16–20 h post insemination, followed by direct fresh culture to day 5 or vitrification of the 2PN cells (**Figure 1**). After warming, fertilized oocytes were cultured in four-well dishes (Nunc, Roskilde, Denmark) or five-well dishes (MTG, Germany) in Global medium supplemented with 7.5% HSA until day 5. On days 2 and 4, the medium was refreshed.

On day 5, the embryo quality was evaluated and blastocysts were selected for embryo transfer. The blastocyst quality was assessed according to the degree of blastocoele expansion and the quality of both the inner cell mass and the trophectoderm according to the classification of Gardner (Gardner et al., 2000). Blastocysts with a degree of expansion of 2, 3, 4 and 5 and with A grading for inner cell mass and trophectoderm or a combination of A and B grading were classified as top-quality blastocysts.

Hormone replacement therapy was administered to each group of patients prior to cryo-embryo transfer. After confirming steroid down-regulation, oestrogen therapy consisting of increasing doses of oestradiol valerate (Progynova; Schering) was started until the appropriate thickness of the endometrium (>8 mm) had been achieved. From that day on, intramuscular or intravaginal progesterone was administered until week 16. Informed consent was signed by all patients.

Vitrification and warming protocols

Vitrification and warming solutions

The non-vitrifying and vitrifying solutions were those described for the vitrification of blastocysts (Vanderzwalmen

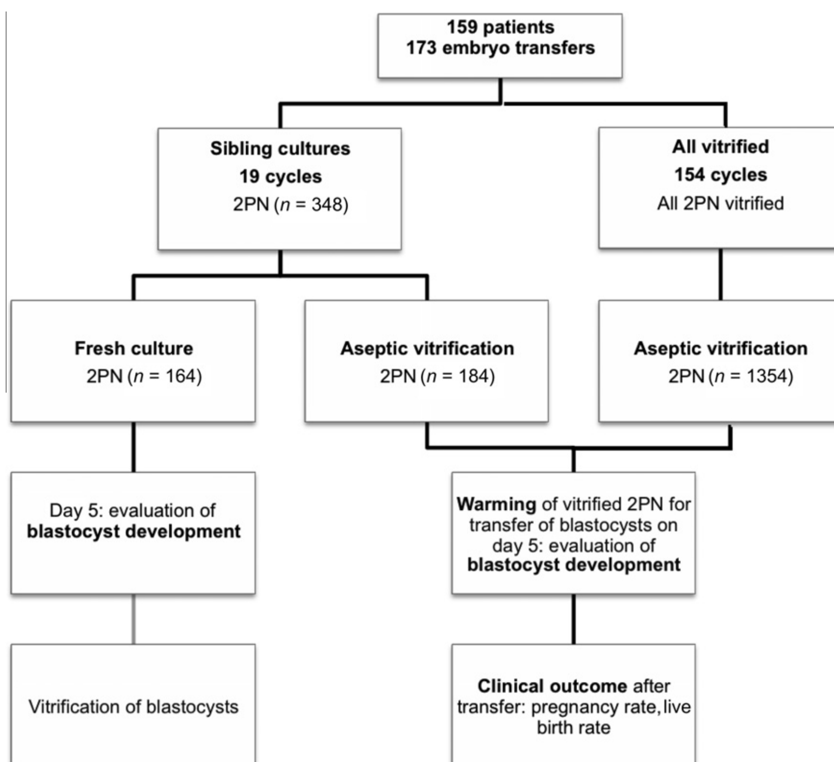

Figure 1 Study design.

et al., 2009). Briefly, two non-vitrifying solutions were prepared in phosphate-buffered saline (Fertipro, Bernem, Belgium) or Global HEPES (LifeGlobal, Ontario, Canada) containing 20% HSA: NVS 5/5, containing 5% (v/v) ethylene glycol and 5% (v/v) dimethylsulphoxide; and NVS 10/10, containing 10% (v/v) ethylene glycol and 10% (v/v) dimethylsulphoxide. The vitrification solution (VS) was composed of 20% (v/v) ethylene glycol and 20% (v/v) dimethylsulphoxide, 25 μmol/l (10 mg/ml) Ficoll (70,000 MW) and 0.75 mol/l sucrose. Warming was performed in five steps in sucrose solution from 1 to 0.125 mol/l in PBS with 10% HSA or with Global HEPES and 10% HSA.

