Large-volume vitrification of human biopsied and non-biopsied blastocysts: a simple, robust technique for cryopreservation

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Abstract

Purpose To evaluate the transition from a proven slow-cooling cryopreservation method to a commercial large-volume vitrification system for human blastocysts.

Methods Retrospective analysis of de-identified laboratory and clinical data from January 2012 to present date for all frozen embryo replacement (FET) cycles was undertaken. Cryopreservation of trophectoderm-biopsied or non-biopsied blastocysts utilized during this time period was logged as either slow-cooling, small-volume vitrification, or large-volume vitrification. Blastocyst survival post-warm or post-thaw, clinical pregnancy following FET, and implantation rates were identified for each respective cryopreservation method.

Results Embryo survival was highest for large-volume vitrification compared to micro-volume vitrification and slow-cooling; 187/193 (96.9 %), 27/32 (84.4 %), and 244/272 (89.7 %), respectively. Survival of biopsied and non-biopsied blastocysts vitrified using the large-volume system was 105/109 (96.3 %) and 82/84 (97.6 %), respectively. Survival for micro-volume biopsied and non-biopsied blastocysts was 16/30 (83.3 %) and 2/2 (100.0 %) respectively. Slow-cooling post-thaw embryo survival was 272/244 (89.7 %). Clinical pregnancy and implantation rates outcomes for non-biopsied embryos were similar between large-volume

and slow-cooling cryopreservation methods, 18/39 (46.2 %) clinical pregnancy and 24/82 (29.3 %) implantation/embryo, and 52/116 (44.8 %) clinical pregnancy and 67/244 (27.5 %) implantation/embryo, respectively. Comparing outcomes for biopsied embryos, clinical pregnancy and implantation rates were 39/67 (58.2 %) clinical pregnancy and 50/105 (47.6 %) implantation/embryo and 4/16 (25 %) clinical pregnancy and 6/25 (24.0 %) implantation/embryo, respectively.

Conclusions The LifeGlobal large-volume vitrification system proved to be very reliable, simple to learn and implement in the laboratory. Clinically large-volume vitrification was as, or more effective compared to slow-cooling cryopreservation in terms of recovery of viable embryos in this laboratory.

Keywords Vitrification · Rapid-cooling · Large volume · Trophectoderm biopsy · Cryopreservation

Introduction

Vitrification techniques for mammalian oocytes and embryos have evolved to use of very small volumes, sub- and microliter volumes to be able to achieve rapid and ultra-rapid cooling rates. However, simple and robust large-volume techniques for the vitrification of embryos; up to 250 μ l, [1], and sperm; up to 0.5 ml [2, 3], have emerged as successful alternatives to the technique-dependent small-volume protocols. For simplicity, the term vitrification will be used in this discussion to include all rapid-cooling and vitrification methods; representations regarding vitrification are made by some commercial manufacturers, and included within the name of said products, declaring them as ‘vitrification’ solutions, but depending on how these solutions are used in the laboratory, the technique may not achieve true vitrification [4]. The methods and fundamental principles of cryopreservation have been discussed at length elsewhere [5–8].

Capsule A commercial large-volume vitrification system was evaluated for biopsied and non-biopsied human blastocysts in this laboratory. Embryo survival was very high: over 95 % of all embryos survived warming and were available for transfer, compared to 84 % and 90 % survival for micro-volume vitrification and slow-cooling techniques, respectively. The LifeGlobal large-volume vitrification system has proven to be very reliable, simple to learn and implement into routine use, and was demonstrated to be as effective or better in terms of recovery of viable embryos, under the conditions of this laboratory.

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Kits for small-volume vitrification and subsequent warming of oocytes and embryos are available from manufacturers who provide detailed instructions, media, and in some cases platforms (platform will be used in this context to include straws, cryotips, cryotops, and other devices or containers). At present there are only two commercial large-volume blastocyst vitrification kits available; the Global® Blastocyst Fast Freeze kit (LifeGlobal Group LLC, Guilford, CT, USA), a methodology modeled after the S³ system developed by Stachecki et al., [1, 9], and the I.C.E. Vitrification System for blastocyst-stage embryos (Innovative Cryo Enterprises, L.L.C., Linden, NJ), which is the updated and rebranded S³ system [1, 9].

