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Validation of microSecure vitrification (μS-VTF) for the effective cryopreservation of human embryos and oocytes ☆

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ABSTRACT

A novel, aseptic closed system vitrification (VTF) technique for the cryopreservation of embryos and oocytes has been developed and clinically validated in this study. It combines the practicality of embryo-containing sterile flexipettes stored safely and securely with 0.3 ml CBS™ embryo straws possessing weld seals. The cooling and warming rates of this double container system were determined using a data logger. Upon direct plunging into LN_2, the flexipettes cool at an average rate of 1391 °C/min, while warming occurs at an average rate of 6233 °C/min in a 37 °C 0.5 M sucrose bath. Direct deposition of the flexipette into a warming bath insured a rapid transition between −100 and −60 °C to minimize potentially harmful recrystalization associated with devitrification. In conclusion, the μS-VTF system has exhibited higher ($p < 0.05$) intact survival, implantation and live birth rates than conventional slow freezing methods. The effective embryo transfer of vitrified blastocysts proved similar to or better than fresh embryo transfer outcomes. The sustained clinical use of μS-VTF has justified a change in our infertility practice.

Capsule: The microSecure vitrification (μS-VTF) procedure is a low-cost, non-commercial, aseptic, closed system that offers technical simplicity and repeatability, while effectively attaining an estimated 4:1 warming-to-cooling rate ratio, which supports excellent embryo survival and sustained viability.

Introduction

The development and use of open system vitrification devices in the 21st century proved highly effective at achieving ultra-rapid cooling rates (e.g., >10–20,000 °C/min), and maintaining human embryo and oocyte viability [5,6,16,17,22,23]. Early success led to the commercial development of numerous high-cost commercial vitrification devices for use in the IVF industry. These devices typically require technical precision and experience to pipette embryos or oocytes in micro-volumes (1 μl or less) onto a secondary device surface. The further reduction of residual volume around the embryo(s)/oocyte(s) represents another variable, but critical, step that allows for maximum cooling and warming rates to be achieved. By striving for ultra-rapid cooling rates, these open system devices are highly dependent on attaining ultra-rapid warming rates to optimize survival of oocytes [21,25]. Conversely, closed devices, which necessarily involve cooling and warming at lower rates, are dependent on the use of a higher intracellular concentration of permeating cryoprotectants to avoid ice formation and achieve high survival rates [30,35]. Therefore, closed devices are more tolerant of slower warming rates to minimize recrystalization events [36]. In both cases, survival is dependent on warming rates and optimized when warming rates exceeded cooling rates to minimize potentially lethal recrystallization events [10–12,29].

In a decade of escalated concern and awareness regarding good tissue handling practices (GTPs), FDA regulations and European Union directives, cryopreservation methods that emphasize secure/aseptic storage, technical ease/repeatability, and consistent outcomes are desirable. Variability in the successful application of commercial vitrification device procedures is attributed to "technical signature", i.e., variation in technical skill between programs and individuals [38]. Ideally, a universal vitrification device for blastocysts that insures technical simplicity, safety and repeatability while routinely achieving high recovery, survival and viability rates post-warming is needed in the assisted reproductive technology industry. Although viral cross-contamination between human

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embryos or oocytes in LN_2 storage has not been documented [7,28], the potential exposure of samples in open devices to various common environmental pathogens (e.g., bacteria, fungi, mycoplasma) in non-sterilized LN_2 creates an undesirable exposure risk for any laboratory [4]. The use of aseptic, closed vitrification devices provides the most reasonable alternative to the safe and secure storage of vitrified gametes and embryos, without having to resort to laborious, costly and impractical decontamination and LN_2 sterilization procedures [4,26,27]. It is worth mentioning that viral cross-contamination of bovine embryos with BVBD and BHV-I, as well as bacteria, has occurred under experimental conditions [3], but that those same types of harmful pathogen have also been effectively controlled in validation studies by CryoBioSystems with their patented ionomeric resin plastic straws which reliably weld seals. Considering that comparative trials between open and closed vitrification devices have exhibited no difference in embryo survival rates or pregnancy outcomes [18,19,24,25], it is puzzling why the ART industry dogma regarding the importance of ultra-rapid cooling rates persists [36].

A novel, aseptic vitrification technique called microSecure vitrification (μS-VTF) was previously validated in the mouse model [32,34], offering technical ease, reliability and cryo-security using two FDA compliant devices. This low-cost, non-commercial vitrification system offered numerous other quality control advantages including a unique tamperproof and internalized dual-colored labeling system [33]. A series of preliminary mouse embryo investigations documented the promising potential of μS-VTF [32], while achieving cooling rates >1100 °C/min and a suboptimal, biphasic warming rate pattern under ambient conditions. The objectives of our four phase validation study was to document the cryophysics of a modified warming protocol and verify the overall clinical efficacy of the μS-VTF procedure in contrast to standard slow freezing protocols.