Carrier devices

Vitrification in an open carrier device (mice experiment) was carried out in hemi-straws (Astro-Med-Tec, Austria). This previously described carrier consists of a large gutter onto which a small quantity of cryoprotectant (<1 μl) containing the zygotes is deposited. An ultra-rapid cooling rate of >20.000°C/min is achieved through the direct contact of the biological material with LN_2. The hemi-straws were subsequently inserted into a larger pre-cooled 0.3 ml straw (Cryo Bio System (CBS), France) (Vanderzwalmen et al., 2003) and closed under LN_2.

The vitrisafe plug (VitriMed, Austria), designed for the aseptic vitrification of blastocysts, was used as a hermetically closed device (Vanderzwalmen et al., 2009). The zygotes were placed into the vitrisafe gutter in a small quantity of vitrification solution (<1 μl). The gutter was inserted in the CBS straw and immediately welded before being plunged into LN_2. This ensured the hermetic isolation of the cells from the liquid nitrogen.

Vitrification and warming procedures

All steps took place at room temperature (22–25°C). Petri dishes with 50 μl of NVS 5/5 or NVS 10/10 covered with oil (Irvine, Santa Ana, USA) were prepared. Mice and human 2PN embryos were exposed in NVS 5/5 for 5 min and in NVS 10/10 for 5 min 30 s. Finally, the zygotes were washed by repeatedly pipetting in the VS before placing them into the gutter (5–10 mice zygotes or 2–4 human zygotes per gutter) on the open or closed carrier device. The time from placing the cells into the VS and loading them into the gutter of both carriers, followed by either plunging them directly into LN_2 or inserting them into the protective straw, welding it and then plunging them into LN_2 was 60 s.

After removing the hemi-straw or the vitrisafe, their gutters containing the 2PN were instantaneously immersed in 1 mol/l sucrose solution at room temperature for warming. After 1 min, the zygotes were transferred to 0.75, 0.5, 0.25 and 0.125 mol/l sucrose for 1, 1, 2 and 2 min respectively. Subsequently, they were directly placed in 500 μl culture medium for 15 min and rinsed twice before further culture.

Clinical outcomes

Sibling analysis of blastocyst development in fresh or vitrified human zygotes

A sibling analysis compared the efficiency of the vitrification procedure in 19 OHSS patients presenting a large number of zygotes (**Figure 1**). For this purpose, the fertilized oocytes were randomly divided into two groups: one half was aseptically vitrified in vitrisafe straws, whereas the other half was kept in culture for 5 days until the blastocyst stage prior to vitrification. When the patients presented for the first cryo-embryo transfer, the vitrified zygotes were warmed and cultured to day 5. In-vitro criteria for survival were established by assessing the percentage of blastocysts compared with that of the fresh control group.

Pregnancy, implantation and birth rate

The pregnancy rate among the patients who received embryos from aseptically vitrified zygote cultures was confirmed by a positive test for βHCG 14 days after embryo transfer. Clinical pregnancy was defined as the presence of a gestational sac at 6 weeks and ongoing pregnancy was defined as the observation of fetal heartbeat by ultrasound 8–12 weeks after embryo transfer. The implantation rate was calculated by the number of fetal heart beats divided by the number of embryos transferred. The results are presented in terms of delivery for the embryo transfers performed in 2009 and 2010.

Statistics

Differences in the implantation rates and pregnancy rates and live births were evaluated using Pearson's chi-squared test. A two-tailed t-test was used to test for differences in blastocyst quality and growth. Differences between the groups were considered statistically significant when the *P*-value was <0.05. Statistical analysis was performed using Statistical Package for Social Sciences version 17.0 for Windows (SPSS, USA).