With increased application of genetic analysis of embryos in some laboratories, e.g. trophectoderm biopsy with subsequent cryopreservation of all biopsied embryos, vitrification of blastocysts immediately after biopsy has become more commonplace. Likewise, in this laboratory, there has been a steady increase in the number of IVF patients desiring genetic analysis of blastocysts, and as such, there was a need to alter the cryopreservation approach to accommodate this clinical paradigm shift. Vitrification had already been in use, but primarily for oocyte banking cycles.

Large-volume vitrification was an attractive alternative to sub- and micro-volume vitrification methods, in that 1) the solutions did not include dimethyl sulfoxide (DMSO), 2) the technique utilized straws already in place with slow-cooling cryopreservation, and as such the loading and handling of the straws was already familiar to the embryologists, and 3) the slower cooling rates and larger volumes of medium used by this approach make this technique less technician-dependent, potentially resulting in a rapid learning curve and more consistent post-warming outcomes.

The primary focus of this report was to provide embryological data, e.g., survival and implantation rates, and secondarily, the comparative clinical outcomes with regard to the transition to the LifeGlobal Blastocyst Fast Freeze system as a replacement for the controlled-rate slow-cooling method [10] that had been in use in this laboratory as the day-to-day cryopreservation system. The authors also note that at the time of this writing, there had been no peer-reviewed published clinical outcome data for either of the two commercial large-volume blastocyst vitrification kits.

Materials and methods

Study data were retrieved by retrospective data mining using a de-identified database. The manuscript does not contain clinical studies or details that might disclose the identity of the patients; therefore patient consent and Institutional Review Board approval was not solicited.

The procedures used for embryo culture have been described in detail elsewhere [11, 12]. Briefly, fertilized ova on

day 1 (day 0=day of oocyte retrieval) were placed into continuous microdrop culture (pre-equilibrated 50 µl of Global medium supplemented with 10 % v/v Life Global Protein Supplement, or pre-equilibrated 50 µl of Global Total medium, LifeGlobal Group LLC, Guilford, CT, USA) under an oil overlay (LiteOil; LifeGlobal Group LLC, Guilford, CT, USA).

Trophectoderm biopsy

All cleavage-stage embryos underwent laser-assisted hatching on day three (day 0=day of oocyte retrieval), using several 670 µs duration pulses (Saturn Active Laser, Research Instruments, U.K.). Blastocysts were biopsied on day five and or day six. Several trophectoderm cells were removed by applying the laser energy across trophectoderm cells held away from the embryo by suction, using several 984 µs pulses. Immediately after biopsy, the trophectoderm cells were pipetted into labeled PCR tubes containing 2 µl of lysis buffer and then frozen in a conventional −32 °C freezer. Biopsied blastocysts were vitrified within 10 to 15 min after the biopsy. Biopsied trophectoderm cells were transported frozen for the determination of chromosomal normality by comparative genomic hybridization (CGH, Genesis-24, Genesis Genetics, Plymouth, MI).

Large-volume vitrification and warming (LVV)

Day five and six blastocysts were vitrified and warmed according to the Blastocyst Fast Freeze and Blastocyst Fast Freeze Thaw kit protocols (LifeGlobal Group LLC, Guilford, CT, USA). Briefly, blastocysts were exposed to Fast Freeze (FF) solution 1 for 5 min, FF solution 2 for 5 min, then FF solution 3 for up to 1 min including the time it took to rinse, load the embryo into the straw, seal the straw, and plunge into a liquid nitrogen bath. Exposure of embryos to the FF solutions occurred at room temperature. CBS High Security 0.3 ml embryo straws (Irvine Scientific, Irvine, CA) labeled with patient identifiers were prepared by loading three 50 µl columns of FF solution 3, separated by 50 µl columns of air. A single blastocyst was cryopreserved per straw. To load the straw the blastocyst was pipetted, using a dissection microscope, from FF solution 3 into the central column of FF solution 3 already in place in the straw; this method of loading allowed visual confirmation of placement prior to sealing and plunging into a horizontal bath of liquid nitrogen. Straws were then quickly removed from the bath and placed in static liquid nitrogen Dewars for storage.