Materials and methods

In our development of the μS-VTF procedure, we obtained IRB approval (Coastal IRB 2008, and Aspire IRB 2009–2012) to conduct a "Comparative Human Oocyte Cryopreservation" study. Investigations began with a series of preliminary validation and verification studies on discarded human immature oocytes that were matured in vitro and abnormal fertilized embryos of various stages (e.g., zygotes, cleavage and blastocysts) to assess the efficacy of μS-VTF using Me_2SO-free S^3/Innovative Cryo Enterprises LLC (I.C.E.) solutions [38]. The basic composition of these Hepes-buffered sterile aqueous solutions includes organic and inorganic salts, non-essential/essential amino acids and human serum albumin. Ethylene glycol, ficoll and sucrose were added to the Oocyte and Embryo I.C.E. vitrification solutions, while glycerol was supplemented to the blastocyst solutions. The exact concentrations of glycol- or glycerol-based cryoprotectants in the oocyte, embryo and blastocyst solutions, respectively, as well as the content of albumin and other macromolecules in the I.C.E. solutions are proprietary commercial properties and have not been disclosed. However, the approximate total content of permeable cryoprotective agents (CPAs) in the final blastocyst vitrification solution is 7.9 M, while the Oocyte solution is 3.6 M with an appreciable amount of non-permeating CPAs. These solutions are readily available for use through I.C.E. (Linden, NJ) for replication of study results.

μS-VTF apparatus

The μS-VTF method combines the use of 300 μm ID sterile flexipettes (Cook Medical, Bloomington, IN) to load vitrification solutions with oocytes or embryos before placing the dried flexipette into a 0.3 ml CBS™ embryo straw with an internalized hydrophobic plug (CryoBioSystems; Irvine Sci., Santa Ana, CA) for secure, long-term aseptic storage in LN_2. In brief, flexipettes that are routinely used in embryology laboratories worldwide are individually extracted from their sterile container and shortened approximately 3 cm from the base and then securely placed onto a Stripper pipettor (preset to 3 μl). Individual colored labels (1.3″ × 0.5″; CL-23, GA International Inc.) were then created, adhered to a 40 mm color coded, weighted ID rod and inserted into the internal labeling end of the CBS™ straw, before being weld sealed under ambient conditions using a Syms 1 sealer (CryoBioSystems), which eliminates technical variation.

Cooling and warming rate determination

The validation of cooling and warming rates (Phase 1) was determined using a DATAQ Instruments Model DI-1000-USB data logger (www.dataq.com) and an Omega 5SRTC-TT-T-30-36 fine (0.13 mm diameter) thermocouple. The thermocouple was threaded into the base of the flexipette filled with 3 μl of I.C.E. vitrification solution ($n = 4$), and the flexipette was then inserted into a 0.3 ml CBS straw, which was then plunged into LN_2 for temperature tracking at 0.2 s intervals. Similar measurements were also taken upon warming, but for warming, the extracted flexipette was immediately allowed to warm in ambient air (preliminary study, Fig. 1) or plunged into a 37 °C solution within a 58 mm petri dish (Figs. 2C and 3). All data points were plotted using SigmaPlot, and mean cooling and warming rates were calculated for each run between 0 and −125 °C and averaged over four independent runs.

Embryo culture and viability testing

All oocytes and embryos were cultured in Global® medium (LifeGlobal, Guilford, CT; 25 μl droplets under oil) using tri-gas mini Sanyo/Panasonic MCO-5 M incubators (5% O_2 and 5.3–6.1% CO_2) at 37 °C under humidified conditions. All mature oocytes were inseminated by intracytoplasmic sperm injection (ICSI) [31], and their fertilization status determined 16–21 h post-ICSI. Normal 2PN zygotes were maintained in extended culture for up to 6 days to promote blastocyst formation, with fresh embryo transfer performed on day 5 and residual fair to good quality blastocysts cryopreserved on days 5 or 6. In vitro matured oocytes and abnormally fertilized zygotes (1PN or 3PN) from consenting patients were assigned to vitrification treatment as oocytes ($n = 21$), zygotes ($n = 43$), cleaved embryos ($n = 28$) or blastocysts ($n = 39$), which may have involved extended culture in research dishes, as described above. Their survival was subsequently assessed in Phase 2 of the validation process.

Prior to implementing vitrification into our clinical practice in November 2008, conventional slow freezing was performed without pre-freeze blastocoele collapse (BL-SF; prior to November 2007) [13,15] or with induced-collapse (CBL/SF; November 2007 thru December 2008) by single pulse laser ablation of a trophectodermal cell using a ZILOS-tk® infrared laser (Hamilton-Throne, Beverly, MA). Conventional BL-SF involved a 5 min equilibration in a PBS stock solution + 20% synthetic serum supplement (SS; Irvine Scientific; Santa Ana, CA), followed 10 min dilutions in 0.8 M glycerol and then 1.6 M glycerol + 0.2 M Sucrose. Individual or paired blastocysts were loaded into and heat sealed in either 0.25 ml IMV straws or 0.3 ml CBS embryo straws. Blastocoele collapsing was not performed on vitrified blastocysts maintained in ICE Me_2SO-free vitrification solutions. With conventional SF, survival rates were classified as transferrable blastocysts possessing >50% intact cells post-thaw. Alternatively, vitrified blastocysts were considered to have survived based on their osmotic responsiveness (i.e., cellular contraction and equilibration) to sucrose