Results

Vitrification of mouse zygotes in open and closed carriers

After collection, mouse zygotes were randomly allocated between the two groups. A total of 53 sibling zygotes were vitrified in open straws allowing for direct contact with LN_2, and 52 zygotes were vitrified in hermetically closed vitrisafe straws (**Table 1**). A total of five assays were performed. The survival rate after warming was 98.1% for both groups. On day 5, the number of blastocysts was 42 in the open straw group (80.8%) and 34 blastocysts hatched on day 6 (81.0%). A total of 43 blastocysts were obtained in the cultures derived from aseptically vitrified zygotes (84.3%) and 36 blastocysts hatched on day 6 (83.7%). No statistical difference between the groups was detected.

Human blastocyst development in fresh cultures versus cultures from aseptically vitrified zygotes

From a total of 499 collected oocytes, 348 zygotes were obtained (**Table 2**). Some of the zygotes was randomly allocated for direct vitrification ($n = 184$) whereas the others were kept in culture until day 5 ($n = 164$). In the subsequent cryo-embryo transfer cycle, the vitrified zygotes were warmed and cultured to day 5. The blastocyst development

Table 1 Blastocyst development of mouse zygotes vitrified in open versus closed straws.

Mouse zygote	*Open straws*	*Aseptically closed straws*
2PN vitrified	53	52
Viable after warming	52 (98.1)	51 (98.1)
Blastocysts	42 (80.8)	43 (84.3)
Fully hatched blastocysts	34 (81.0)	36 (83.7)

Values are *n* or *n* (%). There were no significant differences between type of straw (chi-squared test).

Table 2 Blastocyst development of fresh versus aseptically vitrified sibling zygotes (19 patients).

	Fresh culture	*Aseptic vitrification*
Patient age (years)	32.5 ± 4.2	
2PN	348	
2PN per culture	164	184
Survival after warming	—	176 (95.7)
Blastocysts	45 (27.4)	46 (26.1)
Top-quality blastocysts	29 (64.4)	24 (52.2)

Values are mean ± SD, *n* or *n* (%). There were no significant differences between fresh and vitrified zygotes (chi-squared test).

of the aseptically vitrified zygotes was compared with the sibling zygotes kept in fresh culture. Of 164 2PN in fresh culture, 45 (27.4%) developed to blastocysts on day 5 and 29 (64.4%) of these were classified as top-quality blastocysts. In the vitrified sibling group, 176 2PN of 184 (95.7%) were viable after warming. The developmental potential of the vitrified zygotes to develop to blastocysts (26.1%) and top-quality blastocysts (52.2%) showed no difference as compared with the fresh culture. All blastocysts obtained in the fresh cultures were subsequently cryopreserved.

Survival, blastocyst development, pregnancy and implantation rates after vitrification of zygotes in closed carrier devices

Of 159 patients, 1538 2PN oocytes were vitrified aseptically and warmed for a total of 173 embryo transfers. A total of 1429 zygotes (92.9%) survived the aseptic vitrification—warming process (Table 3). After 5 days in culture, 587 (41.1%) developed to blastocysts, of which 272 (46.3%) showed top quality. Of the 179 warming cycles, six (3.4%) embryo transfers had to be cancelled due to the absence of blastocyst formation. A total of 312 embryos were transferred (mean 1.8 embryos per embryo transfer). Eight weeks after transfer, 65 patients were tested positive for fetal heartbeat (OPR per embryo transfer 37.6%) and a total of 76 embryos implanted (implantation rate per embryo transferred 24.4%).

Table 3 Clinical pregnancies after zygote vitrification with the vitrisafe carrier device (2009—2011; 159 patients).

Parameter	*Sample*
Patient age (years)	34.5 ± 4.2
Warming cycles	179
Cycles with no blastocysts	6 (3.4)
Embryo transfers	173
2PN warmed	1538
2PN intact after warming	1429 (92.9)
Blastocysts	587 (41.1)
Top-quality blastocysts	272 (46.3)
Embryos transferred (*n*, mean per transfer)	312 (1.8)
Clinical pregnancies	78
Per embryo transfer (%)	45.1
Per patient (%)	49.1
Ongoing pregnancies	65
Per embryo transfer (%)	37.6
Per patient (%)	40.9
Implantations	76
Per embryo transferred (%)	24.4

Values are mean ± SD, *n* or *n* (%), unless otherwise stated.