Warming of biopsied blastocysts was accomplished by removal of identified straws from liquid nitrogen and exposing the straw to room air (ambient temperature approximately 27 °C) for 10 s, and then plunging the straw into a horizontal 30 °C water bath for 10 s. Expelled embryos were rinsed through the five Fast Freeze Thaw (FFT) rehydration solutions

per the manufacturer recommendations; FFT 1 for 3 min at room temperature, FFT 2 through FFT 4 for 5 min each at room temperature, and FFT 5 for 5 min, moving the dish from room temperature to a 37 °C surface for gradual warming of the embryo. Rehydrated embryo(s) were rinsed into microdrop culture (pre-equilibrated 50 µl of Global medium supplemented with 10 % v/v LifeGlobal Protein Supplement, or pre-equilibrated 50 µl of Global Total medium, LifeGlobal Group LLC, Guilford, CT, USA) under an oil overlay (LiteOil; LifeGlobal Group LLC, Guilford, CT, USA) and incubated until transfer, approximately 2 to 6 h after thawing. Assisted hatching was performed on all warmed embryos unless the zona had already been breached, e.g., by the embryo itself, or as would have been done to facilitate trophectoderm biopsy.

Micro-volume vitrification and warming (MVV)

Two devices were used for vitrification; HSV High Security Vitrification Straws ($n=8$ cases; Irvine Scientific, Irvine, CA) and the McGill Cryoleaf ($n=9$ cases; Origio, Denmark). The protocol and media used for vitrification and warming were the same for both devices; Vitrification Freeze Solutions for Embryo and Vitrification Thaw Solutions for Embryo (Irvine Scientific, Irvine, CA). Briefly, day five and six embryos were held in the first equilibration medium for 10 min at room temperature, moved to the vitrification medium for 30 s, then placed onto the vitrification device platform in approximately <1 µl medium using a positive displacement pipette. For the HSV straw, the platform was immediately placed inside the protective outer sheath, sealed, then plunged into liquid nitrogen. For the Cryoleaf, the device was plunged into liquid nitrogen, and then keeping the device under liquid nitrogen, the protective outer cover was moved down over the flat blade and locked into position.

Warming embryos vitrified using the HSV device required cutting through the outer sleeve, removing the inner platform, and plunging the tip into the first of four solutions, moving the device back and forth to dislodge the embryo. The embryo was held in the first solution for 1 min at 37 °C, and then moved through the remaining three solutions at room temperature, 4 min per step. Warming embryos vitrified using the Cryoleaf required manipulation of the outer protective sheath of the device under liquid nitrogen to expose the flat blade of the device, then the tip of the blade was plunged into the first of four solutions, moving the device back and forth to dislodge the embryo. The embryo was held in the first solution for 1 min at 37 °C, and then moved through the remaining three solutions at room temperature, 4 min per step. Rehydrated embryo(s) were rinsed into microdrop culture (pre-equilibrated 50 µl of Global medium supplemented with 10 % v/v LifeGlobal Protein Supplement, or pre-equilibrated 50 µl of Global Total medium, LifeGlobal Group LLC, Guilford, CT, USA) under an oil overlay (LiteOil; LifeGlobal Group LLC, Guilford, CT, USA) and incubated

until transfer, approximately 2 to 6 h after thawing. Assisted hatching was performed on all warmed embryos unless the zona had already been breached, e.g., by the embryo itself, or as would have been done to facilitate trophectoderm biopsy.

Slow-cooling cryopreservation and thawing (SC)

Day five and or day six blastocysts (not biopsied) were cryopreserved with a computer-controlled unit according to the protocol of Gardner et al., 2003 [10], using G-Freezekit Blast and G-Thawkit Blast media (Vitrolife, Englewood, CO) supplemented with 20 % v/v Synthetic Serum Substitute (Irvine Scientific, Irvine California) and CBS High Security embryo straws (Irvine Scientific, Irvine, CA). Briefly, embryos were rinsed in the first medium with no cryoprotectants, then moved through two dehydration solutions for 10 and 7 min respectively, after which embryos were pipetted into straws, sealed and cooled according to the protocol. For thawing, embryos were held in air for 10 s, followed by immersion in 30 °C water for 30 s. Embryos were recovered from the straws and rehydrated through a series of four media, 5 min per step. Rehydrated embryo(s) were rinsed into microdrop culture (pre-equilibrated 50 µl of Global medium supplemented with 10 % v/v LifeGlobal Protein Supplement, or pre-equilibrated 50 µl of Global Total medium, LifeGlobal Group LLC, Guilford, CT, USA) under an oil overlay (LiteOil; LifeGlobal Group LLC, Guilford, CT, USA) and incubated until transfer, approximately 2 to 6 h after thawing. Assisted hatching was performed on all warmed embryos unless the zona had already been breached.

