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Research Article

Liquid Nitrogen Vapor Sealing of Straw Containers can be Unsafe and Detrimental to Embryo Survival

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Abstract

Background: Aseptic closed vitrification (VTF) systems have been proving their clinical effectiveness in recent years. Although the risk of pathogenic contamination between samples in liquid nitrogen storage has been a debatable issue among open VTF systems users, there is growing interest to hybridize systems. In short, some open system users aim to achieve the ultra-rapid cooling rates of direct LN_2 exposure and then seal the device into a plastic straw container. Specialized commercial LN_2 baths have been developed to assist in these hybrid-device systems. We strived to 1) determine whether LN_2 vapor sealing of straws presents safety and reliability concerns that create potentially harmful laboratory practices; and 2) reveal a validation method which verifies the competency of seals as a quality control practice.

Materials and Methods: Using a repeated VTF (rVTF) model on research consented, discard embryos, human blastocysts were randomly assigned to either Control (n=19) or ultra-rapid cooling treatment (UR-TRT; n=22). Standard micro Secure-VTF (µS-VTF) warming of flexipettes was first performed without extraction/elution, then dried with sterile gauze. Re-VTF was performed at 1 min post-warming, by either: 1) Control µS-VTF; or 2) UR-TRT where flexipettes were dipped into LN_2 (5 sec), inserted in straws held in LN_2 and sealed closed for storage. Subsequently, all straws were warmed using standard µS-VTF procedures and elution in sucrose solutions. Following isotonic equilibration and 24h *in vitro* culture, blastocyst survival and development, respectively, was assessed.

Results: Following the fatal rupturing of the first 3 UR-TRT straws, warming procedures had to be modified for possible LN_2 accumulation inside straws due to incomplete seals. By allowing for 15 sec of N_2 out gassing, the remaining 19 straws warmed without incident, yet 6 did reveal evidence of LN_2 seepage (41% incomplete seals). No difference in blastocyst survival at 0 h was evident between treatments; however development was reduced in the UR-TRT group at 24 h (86.7% vs. 62.5%).

Conclusion: The hybridization of a UR-closed VTF system has proven to be a potentially unreliable, unsafe and less effective procedure in our rVTF model system. The inability to guarantee complete seals of super-cooled straws or the possible entry of N_2 vapors inside a straw upon sealing creates significant risks which are unnecessary quality control variables absent in standard aseptic, closed VTF methods.

Keywords: Vitrification; Straw sealing; Embryos; Liquid nitrogen vapor; Quality control

Abbreviations

BL: Blastocyst; CBS: Cryo Bio Systems; CSS: Cut Standard Straw; DMSO: Dimethyl Sulfoxide; HSV: High Security Vitrification; LN_2: Liquid Nitrogen; µS-VTF: Micro Secure Vitrification; N_2: Nitrogen; Non-DMSO; BL-VTF: Blastocyst Vitrification with a solution not containing DMSO; rVTF: re-Vitrification; UR-TRT: Ultra Rapid Cooling Treatment; VTF: Vitrification.

Introduction

Following his pioneering embryo vitrification efforts in 1985, Dr. Rall effectively developed a more practical vitrification method for mammalian embryos using a closed straw system containing a less toxic 6.5M glycerol-6% (w/v) bovine serum albumin based solution [1,2]. Over 15 years later, its clinical application with lower molarity, mixed cryoprotective agent solutions began being widely promoted in combination with micro-volume, open device systems [3-5]. A variety of vitrification devices were ultimately introduced into the IVF industry, promoting the concept that ultra-rapid cooling rates in excess of 10,000°C/min were a necessity for vitrification to achieve high survival rates. Ultimately the dogma surrounding the relative importance of cooling rate was put into perceptive by a series of warming rate studies by Seki and Mazur [6-8] clearly exhibiting warming rate as the primary factor influencing vitrification success. Independent of the cooling rate, post-warming survival of embryos

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can only be optimized when the warming rate exceeds the cooling rate.

Among the various devices developed for clinical vitrification, several systems are closed devices aseptically retained within a securely sealed straw at room temperature, including the cut standard straw (CSS) [9], high security vitrification (HSV) [10], Vitrisafe [11,12], and micro Secure vitrification (µS-VTF) [13]. The primary advantage of the latter closed vitirification systems being the safe and secure storage of human gametes and embryos, eliminating possible risks associated with the transmission of pathogens in LN_2, which has been a debated issue [14]. As aseptically closed vitirification systems have now proven to be effective for oocytes and blastocysts [10-13,15], it would seem that any risk of disease transmissions between open sample specimens should be deemed unacceptable by regulatory agencies and professional societies. Although it is possible to cryostore human bio-products in sterilized LN_2 [16], aseptic cryostorage is then dependent on LN_2 vapor storage tanks. Unfortunately, LN_2 vapor storage is not a widely accepted alternative for most IVF laboratories applying vitrification, due to the temperature sensitive nature of vitrified products.