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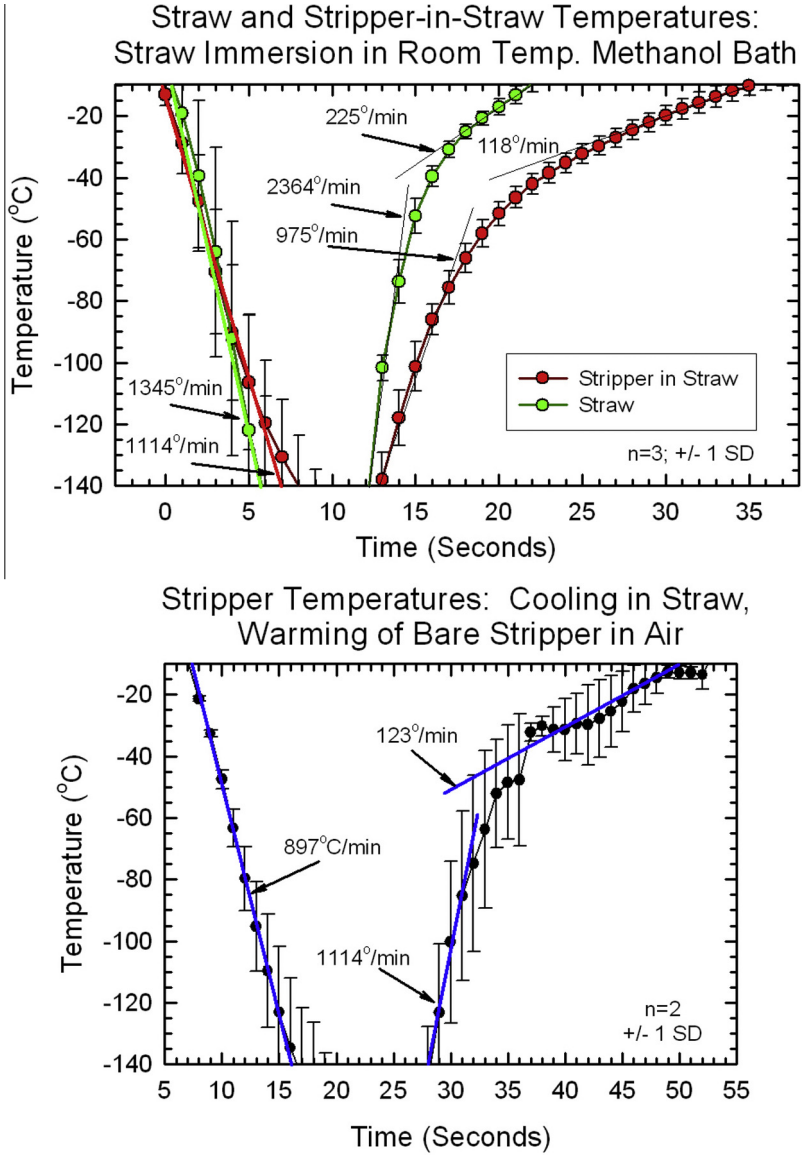

Fig. 1. Preliminary data logger results of μS-VTF straws and flexipettes (i.e., Stripper tip) were used to evaluate their separate and combined cooling rate potential in LN_2 and warming characteristics under room/ambient conditions. Removing the stripper tip from the straw and warming it in air did not significantly improve its warming rate compared to warming it by immersion of the straw in which it was contained. The straw in which it was contained warmed more rapidly than the contained stripper tip due to thermal lag across the stripper tip wall. In all cases, an undesirable slowing in the warming between −100 °C and −60 °C was observed, raising concerns about potential recrystallization and possible cellular damage.

solutions and presence of >95% intact TE cells. Survival for vitrified blastocysts in our system was more of an all or none outcome to the maintenance of cellular integrity. When blastocysts possessed >5% pyknotic cells and failed to contract and/or re-equilibrate in sucrose solutions they were considered as having not survived.

All cryopreserved embryo transfer (CET) cycles involved hormone replacement cycles using oral Estradiol (E_2), E_2 patches or i.m. E_2 valerate followed by i.m. Progesterone (P_4; in oil, 50 mg b.i.d., started when endometrial thickness was $\geqslant$8 mm after documentation of serum P_4 levels $\leqslant$1 ng/dl). All transfers were performed 5 days after P_4 supplement using transvaginal guidance to specifically deposit the embryo(s) 1 cm from the fundus [1] using a Sure View® Wallace® embryo replacement catheter (Smiths Medical Ltd., Kent, UK). The first blood test for β-hCG was drawn 10 days post-embryo transfer, while clinical pregnancies (CP) were documented by the presence of fetal cardiac activity at the 7 week ultrasound. Implantation rates (IR) were calculated by the number of confirmed sacs divided by number of transferred blastocysts. The final determinant of success was the live birth rate. In the validation of the clinical effectiveness of vitrification, all CET cycles (BL/SF, CBL/SF and VTF) from 2008 and 2009 were contrasted by retrospective analysis (Phase 3) and compared to fresh embryo transfer outcomes. In addition, the overall clinical efficacy of μS-VTF application was further assessed over a 3 year period between 2009 and 2011 (Phase 4), and contrasted to our Medical Director's fresh IVF cycle data reported to the Society for Assisted Reproductive Technology (SART). SART is an affiliated sub-society of the American Society for Reproductive Medicine (ASRM) who reports the annual IVF statistics/data of its member clinics to the Center for Disease Control annually as mandated by the federal government.