Embryo transfer

Immediately before transfer, embryos were moved from culture to approximately 3 mL 36 °C Quinn's Sperm Washing Medium (Quinn's Sperm Washing Medium; Sage In-Vitro Fertilization, Trumbull, CT). The catheter (Wallace Sure-Pro, Smiths Medical, Ashford Kent, UK, or Cook Soft-Pass, Cook Medical, Bloomington, IN) was rinsed with a small volume of medium, and the embryos were aspirated into the catheter tip in approximately 15–20 µl volume, located between two small air bubbles. The embryo transfer was assisted using ultrasound guidance.

Uterine preparation prior transfer was accomplished by application of 0.1 mg Vivelle patches (Novartis Pharmaceuticals Corporation, Hanover, NJ, USA), increasing from 1 to 4 QOD by patch day 11, supplemented as needed with 0.1 mg oral estrace to achieve peak serum estradiol targeted to 250 to 300 mIU/ml. On patch day 12, daily injections of progesterone in oil (50 mg/ml) were initiated for 2 days, after which injections increased to 100 mg/ml, with embryo transfer occurring on the sixth day of progesterone administration. Estrogen and progesterone application as described continued until after confirmation of a positive

serum pregnancy test 9 and 11 days after transfer and in the event that a pregnancy was established, continued until serum estradiol and progesterone concentrations indicated independent placental sufficiency.

Statistical analysis

Statistical analysis was performed using Fisher's Exact Test (OpenStat, 2008) with $P < 0.05$ significance.

Results

Data were filtered to allow identification of frozen embryo replacement cycles spanning January 2012 to date, as this time-frame overlaps introduction of the large volume vitrification system into routine use. During this time frame, a total of 1056 blastocysts from 235 patient cycles have been cryopreserved using this method; 589 blastocysts were cryopreserved after trophectoderm biopsy, and 467 blastocysts were cryopreserved without having been biopsied.

The data were first divided into three groups according to the method of cryopreservation; large-volume vitrification (LVV), micro-volume vitrification (MVV), and slow-cooling (SC) (Table 1). The vitrification data were further subdivided between blastocysts that were biopsied or not before cryopreservation (Table 2). Note: MVV was utilized at the initiation of the trophectoderm biopsy program, and then discontinued with introduction of LVV; comparisons and reliance on calculated probabilities involving MVV should be considered with caution in this context. Also, due to lower numbers of data points for some analyses, comparisons and reliance on calculated probabilities should be carefully considered.

Embryo survival after warming and thawing according to cryopreservation technique (not separated for biopsied and non-biopsied embryos, Table 1) was significantly higher for LVV compared to MVV, 187/193 (96.9 %) and 27/32 (84.4 %; $p = 0.0106$), respectively, and between LVV and SC, 187/193 (96.9 %) and 244/272 (89.7 %; $p = 0.0034$), respectively. Embryo survival rates between MVV and SC were not significantly different, 27/32 (84.4 %) and 244/272 (89.7 %; $p = 0.3664$), respectively. Comparing implantation rates between cryopreservation techniques, LVV cycles yielded significantly higher ($p = 0.0095$) implantation rates/embryo compared to SC, 47/187 (39.6 %) and 67/244 (27.5 %).

After subdividing the data between biopsied and non-biopsied embryos within vitrification methods (Table 2), it was found that survival of biopsied and non-biopsied blastocysts cryopreserved using LVV were not significantly different ($p = 0.6986$), 105/109 (96.3 %) and 82/84 (97.6 %), respectively. Survival for MVV biopsied and non-biopsied blastocysts was 16/30 (83.3 %) and 2/2 (100.0 %) respectively.