Based on the growing high-level of success and undeniable security advantages of some aseptic closed systems [10-13], there has been another interesting but potentially disconcerting trend occurring. Some commercial companies, as well as innovative Embryologists, are hybridizing vitirification systems (e.g., Cryotop, CSS, respectively) by attempting to seal a LN_2 exposed open devices into plastic straws. Unlike the safety and security of weld-sealing an ionomeric plastic straw under ambient (20-22°C) conditions, the compliance of super-cooled straws to effective heat sealing may be compromised leading to sub-optimal, unsecure closure. Because embryos do survive vitrification with high efficacy, being relatively unchanged post-warming; it is possible to re-vitrify them with similar efficiency. In turn, we are able to utilize patient consented, vitrified, non-viable aneuploidy embryos as an experimental model to study vitrifcation practices. The objectives of our study were to: (1) assess whether LN_2 vapor sealing of straws is a safe, secure and reliable practice; (2) determine if ambient loading & sealing of flexipettes (µS-VTF; Control) for VTF is equally effective to ultra-rapid cooling and the LN_2 vapor loading/sealing (UR-TRT) of a hybrid device system; and (3) to reveal a validation-verification quality control practices which effectively tests the competency of straw seals.

Materials and Methods

Experimental design

Using a rVTF model on embryos consented by patients for discard research, 41 blastocysts were randomly assigned to either Control (n=19) or UR-TRT (n=22) following standard µS-VTF warming [13]. Randomly applying an apriori arrangement of rVTF treatments, 1 min post-warming each blastocyst-flexipette underwent rVTF by either: 1) Control µS-VTF; or 2) UR-TRT. Survival was assessed at 0 hr and 24 hr post-secondary warming, standard sucrose elution, isotonic equilibration and in vitro culture. Differences in blastocyst survival and formation ($blastocyst_{0hr}$ survival ÷ $blastocyst_{24hr}$ reformation) were assessed by a χ^2 test ($^*p<0.05$).

Additionally, we performed a secondary sealing quality control test to validate and exam the potential inadequacy of heat sealing super cooled/LN_2 vapor exposed straws (n=20) using a water submersion procedure.

Embryo culture

Research embryos were derived from 2PN zygotes fertilized by intra cytoplasmic sperm injection (ICSI) and group cultured in 25uL droplets of Global™ medium (LG; Life Global, USA) supplemented with 7.5% synthetic protein supplement under Ovoil™ (Vitrolife, USA). Embryos were cultured in MCO-5M mini Sanyo/Panasonic tri-gas incubators (5% 0_2/5.3-6.0% CO_2) under humidified air at 37°C. Only good to excellent quality blastocysts (≥3BA/AB or AA) vitrified on either Day 5 or Day 6 was selected for research treatment. Following rVTF, LN_2 storage of at least 30 min and then warming/sucrose dilutions, all embryos were evaluated and returned to LG micro-droplet culture in fresh research dishes for 24 h before re-evaluation and discard.

Micro secure vitrification (µS-VTF) and warming

All blastocysts were vitrified in a hyaluronate-enriched, non-DMSO BL-VTF solution (Innovative Cryo Enterprises, USA). Aseptic µS-VTF was performed using: a 3-step dilution exposure to equilibrium, intermediate and final vitrification solutions for 5 min, 5 min and 1min, respectively. Individual blastocysts were loaded into shortened (i.e., 3 mm cut from the base end) 300 µm ID flexipettes (Cook Medical, USA; 3 µl volume); the flexipettes removed from the pipettor, dried repeatedly on sterile gauze and the flexipettes inserted tip first into internally prelabeled 0.3 ml CBS™ embryo straws [13]. Each straw was weld sealed at room temperature (Control treatment); and plunged directly into LN_2. The cooling rate was ≈1500°C/min, while rapid warming (≈6000°C/min) was achieved by direct placement of each vitrified flexipettes into a 37°C 0.5M sucrose bath [13]. Under standard elution treatment, within 10 sec each blastocyst was pipette directly from the flexipette into an open 200 µl droplet of 1.0 M sucrose solution and then transferred to a 100 µl droplet under oil for 3min. Embryos were then serially diluted in declining sucrose solutions (T2-T4, 3 min each), before isotonic equilibration in Hepes-LG medium. Warmed blastocysts were then cultured in LG medium + protein for 24 hr prior to final evaluation of continued blastocyst development and re-expansion typically characterized by hatching and hatched blastocysts.

