Accepted Manuscript

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PII: S0093-691X(14)00182-4

DOI: 10.1016/j.theriogenology.2014.04.003

Reference: THE 12768

To appear in: *Theriogenology*

Received Date: 12 November 2013

Revised Date: 7 April 2014

Accepted Date: 8 April 2014

Please cite this article as: Sansinena M, Santos MV, Taminelli G, Zaritky N, Implications of storage and handling conditions on glass transition and potential devitrification of oocytes and embryos, *Theriogenology* (2014), doi: 10.1016/j.theriogenology.2014.04.003.

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Implications of storage and handling conditions on glass transition and potential devitrification of oocytes and embryos

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ABSTRACT

Devitrification, the process of crystallization of a formerly crystal-free, amorphous glass state, can lead to damage during the warming of cells. The objective of this study was to determine the glass transition temperature of a cryopreservation solution typically used in the vitrification, storage and warming of mammalian oocytes and embryos using Differential Scanning Calorimetry. A numerical model of the heat transfer process to analyze warming and devitrification thresholds for a common vitrification carrier (open-pulled straw, OPS) was conducted. The implications on specimen handling and storage inside the dewar in contact with nitrogen vapor phase at different temperatures were determined. The time required for initiation of devitrification of a vitrified sample was determined by mathematical modeling and compared with measured temperatures in the vapor phase of liquid nitrogen cryogenic dewars. Results indicated that the glass transition ranged from -126 to -121ºC and devitrification was initiated at -109ºC. Interestingly, samples entered rubbery state at -121ºC and therefore could potentially initiate devitrification above this value, with the consequent damaging effects to cell survival. Devitrification times were calculated considering an initial temperature of material immersed in liquid nitrogen (-196ºC) and two temperatures of liquid nitrogen vapors within the dewar (-50 and -70ºC) to which the sample could be exposed for a period of time, either during storage or upon its removal. The mathematical model indicated samples could reach glass transition temperatures and undergo devitrification in 30 seconds. Results of the present study indicate storage of vitrified oocytes and embryos in the liquid nitrogen vapor phase (as opposed to completely immersed in liquid nitrogen) poses the potential risk of devitrification. Due to the reduced time-handling period before samples reach critical rubbery and devitrification values, caution should be exercised when handling samples in vapor phase.

Keywords: Vitrification, embryo, glass transition, devitrification, liquid nitrogen

1. INTRODUCTION

Low temperature preservation of oocytes and embryos is a fundamental cornerstone of assisted reproductive technologies. Cryopreservation of reproductive cells has been traditionally achieved by slow cooling the samples at specific rates to allow cell dehydration [1,2]. However, the preservation outcome of oocytes and embryos by slow freezing equilibrium protocols is negatively affected by cryoinjury due to formation of intra and extracellular ice crystals, concentration of solutes during the freezing process and prolonged cell exposures to toxic cryoprotectant and chilling temperatures [2–4].

Storage of cryopreserved reproductive cells is conducted in specialized cryogenic, thermally insulated vacuum flask dewars that hold cryogenic fluids such as liquid nitrogen below their boiling point [5]. Smaller to medium-sized tanks (20 to 50 L) used by veterinary practitioners and in some laboratories are routinely filled with cryogenic fluid to maintain adequate chamber temperatures [6]. Even though the recommendation is to maintain the dewars full at all times [5, 7], manufacturers provide guideline static evaporation rates for individual models and suggest close monitoring of liquid nitrogen levels based on specific usage conditions [7].

Cells stored in these containers are kept either immersed in liquid nitrogen or in the immediate vapor phase [5] . Because the temperature of the vapor phase is not a constant (as opposed to liquid nitrogen, -196ºC) a lack of temperature homogeneity within the chamber is observed [8]. Noteworthy, storage recommendations for oocytes and embryos in vapor phase of liquid nitrogen dewars were originally formulated for cells that had been cryopreserved using equilibrium, slow freezing protocols [5, 6]. Cells

cryopreserved under those conditions have been reported to undergo sufficient dehydration and minimal cytoplasm supercooling and thus are less likely to be damaged during warming. However, these recommendations may not be applicable to vitrified material, which has a higher risk of devitrification and can suffer irreversible cryoinjury.

Vitrification, the process of solidification of a sample into an amorphous, glassy-state in absence of intracellular and extracellular ice crystals, requires high concentrations of cryoprotectants, extremely rapid cooling rates and reduced volume handling. In the last decade, vitrification has progressively become the method of choice for the cryopreservation of human oocytes and embryos [9-11] and this trend is now being followed by veterinary and animal science practitioners for domestic and exotic animal species [12, 13].