Table 1 Summary of laboratory and clinical outcomes for all frozen embryo replacement cycles beginning 2012 to date, using three different approaches to embryo cryopreservation

Freezing System	Mean age (s.d)	# thaw cycles	# embryos thawed	# surviving (%) for transfer	# embryos replaced	Positive serum test (%)	Clinical pregnancy per FET (%)	Ongoing pregnancy per FET (%)	Implantation rate/embryo replaced (%)
Large-volume ¹	33.8 (5.1)	106	193	187/193 (96.9) ^a	187 (1.8)	70/106 (66.0) ^a	57/106 (53.8) ^a	48/106 (45.3) ^a	74/187 (39.6) ^a
Micro-volume ¹	31.6 (5.3)	17	32	27/32 (84.3) ^b	27 (1.6)	8/17 (47.1) ^a	4/17 (23.5) ^{b,c}	2/17 (11.8) ^b	6/27 (22.2) ^{a,c}
Slow-cooling	32.0 (5.5)	117	272	244 (89.7) ^b	244 (2.1)	67/116 (57.8) ^a	52/116 (44.8) ^{a,c}	48/116 (41.4) ^a	67/244 (27.5) ^{b,c}

¹ Represents vitrification (rapid-cooling) by two different approaches, according to final volume of vitrificant
Different superscripts within columns are significantly different at $p < 0.05$

Table 2 Summary of laboratory and clinical outcomes for biopsied and non-biopsied embryo frozen embryo replacement cycles beginning 2012 to date, for the two vitrification methods employed during this time period

Freezing System	Mean age (s.d)	# thaw cycles	# embryos thawed	# surviving (%) for transfer	# embryos (mean) replaced	Positive serum test (%)	Clinical pregnancy per FET (%)	Ongoing pregnancy per FET (%)	Implantation rate/embryo replaced (%)
Large-volume¹									
Biopsied	34.1 (4.8)	67	109	105 (96.3) ^a	105 (1.6)	47/67 (70.1) ^a	39/67 (58.2) ^a	34/67 (50.7) ^a	50/105 (47.6) ^a
Non-biopsied	33.2 (5.7)	39	84	82 (97.6) ^a	82 (2.1)	23/39 (59.0) ^a	18/39 (46.2) ^a	14/39 (35.9) ^{a,c}	24/82 (29.3) ^b
Micro-volume¹									
Biopsied	32.3 (4.8)	16	30	25 (83.3) ^b	25 (1.6)	8/16 (50.0) ^a	4/16 (25.0) ^b	2/16 (12.5) ^{b,c}	6/25 (24.0) ^b
Non-biopsied	22	1	2	2 (100.0)	2 (2.0)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/2 (0.0)

¹ Represents vitrification (rapid-cooling) by two different approaches, according to final volume of vitrificant
Different superscripts within columns are significantly different at $p < 0.05$

There was only one instance where embryo transfer could not be performed due to non-survival of a single embryo in the SC group.

Clinical and ongoing pregnancy rates for biopsied vs. non-biopsied embryos after LVV were similar; clinical pregnancy rates of 39/67 (58.2 %) and vs 18/39 (46.2 %, $p=0.3126$), respectively, and ongoing pregnancy rates of 34/67 (50.4 %) and 14/39 (35.9 %, $p=0.1603$), respectively. Implantation rates/embryo for biopsied embryos were significantly higher ($p=0.0156$), 50/105 (47.6 %) vs. non-biopsied embryos, 24/82 (29.3 %), within the LVV category.

Discussion

As with many commercial culture media products, the exact components and concentrations of the components that make up the LifeGlobal Blastocyst Fast Freeze kit and the I.C.E. Vitrification solution for blastocyst-stage embryos were not made public; however what is common to both of these commercial kits is that 1) DMSO is not used, and 2) both list ethylene glycol, glycerol, and sucrose as cooling and warming solution components.