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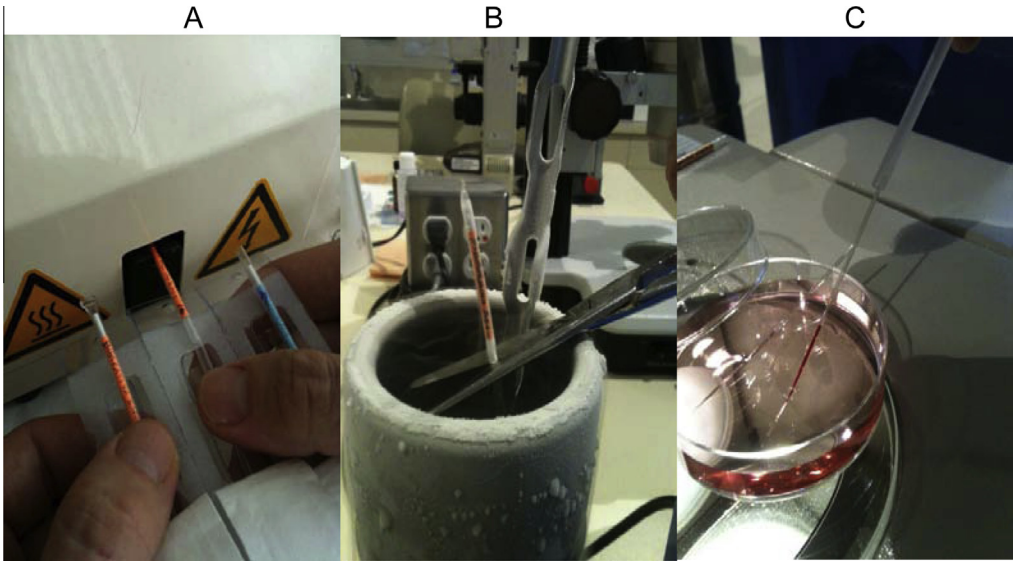

Fig. 2. In frame A, pre-labeled CBS embryo straw awaits open-ended (right straw) within the confines of its sterile packaging during vitrification set-up. The central straw is being reliably sealed in an automated Syms 1 sealer, with the completed weld seal seen on the straw to the left. Note that the tamperproof internalized labels can be different colors and secured on different colored weighted ID rods. In 2B, the frozen μS-VTF straw is secured with heavy-duty surgical scissors just below the internal hydrophobic plug separating the dual-colored identity label from the vitrification tip, which is safely maintained in sub 150 °C conditions. For extraction and warming, the straw is lifted from the LN_2, cut in a horizontal position and then tilted downward to release the contents as per 2C. Frame 2C shows a vitrified flexipette, containing 3 μl of red dye, free-falling from the opened CBS embryo straw into its target, a petri dish filled with warm medium to serve as a thaw bath. Note that a secondary, typical flexipette (without dye) rests securely against the dish edge while warming, prior to being attached to a Micro-Cap pipettor to gently evacuate contents into 1.0 M sucrose in a separate multi-well elution dish.

Vitrification and warming procedures

The vitrification procedure has been described in detail previously [32,33] (Fig. 2). All handling procedures were performed at room temperature (20–24 °C) using a 3-step vitrification solution addition and 5-step dilution procedure, as described in detail elsewhere [32]. For vitrification, all oocytes and embryos (day 1 and 3) were placed into V1 and V2 solutions for 5 min and 2 min, respectively, and 5 min and 5 min for blastocysts. Following V2 equilibration, embryos and oocytes were pipetted into the final V3 solution for 1–1.5 min, respectively, and loaded into a 3 μl column in the flexipette. The tip was simply removed and wiped dry on sterile gauze, and then inserted completely into the inner lumen of the open-ended straw before final weld sealing and direct placement into LN_2 storage.

Prior to cooling, the outer CBS straws were heat sealed as described in Fig. 2A. For warming, the identification of a patient's colorized straw label (with the aseptic contents submerged in LN_2) was safely and effectively performed by grasping the μS-VTF straw with large surgical scissors (Fig. 2B) just below the internal plug. Once the ID was confirmed, the straw was quickly pulled up into ambient air and a "cut-tilt-tap" technique applied (refer to YouTube video, "microSecure vitrification warming"). The straw was cut in a horizontal position while holding the non-labeled end and then tilted downward (45–60° angle) over a warm (37 °C) 0.5 M sucrose bath, tapped gently and the vitrification tip was allowed to free-fall directly into the 58 mm diameter petri dish for rapid warming (Fig. 2C). Within 10 s, the flexipette was removed from the bath and its contents expelled into a 1.0 M sucrose solution. The cryoprotectants were then eluted from the oocytes or embryos in a series of declining sucrose concentrations (e.g., 1.0 M to 0.5 M to 0.25 M to 0.1 M; proprietary solutions: T1–T4; 5 min/step) at room temperature (20–24 °C) before a 5 min isotonic equilibration in culture medium at 37 °C, as described elsewhere [32,33]. The routine thawing of slow frozen blastocysts has been described previously [15].

Pregnancy outcomes were evaluated by the determination of +β-hCG levels, clinical pregnancy, implantation and live birth rates. In Phase 3, the cryopreservation treatments were all compared to a fresh blastocyst embryo transfer group with rate (%) differences determined using Chi-square analysis.

Results

Phase 1: warming rate validation

In the validation of a modified warming procedure for μS-VTF, it became clearly evident that the immediate submersion of the flexipette into a warm bath significantly enhanced the warming rate by approximately 600%, from 1114 °C/min (Fig. 1) to a mean of 6233 °C/min (Fig. 3), compared to preliminary measurements under ambient air exposure (Fig. 1). More importantly, it eliminated the biphasic pattern of warming previously observed, where a significant decline in warming rate was evident by −100 °C with a distinct shift seen between −60 °C and −40 °C (Fig. 1). Significant variation recorded in warming run 2 was due to a slight delay in plunging of the tip into the warming bath. Overall the warming rate for this sample was still more than 2-fold greater than the cooling rate and, more importantly, the warming rate above ≈ −90 °C was just as rapid as the other samples. It is doubtful that slower warming below −90 °C would be harmful to the vitrified oocytes and embryos. The calculated cooling rate of 1391 °C/min was consistent with previous measurements. Thus, we verified that the mean warming rate is 4.5-fold greater than the cooling rate.