Devitrification is defined as the process of crystallization in a formerly crystal-free, amorphous glass solution [12, 14-16]. Early experiments to study the warming behavior of vitrified aqueous solutions were conducted by Luyet [12] and Luyet and Rasmusen [13,14] using differential thermal analyses to detect enthalpy changes associated with transition events [17,18]. Unlike melting point, devitrification phenomenon has been described not as an individual point but as a temperature range determined by the composition of solution, presence of nucleating particles, among other factors [14, 15, 18-21].

The devitrification of the intracellular solution and the surrounding extracellular medium can lead to significant damage during the warming of cells [20, 21]. Several authors have indicated that above the glass transition temperature of the cytoplasm (approximately -120 to -130℃), the vitrified

cytoplasm of oocytes and embryos could enter a liquid transition, promoting devitrification and subsequent ice nucleation and crystallization [22-24]. There are limited reports on glass transition temperatures of cryopreservation solutions [20–22] and those available are mostly water-sugar solutions and not the complex mixtures of balanced salt solutions, permeating and non-permeating cryoprotectants used in current oocyte and embryo vitrification protocols [25–27].

To date, there are no reports on glass transition temperatures of vitrification solutions used in the storage of oocytes and embryos. This information would be of value to calculate critical devitrification thresholds and update recommendations for the storage of vitrified oocytes and embryos. Therefore, the objective of this study was to determine the glass transition temperature of a cryopreservation solution typically used in the vitrification, storage and warming of mammalian oocytes and embryos. In order to analyze devitrification thresholds, a numerical modeling of heat transfer for a common vitrification carrier (open-pulled straw, OPS) was conducted. Finally, the implications of these results on specimen storage and handling conditions in nitrogen vapor phase were discussed.

2. MATERIALS AND METHODS

2.1 Measurement of the glass transition temperature (Tg) of the vitrification solution by differential scanning calorimetry (DSC).

Current vitrification protocols require that cells be successively moved through increasing cryoprotectant concentrations (permeable and non-permeable) prior to their vitrification by direct plunging into liquid nitrogen and

long-term storage. Therefore, the glass transition temperature (Tg) of the final vitrification solution routinely used in our laboratory was determined by differential scanning calorimetry (DSC).

The Tg of the vitrification solution consisting of 2.8 M Me_2SO (Sigma D2650) + 3.6 M EG (Sigma102466) and 0.65 M trehalose (Sigma T3663) in TCM199 (Invitrogen 12350-039) with 10% v/v Fetal bovine serum (Invitrogen 10100139, Australia) was measured using a differential scanning calorimeter (TA Instruments, New Castle, Delaware, USA) model Q100 controlled by a TA 5000 module with a quench cooling system under a nitrogen atmosphere. Samples of vitrification solution were enclosed in sealed aluminum pans and quench cooled up to -150ºC. An empty pan was used as a reference sample. Pans were heated at 2 ºC/min from -150 to 20 ºC, with isothermal periods at the initial and final temperatures. Distilled water was also scanned using the same program to verify equipment calibration. The step change visualized in the heat flow curve as a function of temperature corresponds to a second order transition (glass transition temperature, Tg). In the present work the midpoint temperature in the step curve of the thermogram was defined as Tg [28].

2.2 Mathematical modeling of devitrification thresholds

2.2.1 Numerical Modeling of the warming process of OPS.

The initiation of devitrification in vapor phase was analyzed conducting a mathematical modeling of devitrification thresholds for a commonly used vitrification support (open-pulled straw, OPS) loaded with vitrification solution. The OPS consists of a French polypropylene straw pulled under heat to

reduce its internal diameter, therefore minimizing the loading volume of solution. OPS as well as other devices such as cryotop, cryoloop, cryotip, etc., is a reduced volume since only the tip of the OPS is loaded with minimal volume of aproximately 1-3 microliter containing the oocytes/embryos by capillary action [12].

When the OPS is placed at a certain height over the liquid nitrogen it begins warming, as the height increases the temperature of the nitrogen vapor increases (higher values of T_v=vapor temperature). If the OPS system reaches the temperature of the glass transition (Tg), the vitreous biological solution has a greater risk of suffering damage since it enables the transition into a rubbery state which in turn allows the formation of ice crystals (devitrification).

In order to quantify this critical time the numerical modeling of the warming process was carried out using the finite element software COMSOL Inc. The partial differential equation that describes the heat conduction process of OPS when they are lifted from the liquid nitrogen and maintained at a certain height over liquid nitrogen under nitrogen vapor is given by Eq.1.

$$\rho(T)\, Cp(T)\, \partial T/\partial t = \nabla .\, (k\,(T)\, \nabla T) \quad (1)$$

The full description of the OPS system was described in detail in Sansinena et al., 2011 [29]. The initial condition of the OPS system for the warming process is T=-196℃ at t=0 for the straw an d the solution domain when it is immersed in the liquid nitrogen.