Deliveries and take-home baby rate

Between January 2009 and December 2010, 91 embryo transfers of blastocysts from aseptically vitrified 2PN were performed in the study institute (Table 4). Only in four warming cycles (4.2%) was no embryo transfer performed due to a lack of blastocyst development. In the 91 cycles, 165 blastocysts were transferred (mean 1.8 blastocysts per embryo transfer). An OPR per embryo transfer of 40.7% was obtained, as a result of which 32 patients (36.8%) delivered. No malformations in any of the 38 babies born were reported. An implantation rate of 23% per embryo transferred was noted.

Outcome according to blastocyst quality

Blastocyst quality plays a crucial role in the potential to implant. Therefore, the data were analysed in respect to the quality of the embryos transferred (Table 5). At least one top-quality blastocyst was transferred in 128 embryo transfers (73.9%). In 45 embryo transfers (26.0%), only non-top-quality blastocysts were available for transfer. In the group of top-quality blastocysts, the OPR per embryo transfer was 42.2%, which was significantly higher ($P < 0.05$) than in the non-top-quality blastocyst group (24.4%). The implantation rate was 27.4% in the top-quality blastocyst group as compared with 15.4% in the non-top-quality group. The differences were also statistically significant ($P < 0.05$).

Discussion

The objective of the present work was to establish a safe vitrification method for zygotes from patients where a fresh embryo transfer is not advisable due to different medical indications. With this technique, high survival rates after

Table 4 Babies born after zygote vitrification in closed cooling conditions (2009–2010; 87 patients).

Parameter	*Sample*
Patient age (years)	33.4 ± 4.8
Warming cycles	95
Cycles with no blastocysts	4 (4.2)
Embryo transfers	91
2PN warmed	869
2PN intact after warming	8149 (3.7)
Blastocysts	3013 (7.0)
Top-quality blastocysts	1354 (4.9)
Embryos transferred (*n*, mean per transfer)	165 (1.8)
Clinical pregnancies	37
Per embryo transfer (%)	40.7
Per patient (%)	42.3
Live births	32
Per embryo transfer (%)	35.2
Per patient (%)	36.8
Babies born	38
Implantations	38
Per embryo transferred (%)	23.0

Values are mean ± SD, *n* or *n* (%), unless otherwise stated.

warming and implantation rates comparable to fresh embryo transfer were achieved. Therefore, cryopreservation can be performed with complete isolation of the biological material from LN_2 using the vitrisafe straw.

Prior to introducing the closed device for human zygotes, this study conducted a sibling analysis of the post-warming survival rates and blastocyst development of mouse zygotes after vitrification in open and closed conditions. No difference was noticed in the outcome of embryo development. Subsequently, the efficiency of the closed device was evaluated by comparing the developmental capacity of vitrified and non-vitrified human zygotes in sibling cultures to form blastocysts. No statistically significant difference in the rate of blastocysts and top-quality blastocysts was noticed between the vitrified and non-vitrified group, suggesting no impairment of the developmental potential due to the vitrification process.

These findings are reinforced in the clinical results, covering a period of 3 years, which show that no blastocysts were observed in only 3.4% of 179 vitrification–warming cycles. After warming, 92.9% of the zygotes were viable and divided, resulting in a blastocyst rate of 41.1% and an OPR per embryo transfer of 37.6%. The delivery rate calculated for embryo transfer in 2009 and 2010 was 35.2%, and a delivery rate of 36.8% per patient was obtained. A total of 38 babies were born, of which six were twins and no malformations were reported. The quality of blastocysts was identified as a predictive factor for the success of the cryo-cycle (as in fresh cycles). When at least one top-quality blastocyst was transferred, a significantly higher OPR was found as compared with non-top-quality blastocyst embryo transfer. These findings correspond to earlier publications showing the importance of embryo quality for embryo transfer (Ebner et al., 2009).