Phase 2: survival and developmental competence of abnormal human embryos

Our initial observations of the applied effectiveness of μS-VTF on oocytes, zygotes and embryos (Table 1), revealed a high level of survival (95–100%) independent of the developmental stage

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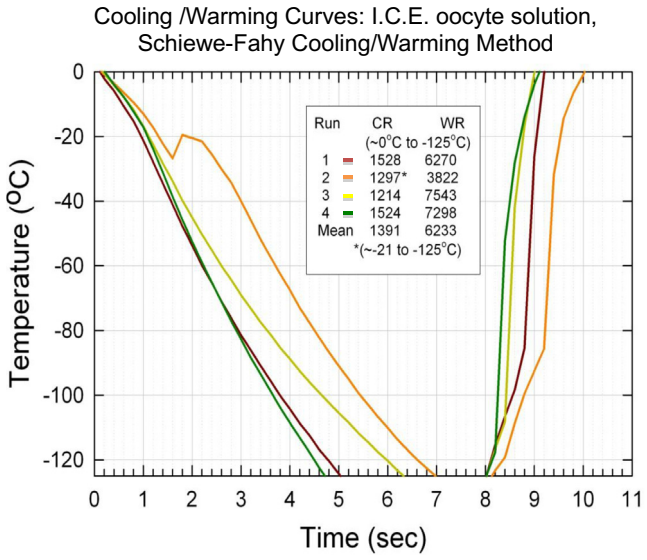

Fig. 3. Final data logger results of μS-VTF straws and flexipettes combined for cooling rate assessments in LN_2 and the warming characteristics of the extracted flexipettes plunged directly into a warm (37 °C) bath (Fig. 2C). The rapid cooling rate remained consistent at 1214 °C to 1528 °C/min, whereas the direct warming of the vitrified flexipette in a 37 °C medium bath achieved a mean rate of 6233 °C/min. The 4-fold increase in the rate of warming over cooling alleviated the prior concerns of recrystallization events occurring upon devitrification.

Table 1
Preliminary evaluation of the cellular survival of microSecure vitrified human embryos derived from immature eggs and abnormal zygotes.

Developmental stage	No. vitrified	No. recovered	No. survived (%) **partial: ⩾50%/75%	No. complete survival (%)
IVM MI/MII Oocytes	21	21	20 (95%)	20 (95%)
Pronuclear*	43	43	42 (98%)	42 (98%)
Cleavage* (6c–8c)	28	28	28/23 (100%/82%)	19 (68%)
Blastocyst*	39	39	39/39 (100%/100%)	37 (95%)

* All tested research embryos were deemed abnormal based on fertilization assessments.

** Partial survival was based on the presence of pyknotic/degenerate cells, which was less than 50% or 25% of the cellular mass.

(Fig. 4). Furthermore, the μS-VTF device proved to be highly reliable at routinely recovering its vitrified contents, with all 131 oocytes and embryos successfully recovered (100%). Only the day 3 cleavage-stage embryos were susceptible to partial damage of some blastomeres (Fig. 4D), with 68% and 82% maintaining complete or ⩾75% cellular integrity, respectively. The developmental potential of these abnormal fertilized embryos to grow to the blastocyst stage was assessed following vitrification. 8% of the zygotes and 10% of the cleavage stage embryos developed to the blastocyst stage, which was similar to the 12% blastulation rate observed in a fresh control group of abnormal zygotes ($n = 66$ embryos).

Phase 3: retrospective parallel clinical testing of cryopreserved human blastocysts

The encouraging findings of Phase 2 allowed us to move forward with the incorporation of vitrification into our routine blastocyst cryopreservation program. The Phase 3 validation findings comparing pregnancy outcomes of good prognosis patients (⩽34 years old) having fresh embryo transfers ($n = 283$) to the cryopreserved embryo transfers ($n = 136$) over a 2 year period (2008–2009), revealed a consistent trend of improvement in cryopreservation outcomes. Although no differences were observed in total survival rates between treatments (89–92%; Table 2), as defined as a transferable embryo (>50% intact cellular mass), it is clearly evident by pregnancy outcomes that survival differences did exist. Finally, μS-VTF was validated to be the most effective procedure at preserving the overall viability of blastocysts, with no differences exhibited in live birth rates or the implantation potential of the vitrified embryos compared to fresh control blastocysts.

Phase 4: verification of microSecure's clinical effectiveness

The efficacy and importance of μS-VTF in our clinical practice has become increasingly obvious (Table 3). All aspects of pregnancy outcome assessment in women <38 years old ($n = 209$) were comparable to expectations for fresh embryo transfer results (Table 4). Surprisingly, vitrified pregnancy outcomes were exceptionally high in the older age women (38–43 years old; $n = 54$), with a mean live birth rate of 53.7%. In either case, the effectiveness of μS-VTF was clearly proven. After modifying the warming procedure, there was a general improvement in both survival and pregnancy outcomes after 2009 (Table 3). Survival improved ($p < 0.05$) from 86.4% to over 94%, with the exception of a small group of older age women ($n = 20$; 88.5%) in 2010, which averaged out after 2011 when survival in that group was 98.3%. Not unexpectedly, more spontaneous miscarriages were observed in the older-aged patients (13%) than women 37 years old or younger (4.7%).