The convective boundary equation is - $k(\nabla T.n) = h (T-T_v)$ for t>0 at the surface of the straw that is exposed to the nitrogen vapor, k is the thermal conductivity of polypropylene, h is the surface heat transfer coefficient and T_v is the temperature of the nitrogen vapor over liquid nitrogen.

Two different T_v values (-70 and -50℃) were considered for the calculation of the critical time needed for the system to reach the following final temperatures -100, -120, -130℃, which are va lues close to the glass transition temperature of the biological fluid in the straw.

2.2.2 Heat transfer coefficient (h).

Depending on the temperature and nature of vitrified material, devitrification of specimens may occur when samples are exposed to liquid nitrogen vapors within the storage tanks. Because heat transfer coefficients for this system are not available, literature values for heat transfer coefficients (free convection) in air (78 % nitrogen) were used for the calculations (10 and 15 W/m^2K) as previously reported by Santos et al. [30].

2.3 Measurement of temperatures in nitrogen vapor phase of cryogenic dewars under various conditions

The temperature inside a typical cryogenic storage dewar was measured in triplicates. Temperatures with full and half-full liquid nitrogen loads were measured in triplicates for a 20-L dewar (MVE XC20, Millenium 2000, Chart Biomedical, GA, USA). Also, temperatures of nitrogen vapor phase immediately after raising and lowering canisters were obtained. Temperatures were recorded using a Testo 735-1 measuring instrument (Testo AG,

Lenzkirch, Germany), fitted with a type T copper-nickel immersion probe (-200 to + 40℃). The thermocouple was previously calibra ted using literature reference fixed-points.

3. RESULTS AND DISCUSSION

3.1 Measurement of Glass transition and devitrification temperatures of a vitrification solution by differential scanning calorimetry (DSC)

The glass transition temperature of a vitrification solution commonly used in which oocytes and embryos are later stored was determined by DSC. Since the majority of oocytes and embryos are vitrified and stored in vitrification supports individually (one oocyte/embryo is loaded, vitrified and stored per support device), the glass transition temperature of the system is dominated by the glass transition of the surrounding medium and the contribution of the cytoplasm assumed to be negligible. A schematic representation of heat flow process is presented in Figure 1.

Figure 1. Schematic representation of heat flow process described by DSC analysis.

Glass transition (Tg) and devitrification (Td) temperatures are shown in Tables 1 and 2, respectively. The Tg values of the solution containing oocytes and embryos ranged from -126 to -121ºC (Table 1). Devitrification process initiates at -109 and is completed at -97ºC. It should be noted (Table 2) that the samples enter a rubbery state after -121ºC and therefore could potentially initiate devitrification and crystallization of ice (freezing) with the consequent damaging effects of cell survival. The DSC peaks obtained for the vitrification solution analyzed are presented in Figure 2.

Table 1. Glass transition temperatures (Tg) obtained for the vitrification medium used for oocyte and embryo cryopreservation.

Vitrification/storage Glass transition		
Onset ℃	**Tg** ℃	**End** ℃
-126.81	-124.49	-121.06
-92.81	-91.02	-87.58
-67.73	-65.9	-63.79

Table 2. Devitrification, melting temperatures and exothermal heat of devitrification for the solution analyzed.

Vitrification/storage medium	**Onset**	**Peak**	**End**	**∆H**
	℃	**℃**	**℃**	**J/g**
Devitrification	-106.11	-102.63	-97.53	17.7
Melting	-46.77	-32.5	-27.37	30.4

Figure 2. Differential scanning calorimetry (DSC) heat flow process for oocyte and embryo vitrification/storage medium.

3.2 Mathematical modeling of devitrification thresholds

Devitrification times in seconds for an arbitrarily chosen, commonly described vitrification support (open-pulled straw, OPS) were calculated considering an initial temperature of material immersed in liquid nitrogen (-196ºC) and two possible temperatures of liquid nitrogen vapors (Tv) within the dewar (-50 and -70ºC) to which the sample could be exposed for a period of time, either during storage or upon its removal. Time in seconds needed for the OPS to reach -100, -120 and -130ºC (arbitrary temperatures close to the glass transition values measured by DSC for oocyte and embryo vitrification solution) are shown in Table 3. Results indicate that for the selected heat

transfer coefficients and external temperatures of nitrogen vapors over liquid nitrogen, samples could reach glass transition temperatures and undergo devitrification between 30 and 104 seconds.

Table 3. Time (in seconds) required for an OPS to go from an initial temperature (Ti) of -196ºC to several final temperatures (Tf) considering two external vapor temperatures (Tv) of -70 and -50ºC and two heat transfer coefficients (h).