Despite the long-standing use of DMSO as a single, or cryoprotectant, there have been concerns regarding its potential for toxicity and intra-cellular perturbations in a variety of cell types [13]. For example, both DMSO and ethylene glycol were found to induce a transient increase in intra-cellular calcium in mouse oocytes [14] and further, DMSO was responsible for release of calcium from intracellular stores, as opposed to the influx of calcium from extracellular culture media generated by ethylene glycol exposure. For mouse oocyte cryopreservation, the potential for cryoprotectant toxicity was reduced by eliminating or combining cryoprotectants, and exposing oocytes to these agents at room temperature rather than 37 °C [15]. In regard to post-fertilization embryonic cells, recent reports demonstrate cellular/genetic perturbations following DMSO exposure for mouse blastocysts [16], mouse embryoid bodies [17], and human induced pluripotent stem cells [18]. It is prudent, in the context of this discussion and its relevance to LVV, to note that unlike many MVV techniques, the commercial LVV kits do not use DMSO and require by protocol, that the blastocysts be exposed to the cryoprotectant solutions at room temperature.

Cooling rates are multifactorial [19–21], and are influenced by vitrificant solution composition, platform style, e.g. open or closed, physical characteristics, e.g. surface area and shape (cylinder, drop, film, etc.), and the temperature of the refrigerant that the platform is exposed to, e.g. liquid nitrogen (−196.0 °C) vs. slush nitrogen (−205 to −210.0 °C). The S³ studies investigated several medium volumes from 10 to 250 µl [9, 22]; a 0.25 ml straw platform and the maximum medium volume a cooling rate of <100 °C/min was cited.

Vanderzwalmen et al., 2002 [23], demonstrated a cooling rate of near 2000 °C/min for a 0.25 ml straw with a central column of vitrification solution approximately 15 mm in length (approximately 40 µl), and Kuwayama et al., 2005 [24], reported a cooling rate of 4460 °C/min using a 0.25 ml straw with 25 µl vitrification medium. Sansenena et al. 2012 [20], using heat transfer modeling found that filled 0.25 ml straws were estimated to have cooling rates of 936 °C/min and 1418 °C/min using liquid nitrogen and slush nitrogen, respectively.

There have been several publications supporting the concept that very rapid warming rates can be used to achieve high oocyte and embryo survival rates, in spite of the type of platform used and cooling rate achieved [25–28]. Seki and Mazur, 2009 [25], using 0.25 ml straws with 50 mm columns of medium (approximately 100–120 µl), with no additional covering over the straw, placing the straw on a Styrofoam raft floating on liquid nitrogen for 6 min before plunging, achieved cooling rates of 1827 ± 214 °C/min. Warming rates of as high as 2950 ± 119 °C/min were achieved by holding the straw in air for 10 s, then placement into a 25 °C water bath.

The platform chosen for use with human blastocysts in this laboratory was the CBS High Security 0.3 ml embryo straw with three 50 µl columns of the final LVV solution separated by 50 µl of air. Though the manufacturer (LifeGlobal Group LLC, Guilford, CT, USA) did not make representations for, or disclose cooling and warming rates, based on data described in other studies it was assumed then that the platform and technique utilized in this laboratory would likely have achieved cooling rates near 2000 °C/min, and warming rates greater than 2900 °C/min. Consistent glass formation, whether true vitrification or rapid freezing, was evident by visual confirmation of clear, non-opaque columns of medium in the straw after plunging into a horizontal bath of liquid nitrogen.

The embryological and clinical outcomes experienced by this laboratory with LVV are similar to limited data as presented in other publications where the S³ vitrification technique was used [1, 9, 22]. Only one study [29] presented in vitro data (no clinical outcome data) comparing the Global® Blastocyst Fast Freeze system to an alternate vitrification system; post-warming survival, re-expansion, and numbers of cells in human blastocysts were found not to be significantly different between the two vitrification systems, and importantly, cell numbers were not significantly different between the two systems and compared to non-cryopreserved controls.

Comparing cryopreservation methods for blastocyst-stage embryos that were not biopsied there was no difference in implantation rate/embryo ($p=0.7768$), or clinical and ongoing pregnancy rates/cycle between vitrification and slow-cooling ($p=0.3479$ and $p=0.5766$, respectively) indicating that for routine cryopreservation, LVV was just as effective as the SC technique.

Comparisons between MVV and LVV outcomes for these data were not conclusive, though there were apparent differences in embryo survival and clinical outcomes; the inherently more difficult technical requirements, and the smaller number of cycles for the MVV cycles should be taken into consideration, noting also that the MVV approach was discontinued in favor of the more simple LVV method.