Conclusion

Our study has verified the clinical efficacy of a non-commercial, aseptic closed vitrification system called microSecure for human embryos. This device offers an unparalleled combination of safety, security, simplicity, reliability and repeatability between technicians unavailable in commercial vitrification devices [33]. Only the High Security Vitrification (HSV) straw (CryoBioSystems, France) and VitriSafe device (IVF Distribution GmbH, Bregenz, Austria) offer similar quality control advantages, but they do not have internal labeling and require the additional pipetting of embryos and oocytes onto a carrier surface which introduces technical variation. Of critical importance, this study has validated the cryophysical properties of Me_2SO-free metastable vitrification solutions in the μS-VTF device during cooling and warming. The cooling rate of our closed straw system (1214–1528 °C/min) is consistent with other reported closed straw devices [36,39] being rapidly plunged directly into LN_2. Knowing that post-vitrification survival is dependent on warming rates relative to cooling rates [29], it was critical for us to develop a warming strategy which optimized the warming rate. This was achieved by removal of the embryo/oocyte-containing vitrified flexipette from the external straw just prior to rewarming and direct immersion of the flexipette into a warm sucrose solution using a cut-tilt-tap technique [32], in contrast to ambient air exposure. In this way, we achieved a >6000 °C/min warming rate which was >4 times greater than the cooling rate, thus greatly reducing concerns about devitrification mediated recrystallization [10,40]. Seki and Mazur [36] demonstrated the importance of warming rates over cooling rate in a closed straw experimental model with mouse oocyte survival being maximized with a warming rate of 2950 °C/min when cooling proceeded at 522 °C/min or 1827 °C/min. The use of a 0.5 M sucrose solution for warming was a quality control measure, to protect against the possible inadvertent expulsion of an embryo

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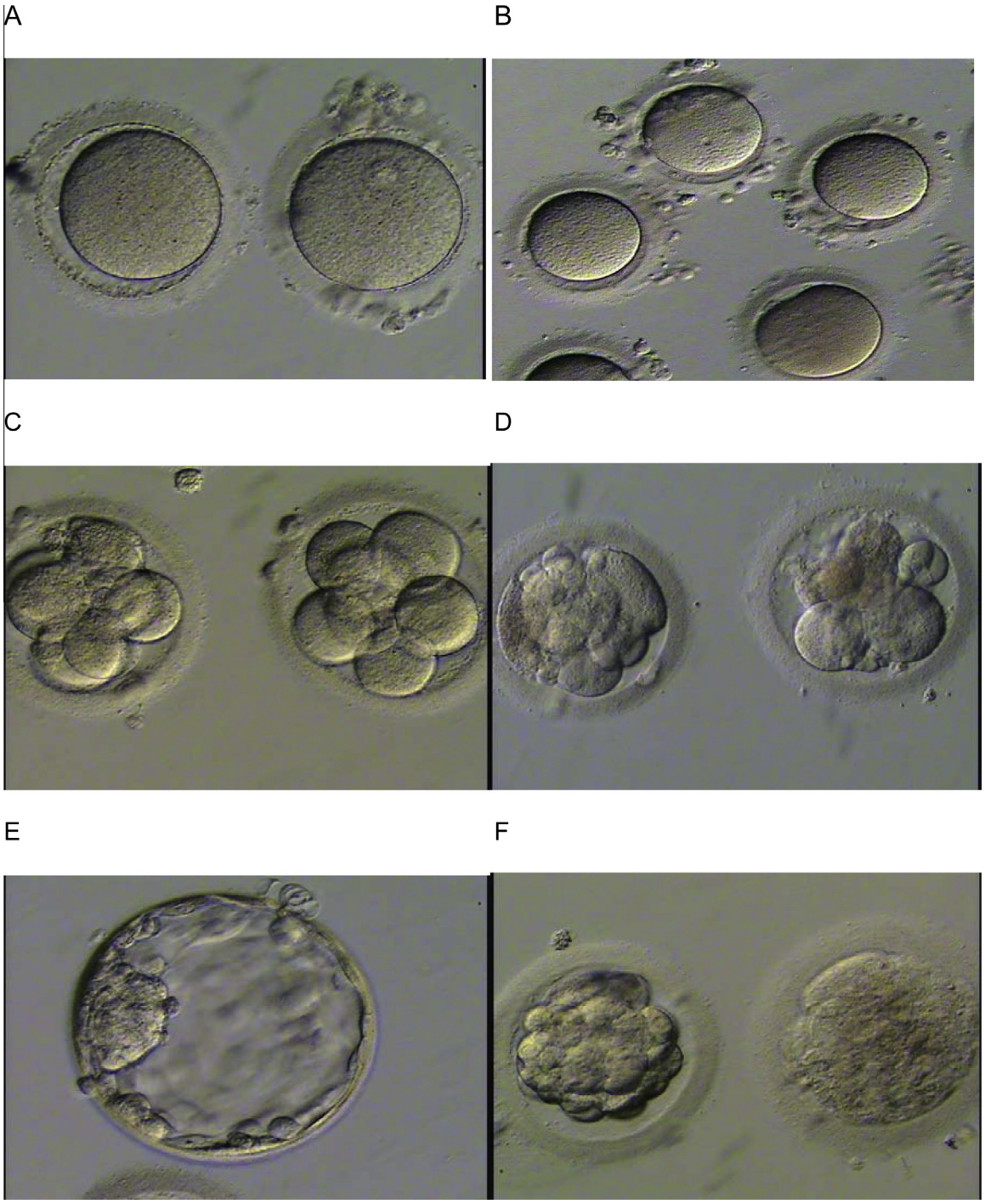

Fig. 4. The post-vitrification survival of different developmental stages is depict as complete for human oocytes [A], 8-cell embryos [C] and a blastocyst [E], being indistinguishable from fresh oocytes and embryos. The partial survival of cleavage-stage embryos [D], being >75%, is clearly shown by the darken presence of pyknotic blastomeres. A few pyknotic blastomeres are also seen in a blastocyst [F, left side] on its upper pole, in contrast to the completely lysed blastocyst to its right. Furthermore, a small percentage of oocytes do not survive warming and elution steps [B], as evidenced by the lack of cellular responsiveness to osmotic changes and/or the eventual darkening/degeneration before or after isotonic equilibration.

or oocytes during the initial warming step, since the flexipette remains open-ended during vitrification. In the hundreds of warming procedures performed in this validation study, the latter incident was not observed. However, clinical application to-date has on rare occasions discovered an apparent missing blastocyst safely eluted in the sucrose bath, reminding us to adhere to standard protocol to not load embryos or oocytes too close to the tip.