For rVTF treatment, following the 10 sec rapid warming, the pipettes were dried and either randomly assigned to control or UR-TRT rVTF. The flexipettes containing Control blastocysts were directly reinserted into a new, treatment labeled CBS embryo straw and weld sealed using a Syms 1 automated sealer at room temperature (20-22°C). In the UR-TRT group, the flexipette was secured and placed directly into LN_2 (i.e., within a 0.5 L dewar flask), while the

Table 1: UR-TRT group compared to the control group.

Observations	µS-VTF Control	Ultra-Rapid TRT	P-value
# BL rVTF/warmed	19	19$^{\Psi}$	N.S.
# Survived (0hr)	15 (78.9%)	16 (84.2%)	N.S.
# Survived (+24hr)	13 (68.4%)	10 (52.6%)	p<0.10
BL Reformation (+24hr)	13/15 (86.7%)*	10/16 (62.5%)	p<0.05*

$^{\Psi}$ 3 UR-TRT straws exploded before warming procedure modifications (15 sec of N_2 out gassing).

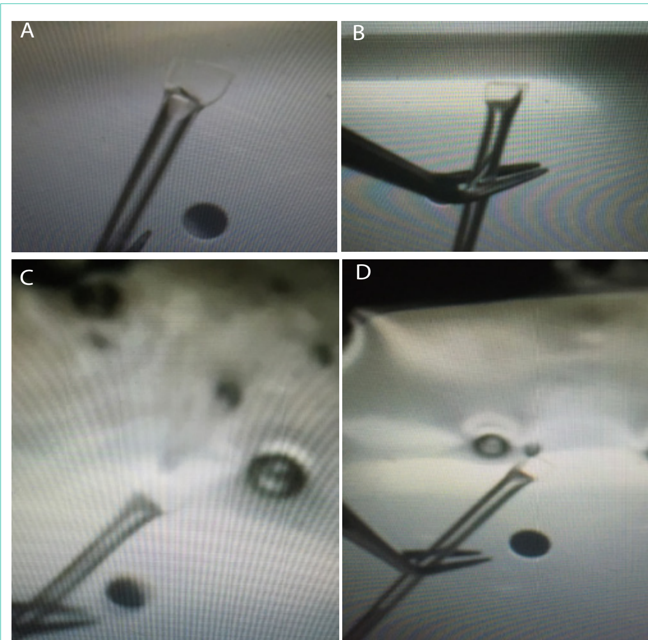

Figure 1: A post-study quality control validation test was performed on the patency of straw seals (n=20) created under the experimental LN_2 vapor conditions. The straws were not submerged in LN_2, but instead the straws were directly placed underwater to inspect the seals. Air in a straw with a complete seal (Figure 1A) can be seen as a straight backline, whereas the partial-complete seal (Figure 1B) reveals air seeping up the right-side edge of the seal. Incomplete seals are overtly apparent by the rapid bubbling of escaping air from the straw (Figure 1C), which tapered down to a slow release (Figure 1D). Note, no explosive events occurred because ambient air was allowed to freely escape, as opposed to LN_2 being converted to a rapidly expanding N_2 gas, resulting in over-expansion of the inner straw lumen.

new; labeled straw was supported in LN_2, with its open end in the vapor layer (approximately 3 cm above liquid). The super cooled flexipette was then carefully lifted into the LN_2 vapor phase and inserted into the lumen of the straw. Each UR-TRT straw was then lifted up (halfway submerged in LN_2; 5-6 cm) and sealed with a hand sealer at least 2-3 times (with 180° rotations) until an overt, adherent flattening was observed. To validate the competency of straw seals, it is an effective quality control practice to use a water submersion test. Therefore, using 20 empty “air-filled” straws exposed to LN_2 vapor sealing conditions, each straw with an apparent intact seal was placed underwater in a water bath. Each straw was then assessed as having either a complete (normal, 180° intact), nearly incomplete (sealed at upper edge but overt air present on one side) or incomplete seal (air bubbling detected).

Results

The first 3 UR-TRT straws exploded upon ambient air extraction and cutting, forcing us to modify our warming process to account for possible LN_2 accumulation inside straws due to incomplete seals. By allowing for 15 sec of N_2 out gassing, the remaining 19 straws warmed without incident, yet 6 did reveal evidence of LN_2 seepage (41% incomplete seals). No problems were experienced in the warming of control straws, and no difference in 0 h blastocyst survival being evident between treatments (Table 1). However, survival at 24 h tended to be lower and sustained blastocyst development was reduced in the UR-TRT group compared to the control group (Table 1).