In regard to the outcome data for biopsied embryos, and despite the additional manipulation and loss of cells required for genetic testing, the transfer of euploid embryos combined with use of the LVV system demonstrated overall statistically improved implantation rates compared to non-biopsied embryos, maintaining similar clinical pregnancy rates while reducing the number of embryos transferred per cycle. The use of genetic analysis of blastocysts by CGH in this center to date, has demonstrated an overall euploid frequency of 48.2 % (371/770). By day of biopsy, the frequency of euploid blastocysts was 54.7 % (196/358) on day five, and 42.5 % (175/412) on day six. If these figures are representative of all cryopreserved, non-biopsied embryos, over half of these embryos would be expected to be chromosomally aneuploid.

Implantation rates for biopsied embryos (vitrified by either LVV or MVV methods) did not meet expectations, as compared to data published by other centers, for example 65.1 % implantation per embryo [30] and 60.0 % implantation per embryo [31]. Trophectoderm biopsy was introduced at the end of 2011, and by year, to date, the percent of retrieval cycles utilizing trophectoderm biopsy were: 2011, 5/143 (3.5 %); 2012, $n=36/173$ (20.8 %); 2013, 69/160 (43.1 %); and 2014, 71/96 (74.0 %). Clinical pregnancy and implantation rate outcomes will be re-evaluated as the numbers of cycles, and experience increase.

The decision to adopt the Global® Blastocyst Fast Freeze system was based on multiple factors: 1) having a proven, closed platform already in use in this laboratory since 2003, where the CBS High Security straw composition and sealing method rendered the platform highly resistant to leakage [32–36], 2) the larger volume of medium (50 µl) for vitrification and a simple protocol for cryoprotectant addition and dilution allowed for a consistent technical transition, 3) the use of straws allowed for no changes in storage efficiency (seven straws per 13 mm goblet) or ease of handling, and 4) potentially improved safety for vitrified embryos during storage, shipping in nitrogen vapor and the concomitant multiple temperature transitions that occur during shipping and receiving. This latter aspect was important, as concerns have been raised over the possibility of inadvertent devitrification during handling, and long- or short-term storage in liquid nitrogen vapor. Micro-volume vitrification platforms might be more susceptible to temperature excursions [37, 38]. A recent report by Sansenena et al., 2014 [39], demonstrated substantial temperature excursions within the head space of the liquid nitrogen storage Dewar, where temperatures changed widely

during canister removal and replacement. Embryos could be exposed to temperatures that would exceed glass transition temperature where the viscosity state could move from glass to rubber, hence increasing the risk of devitrification. External heat transfer coefficients for four micro- to very small-volume vitrification devices were modeled based on the physical device specification, e.g. a film vs. a long thin column using the same vitrification solution [20]; a film had the fastest modeled heat transfer coefficient compared to a small thin column device, indicating that the physical structure of a film favored the fastest cooling rate. But importantly, it is conceivable that this same heat transfer coefficient would work against the device, where devitrification would be a greater risk for micro- and very small volume vitrified samples.

The heat transfer modeled data are not yet available for the current system, however this laboratory is pursuing such data with the facility studying glass transition and devitrification as described by Sansinena et al., 2014 [39], where the data will be used to better evaluate handling, storage and shipping protocols. There are data for temperature transitions for semen straws and vials, where 0.5 ml semen in 1.0 ml cryovials took approximately 80 s to reach -80°C from -196°C in room air, compared to approximately 40 s and 15 s for 0.5 ml and 0.25 ml straws, respectively [40], effectively demonstrating the value of larger sample volumes on reducing the warming rate. A similar protection against devitrification might be observed, comparing a 50 μl column of medium to micro-volume vitrification platforms.

In conclusion, the data demonstrated that the transition to LVV as described above, despite the limitation of smaller numbers of cycles to date, was successful from a clinical standpoint, e.g., LVV yielded similar or better recovery of viable embryos and similar clinical outcomes compared to the slow-cooling system that had been in use for day-to-day cryopreservation of blastocysts. Further, the transition within the laboratory was a success as the technique for LVV was simple, and less technically demanding compared to the earliest MVV methods used [41] and recently investigated in this laboratory.

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