High levels of complete oocyte and embryo survival were attained in all of our Phase 2 developmental treatments, being consistent with summarized results reported by Edgar and Gook [9]. Although we did not fertilize oocytes in this experimental phase, we subsequently confirmed the clinical efficacy of μS-VTF in a randomized controlled trial ($n = 13$ donor egg recipients), using a non-Me_2SO, glycol based solution. Post-warming, all 122 mature oocytes were recovered, 106 survived (87%; Fig. 4A and B), 87 two pronucleate zygotes were produced (81% normal fertilization). Upon transfer of Day 3 or Day 5 embryos (2.3 mean embryos/transfer; total: $n = 30$), 7 clinical pregnancies

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Table 2
Optimizing blastocyst (BL) cryopreservation with microSecure vitrification (μS-VTF) versus conventional slow freezing without (BL/SF) or with pre-freeze blastocoele collapse (CBL/SF) for cryopreserved embryo transfer cycles performed in 2008 and 2009.

Patient Grp/treatment	BL Thawed	BL-ET (%survived)	No. of ET (μBL/pt.)	Pregnancy outcomes			
				+β-hCG (%)	Clin. preg (%)	Live birth (%)	Impl. rate (%)
⩽34 year old							
Control	0	605	283 (2.1)	215 (76%)[a]	198 (70%)[a]	181 (64%)[a]	274 (45%)[a]
BL/SF	124	114 (90%)	47 (2.4)	23 (49%)[b]	14 (30%)[c]	11 (23%)[c]	12 (11%)[c]
CBL/SF	148	136 (92%)	52 (2.4)	36 (70%)[a]	26 (56%)[b]	21 (40%)[b]	29 (21%)[b]
μS-VTF	98	87 (89%)	37 (2.4)	27 (73%)[a]	24 (65%)[a]	22 (59%)[a]	34 (39%)[a]

[a,b,c] Different superscripts within columns reveal differences ($p < 0.05$).
Abbreviations: BL-ET. – No. of blastocysts transferred; μBL/pt. – mean No. of BL transferred per patient; +β-hCG – No. positive pregnancy test; Clin. Preg (CP) – No. of clinical pregnancies; Impl. Rate – No. blastocysts resulting in a fetal sac with a heart beat.

Table 3
Clinical outcome data of vitrified ET cycles by age.

Age group	Year	No. thawed	No. survived (%)	No. of BL	No. of Impl.	No. of transfers (μBL/pt)	No. of +β-hCG	No. of CP (%)	No. of LB (%)
<38	2009	140	121 (86.4)	119	38 (31.9)	50 (2.4)	34 (68.0)	27 (54.0)	25 (50.0)
Years	2010	169	159 (94.1)	159	68 (43.0)	80 (2.0)	59 (73.8)	50 (62.5)	46 (57.5)
Old	2011	140	135 (96.4)	132	68 (51.5)	79 (1.7)	59 (74.7)	51 (64.6)	47 (59.5)
	Sub-total	449	415 (92.4)	410	174 (42.4)	209 (2.0)	152 (72.7)	128 (61.2)	118 (59.5)
38–43	2009	23	20 (87.0)	18	2 (11.1)	7 (2.6)	5 (71.4)	3 (42.9)	2 (28.6)
Years	2010	52	46 (88.5)	46	12 (26.0)	20 (2.3)	15 (75.0)	12 (60.0)	9 (45.0)
Old	2011	59	58 (98.3)	50	30 (60.0)	27 (1.9)	23 (85.2)	21 (77.8)	18 (66.7)
	Sub-total	134	124 (92.5)	114	44 (38.6)	54 (2.1)	43 (79.6)	36 (66.7)	29 (53.7)
Overall	Total	583	539 (92.4)	524	218 (42.0)	263 (2.0)	195 (74.0)	164 (62.0)	147 (56.0)

∗2009 data was influenced by the original ambient air thawing protocol, changed to direct-rapid thaw in a warm Sucrose bath by early 2010.
∗∗100% recovery rates were attained.
∗∗∗Survival was defined as ⩾95% intact cells and an embryo displaying overt osmotic changes and re-equilibration.
Abbreviations: No. of Impl. – No. of BL implanting; μBL/pt – mean No. of BL transferred per patient; No. of +β-hCG – No. of positive pregnancy tests; No. of CP – No. of clinical pregnancies; No. of LB – No. of live births.

Table 4
Clinical outcome SART Data* of Fresh ET cycles by age.