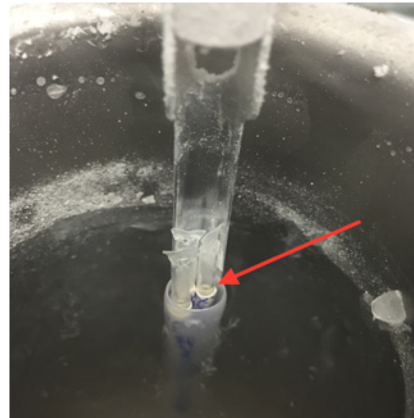

Figure 2: Upon inspecting a group of hybrid –vitrified straws transferred to our lab in the spring of 2016, we observed LN_2 present in the inner lumen of all seals (see arrow), which appeared to possess closed seals. The originating lab failed to provide accurate procedural paper work mentioning the latter potential flaw in their ‘cut-straw, rapid plunge, vapor sealed-closed straw system’, and how to safely warm incompletely sealed straws.

In a secondary “sealing” quality control examination, all the seals appeared visually complete. However only 70% actually were complete (Figure 1A), of which 30% of those were nearly incomplete (i.e., partial) exhibiting a distinct air bubble progressing up the sidewall of the seal (Figure 1B). The remaining 30% of the straws displayed incomplete seals, evidenced by overt air bubbling underwater (Figure 1C) which dissipated to a slow release of bubbles (Figure 1D).

Discussion

The application of standard µS-VTF warming practices to UR-TRT straws proved unreliable. The fatal rupturing of the first 3 UR-TRT straws was caused by the rapid vaporization of LN_2 (at 21°C) which had seeped into the straws due to incomplete seals. We performed a secondary sealing test to exam the inadequacy of heat sealing super cooled/LN_2 vapor exposed straws (Figure 1) using a water submersion test. Although all seals appeared normal, only 40% actually were complete, while another 30% were intact but nearly incomplete. Finally, a third of the straw seals were inadequate and at risk of an explosive outcome under standard rapid warming conditions. Fortunately, the explosive pressure build-up was avoided in our primary study, following the former initial incidents, by allowing the N_2 gas to escape the leaky seal by holding the straw gradually above the dewar flask vapor phase for 15 sec before cutting the straw. By allowing for N_2 out gassing before cutting the straw near the inner plug, and the flexipettes were safely released by tilting the straws downward over the warm sucrose bath. With a slight tap to the straw, all 38 flexipettes were released, rapidly warmed and all the embryos were recovered. The reliable recovery (100%) of vitrified embryos has been a superior characteristic of the µS-VTF system [13].

Once recovered, we did not observe any advantage to the ultra-rapid cooling of flexipettes, in contrast to the slower cooling achieved in the insulated environment of a CBS embryo straw. Some decline in embryo survival was observed in both groups (16-21%) following rVTF. The reduced development of the UR-TRT blastocysts at +24 h was likely an artifact of the rVTF model used. The slower cooling rate of an aseptic closed VTF device tolerates the slower warming

rates inherent to the system applying safe lab practices to effectively retrieve and warm the device. Based on recent clinical experience, a more optimal developmental outcome may have been achieved by cutting the straws in LN_2 and somehow retrieving the flexipette before ambient exposure and rapid warming in a warm bath. Hybrid-vitrified cut straw devices were recently transferred from another ART Lab in southern California to our laboratory. Upon inspection, ID confirmation and secured storage, it was noticed that LN_2 was bubbling inside each of 10+ straws from 3 separate shipments (Figure 2). Using the current warming practices of the commercial Cryotop-closed VTF hybrid system, we successfully warmed the former flawed straw devices by retrieving the device inside LN_2. Even then, this successful warming practice would be device dependent to a system capable of achieving ultra-rapid warming to prevent recrystallizing ice formation upon devitrification. As shown by Mazur and Seki [6-8], and discussed in a slow warming model by Wowk [17], the latter rate dependent event outcome is further dependent on the stability of the VTF solution used (i.e., concentration of the cryoprotective agents used). It has been proposed that commercial 30-32% (v/v; 4.8-5.2M) VTF solutions are relatively unstable in a slow warming model, which would put most hybrid-VTF systems at increased risk of user variation in warming practices having an adverse effect. In this study, our own metastable VTF solutions (>7.9M) displayed the risk of reduced blastocyst post-warming viability due to warming variation.

The handling of VTF devices and open straws in LN_2, and LN_2 vapor, is performed at increased risks to the safety of the Embryologist. Furthermore, the embryos themselves are susceptible to unexpected destruction if a hybridized VTF straw is warmed to quickly before extracting the device. This study has proven that UR-closed VTF may be a potentially unreliable, unsafe and less effective procedure. The inability to guarantee complete seals of super-cooled straws or the possible entry of N_2 vapors inside a straw upon sealing create significant risks which are unnecessary quality control variables absent in standard aseptic, closed VTF methods. As standard aseptic, closed VTF systems (e.g., HSV, µS-VTF, Vitrisafe) have proven to be highly effective for the cryopreservation of oocytes and embryos; it is time that the ART industry begins recognizing quality control variables and avoidable risks associated with flawed device concepts.

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