In order to comply with European (European Parliament and the Council of the European Union, 2004, 2006) as well as with US Food and Drink Administration (Food and Drink Administration, 2007, 2004, 1997) directives on tissue and cell storage, requiring that gametes and embryos are protected from any possible contamination with pathogens during vitrification and storage, it is mandatory to switch from an open vitrification carrier device to a protocol that entails complete isolation of the biological samples from LN_2 during both the cooling process as well as storage by hermetically isolating the embryos from LN_2 in the tanks. Until recently, however, it was believed that the reduction in the cooling rate associated with closed carriers impaired embryo viability. Although reports of successful vitrification protocols using closed devices are increasing, it is still widely believed that reduced cooling conditions may profoundly impair results. Some studies show that the vitrification of blastocysts in closed carriers achieves promising IVF results in clinical studies (Kuwayama et al., 2005; Vanderzwalmen et al., 2009; Fasano et al., 2010; Stachecki et al., 2008). It may be deduced from the present study that, despite the reduction in cooling rates to less than 2000°C/min in the carrier used here, survival rates of more than 90% can be obtained, as well as an embryo development comparable with fresh cultures and high implantation rates.

Although no direct comparison between the results obtained after fresh and cryo-embryo transfer after zygote vitrification are possible – because cryopreservation was always medically indicated in these patients – the data clearly show that no decrease in the developmental potential of the embryos was found between the two groups. When transferred to a well-prepared uterine environment, the embryos from fresh or cryopreserved cultures had the same chance of successful implantation. The clinical pregnancy and implantation rates for 2633 patients with fresh embryo transfer in this centre during the same time period were 44.2% and 23.3% per embryo transfer, respectively, as compared with 45.1% and 24.4% in 159 patients with embryo transfer from vitrified zygotes. No statistically significant differences in the miscarriage and birth rates between the groups were observed.

However, a large majority of assisted reproduction practitioners continue to focus their attention on the cooling rate and claim that an ultra-rapid cooling rate is mandatory without being fully aware of the importance of the warming rate. It has become obvious that the warming rate may play a more essential role in modulating survival rates after vitrification than the cooling rate. Warming rates over 25,000°C/min are obtained with the vitrisafe device when the zygotes are directly plunged into at least 500 μl sucrose solution.

A high warming rate prevents the vitreous water from crystallizing during the warming phase (Seki and Mazur, 2008, 2009) and these authors conclude that a warming process that is too slow is potentially lethal, as this may cause the formation of ice crystals in lethal quantities. In fact, during the process of warming, cells first devitrify when they are warmed above the glass transition temperature. If the warming rate is not high enough, the supercooled liquid is transformed with great rapidity into small ice crystals. Given appropriate time or low warming rates, the small ice crystals are subjected to the phenomenon referred to as (re)crystallization. This may in part explain why closed carrier systems with their slower cooling rates can still be

Table 5 Clinical outcomes in relation to the quality of blastocysts transferred.

	Quality of blastocysts transferred	
	Top-quality (117 patients)	*Non-top-quality (42 patients)*
Patient age (years)	34.2 ± 4.4	34.6 ± 4.7
Embryo transfers	128 (73.9)	45 (26.0)
Embryos transferred (*n*, mean per transfer)	234 (1.8)	78 (1.7)
Clinical pregnancies[a]	66	12
Per embryo transfer (%)	51.6	26.7
Per patient (%)	56.4	28.6
Ongoing pregnancies[b]	54	11
Per embryo transfer (%)	42.2	24.4
Per patient (%)	46.2	26.2
Implantations[c]	64	12
Per embryo transferred (%)	27.4	15.4

Values are mean ± SD, *n* or *n* (%), unless otherwise stated.
[a]Chi-squared = 8.3, $P < 0.01$.[b]Chi-squared = 4.5, $P < 0.05$.
[c]Chi-squared = 4.5, $P < 0.05$.

successfully applied as long as the system parameters permit sufficiently fast warming rates.