	2009	2010	2011	Total
<38 years old				
No. of ET cycles	144	154	152	450
Live birth rate	48.4%	53.9%	50.0%	50.8%
Implantation rate	32.8%	38.7%	39.2%	37.0%
38–42 years old				
No. of ET cycles	107	133	130	370
Live birth rate	21.1%	31.1%	29.4%	27.6%
Implantation rate	12.0%	14.7%	19.3%	15.5%

* Weighted values represent combined age grouping acquired from SART statistics for the Southern California Center for Reproductive Medicine, Newport Beach, CA.

and 6 live births (54%/43%, respectively) produced 10 healthy babies [33]. The latter oocyte vitrification outcomes are comparable to those reported with open device-EG/Me_2SO systems [5,6,25] and a closed system [25]. In Phase 2, the viability of vitrified embryos was partially confirmed. Abnormal zygotes and cleaved embryos following vitrification developed to the blastocyst stage at a comparable rate to non-vitrified (fresh) abnormal zygotes. Although the day 3 cleavage-stage embryos only experienced 68% completely intact cell survival, this finding was consistent with the 74% occurrence reported by Balaban et al. [2] for vitrified day 3 embryos. In both cases, the overall survival of 95–100% survival achieved was a significant improvement over typical, historic slow freezing success.

The retrospective analysis of our blastocyst freezing success in Phase 3 revealed that our adoption of technical advances (i.e., pre-freeze blastocoele collapsing and then vitrification) significantly improved pregnancy outcomes. Although blastocysts appeared to survive conventional slow freezing fairly well, being completely intact 60–70% of the time, it is apparent that significant harm to the trophectoderm layer seemingly impaired both implantation and overall pregnancy success. In contrast, slow frozen collapsed blastocysts (CBL/SF) effectively preserved the trophectoderm, with +β-hCG outcomes being similar to μS-VTF and the fresh control groups. However, the fewer clinical pregnancies in CBL/SF treated embryos compared to μS-VTF suggests that there may have been an impact on inner cell mass viability following slow freezing. By incorporating pre-freeze blastocoele collapse we apparently better sustained the functionality of the trophectoderm layer post-thaw, which led to greater implantation potential. The positive effect of blastocoele collapse on survival and implantation was consistent with other reports [14,39]. However, even though a healthier trophectoderm led to greater implantation success, the maintenance of clinical pregnancies was still reduced in the CBL/SF treatment group, due to probable unnoticed cryoinjury of the inner cell mass of these intact blastocysts. As blastocyst vitrification evolved, blastocoele collapse was found to be advantageous by investigators using EG/Me_2SO vitrification solutions with open [23] and closed systems [20,39]. Yet, Stachecki and coworkers [38] found their non-Me_2SO, glycerol based VTF solution did not require physical collapse of the blastocoele cavity to yield high survival rates and pregnancy outcomes. In fact, our experience with μS-VTF using I.C.E. blastocyst vitrification solutions yielded complete survival of the trophectodermal layer over 92% of the time, with the occasional appearance of non-viable, pyknotic nuclei as described by Ebner et al. [8].

The increased intracellular concentration of cryoprotective agents associated with vitrification appears to have improved cryoprotection of the inner cell mass of blastocysts, producing cryopreserved embryo transfer pregnancy rates approaching those of good prognosis (⩽34 y.o.) fresh blastocyst embryo transfers. It is

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interesting to note that the level of pregnancy success we were able to achieve in an aseptic, closed vitrification system using a glycerol-based, non-Me_2SO solution between 2009 and 2011 ($n = 263$ vitrified embryo transfer cycles) was comparable to another closed carrier system [20], only when they artificially collapsed their blastocysts prior to vitrification in EG/Me_2SO solutions (2010–2012; $n = 276$ vitrified embryo transfer cases). In fact, when they did not collapse the blastocoele cavity they observed similar overt survival of blastocysts (98.6–98.9%), but significantly reduced +β-hCG outcomes (50.7% vs. 75%), clinical pregnancies (43.2% vs. 63.4%), live birth rates (35% vs. 58.7%) and implantation rates (32% vs. 46.7%). Similar pregnancy outcomes and good survival rates (82–84%) with non-collapsed blastocysts were recently exhibited in a prospective randomized study showing no difference between an open or closed VitriSafe (VitriMed, Austria) device [24]. However, several studies do indicate that blastocoele collapse is useful to optimize a EG/Me_2SO vitrification protocol [14,20,23]. Our μS-VTF/I.C.E. system optimized +β-hCG outcomes (74%), clinical pregnancies (62%), live birth rates (56%) and implantation rates (42%) without the need for pre-vitrification collapsing of the blastocoele cavity. This phenomenon is likely attributed to the lower permeation rate of glycerol having a sustained effect on water efflux at the time of rapid cooling. The benefit of the μS-VTF/I.C.E. blastocyst vitrification system was particularly apparent in women $\geqslant$38 years old who seem to benefit from the use of their embryo(s) in a non-hormonally stimulated CET cycle. Shapiro and coworkers [37] showed that CET cycles provide optimum uterine receptivity conditions to maximize implantation and pregnancy outcomes. The impressive rise in live births we observed in 2011 (Table 3) is partially attributed to our initial efforts to biopsy blastocysts and subsequently cryo-store only euploid embryos. In either case, CET pregnancy rates were appreciably higher than similar fresh embryo transfer cycles.

Over a 4 year period, we successfully completed a validation–verification study that extensively documented the cryobiology of a novel aseptic closed vitrification system for human oocytes and embryos. μS-VTF has proven to be a clinically competent procedure worthy of worldwide ART industry acceptance. As a non-commercialized, FDA compliant approach, μS-VTF offers a significant cost-savings over common marketed devices for oocyte and embryo storage, and is a technically simple approach optimizing quality control considerations of labeling, weld sealing, aseptic storage and user repeatability.

Conflict of interest

M.C.S., S.Z., R.E.A. and G.M.F. have no commercial conflict of interest to disclose in the application of this novel procedure.

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