It is well known that, for any given concentration of cryoprotectants, the critical warming rates are much higher than the critical cooling rates (Fahy et al., 1987). Consequently, the minimal concentration of cryoprotectants to prevent crystallization during warming must be higher than during cooling, and cooling and rewarming speeds must be constant. This means that it might be easier to maintain a vitrified state during the cooling than during the warming process for the same concentration of cryoprotectants. If the warming rate is reduced by using devices that separate the drop containing the embryos, higher intracellular concentrations of cryoprotectants are needed in order to reduce the likelihood of recrystallization, which may be toxic for the further development.

This report shows that aseptic vitrification is a safe method for clinical application, making it much easier to prevent contamination during cryopreservation and storage. Cobo et al., 2011 proposed filtration through ceramic filters or UV-treated LN_2 when using open straws in order to ensure that the LN_2 into which the samples are plunged fulfils safety criteria. The theoretical risk of cross-contamination in LN_2 containers even at −196°C has been widely debated (AbdelHafez et al., 2011). It is well known from cryobanking or the storage of cultured cells that contamination of LN_2 with micro-organisms, for example, mycoplasma or *Escherichia coli*, is quite common (Bielanski, 2012). Although the risk of cross-contamination is merely a theoretical one and no contamination has been reported for cryopreserved oocytes and blastocysts, cross-contamination in human bone marrow has been reported in a case report addressing the safety management of assisted reproduction (Hawkins et al., 1996). Storage in LN_2 vapour tanks has been proposed to prevent cross-contamination during storage (Cobo et al., 2010). However, even LN_2 vapour is reported to be a risk factor for pathogen transfer (Grout and Morris, 2009). Additionally, temperature variations within the vapour might impair the viability of cryopreserved gametes and embryos.

During long storage, not only the problem of pathogen contamination has to be taken into account but also the substantial problem of contagion with detrimental particles that may appear in LN_2. Reactive chemical compounds in LN_2 might induce biophysical injuries of the cryopreserved samples. Methods to sterilize the LN_2 prior to contact with the gametes or embryos with UV light might theoretically provoke the production of reactive compounds, leading in turn to an increased risk of damage due to the physical and chemical properties of irradiated LN_2 when not carefully performed. In a nitrogen-saturated environment, it was shown that the risk of radical formation due to UV sterilization is nil (Parmegiani et al., 2011).

However, even the standard storage conditions and refilling of the tanks can pose a potential hazard when oxygen from surrounding air condenses and mixes with LN_2 during the regular opening of the nitrogen tank for routine refilling or whenever straws are added or withdrawn. Although it is generally assumed that thermally driven reactions do not occur in cells at −196°C, it has been reported that, in the case of radiation of an LN_2/oxygen mixture, a synthesis of oxygen radicals resulting from ozone formation and decomposition cannot be excluded and is even enhanced by the catalytic effect of nitrogen. A recent publication reports that mouse oocytes show impaired survival, fertilization rates and embryonic development after prolonged contact with LN_2 (Yan et al., 2011). These findings are of special interest as the biological sample in the open devices commonly used is constantly exposed to LN_2 since the plug is inserted into the outer straw in the LN_2 bath. As a result, not even the avoidance of cross-contamination in the tanks by storage of straws in nitrogen vapour can sufficiently protect the samples. In view of the different theoretical and hypothetical dangers that may be present during the cooling process and storage, it is therefore recommended that only a closed system can be used to guarantee aseptic storage conditions.

In conclusion, in order to prevent gametes and embryos from contamination with pathogens or chemical compounds in LN_2 and in order to adhere to statutory requirements, closed vitrification devices should be used to separate the probes from LN_2 during the whole process. This study shows that vitrification in aseptic devices is safe and does not

impair embryo viability, developmental potential or the implantation rate. The establishment of a successful aseptic vitrification protocol might change the transfer strategy and encourage cryo-embryo transfer in the hope of increasing clinical pregnancy rates in those patients assessed with a suboptimal uterine environment during the fresh cycle (Cohen et al. 2012).

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