Time (s)	Tv = -70℃			Tv =-50℃		
T final / h (W/m^2K)	*-100℃*	*-120℃*	*-130℃*	*-100℃*	*-120℃*	*-130℃*
10	104	67	54	79	54	44
15	70	46	37	53	36	30

3.3 Measurement of temperatures in nitrogen vapor phase of cryogenic dewars under various conditions

Temperatures of nitrogen vapor phase inside a cryogenic dewar under full, half-full and immediately after raising and lowering of canisters are presented in Table 4. The measured temperature gradients for a full and half-full dewar under normal operating conditions were similar. However, it is important to point out that after raising and lowering of the canisters the new N_2 vapor temperature distribution in the (vertical direction) axial direction exhibits higher temperatures compared with the N_2 temperature profile in a full container (Table 4). The N_2 vapor temperatures established after external disturbances is a critical variable which can increase the risk of damage to a vitrified sample.

A schematic representation of the nitrogen levels inside 20-L dewar is

presented in Figure 3. For a sample stored in vapor phase at 24 cm from the neck of the dewar, this transient temperature-mixing effect would result in nitrogen vapor temperature of -99ºC. This value is well above -121 ºC, in which a vitrified sample could enter rubbery state followed by devitrification followed by immediate ice crystallization. Temperature values in vapor phase of full and half-full cryogenic dewar in relation to critical rubbery and devitrification range are presented in Figure 4.

Table 4. Measurement of temperatures of vapor phase of cryogenic dewars under full, half-full conditions and immediately after raising and lowering storage canisters.

	Full dewar (LN_2 level 30 cm from top of neck)	**Half-full dewar (LN_2 level 44 cm from top of neck)**	**Half-full dewar immediately after raising and lowering canister**
Distance (cm) from top of dewar	*Mean*[a] ± *SD*	*Mean*[a] ± *SD*	*Mean*[a] ± *SD*
2	22.07 ± 0.06	21.62 ± 0.6	12.10 ± 0.89
4	17.80 ± 0.35	16.51 ± 0.59	9.05 ± 0.75
6	3.67 ± 0.25	3.63 ± 0.28	8.25 ± 0.96
8	-17.37 ± 0.45	-19.43 ± 0.92	3.59 ± 1.21
10	-41.77 ± 0.40	-44.00 ± 0.65	-1.84 ± 1.32
12	-69.50 ± 0.87	-66.07 ± 0.95	-8.56 ± 1.56
14	-97.87 ± 1.42	-95.63 ± 1.06	-13.89 ± 1.87
16	-129.03 ± 1.35	-125.06 ± 0.87	-29.08 ± 1.09
18	-165.03 ± 0.87	-160.10 ± 0.79	-45.03 ± 1.05
20	-187.07 ± 0.21	-181.86 ± 1.34	-67.67 ± 0.90
22	-191.47 ± 0.78	-189.56 ± 1.09	-82.52 ± 0.93
24	-194.83 ± 0.06	-192.10 ± 1.15	-99.22 ± 0.81
26	-196.13 ± 0.15	-195.46 ±1.07	-114.05 ± 0.56
28	-196.11 ± 0.09	-196.19 ± 0.95	-145.39 ± 0.71
30[b]	196.09 ± 0.10	-196.21 ± 0.76	-159.01 ± 0.65
32	-196.03 ± 0.17	-196.14 ± 1.10	-173.28 ± 0.39
34	-196.01 ± 0.08	-196.27 ± 0.86	-194.17 ± 0.51
36	-196.03 ± 0.13	-196.16 ± 0.94	-196.28 ± 0.39
38	-196.00 ± 0.07	-196.20 ± 0.80	-195.12 ± 0.22
40	-196.05 ± 0.12	-196.18 ± 0.89	-196.09 ± 0.40
42	-196.06 ± 0.09	-196.24 ± 0.39	-196.11 ± 0.46
44[c]	-196.04 ± 0.07	-196.27 ± 0.52	-196.20 ± 0.31

[a] Measured in triplicates
[b] Liquid nitrogen level measured from top of neck in full dewar (30 cm)
[c] Liquid nitrogen level measured from top of neck in half-full dewar (44 cm)

Figure 3. Schematic representation of nitrogen levels inside 20-L dewar, values expressed in mm. Letters indicate the depth at which sample would reach devitrification values in full and half-full dewar conditions (A) and after undergoing external disturbance (for example raising and lowering of a canister) (B).

Figure 4. Temperature values in vapor phase of full and half-full cryogenic dewar in relation to critical rubbery and devitrification range.

4. DISCUSSION AND CONCLUSION

Correct storage management of cryopreserved material is a fundamental aspect of cell survival and viability after thawing or warming. Rapid cooling, vitrification protocols have now mostly replaced slow-cooling equilibrium protocols in the cryopreservation of human oocytes and embryos due to improved viability and development after warming [31]. This improved cell survival determines that vitrification is also progressively becoming the method of choice for cryopreservation of domestic and exotic animal species. The shift in cryopreservation techniques determines animal practitioners are increasingly storing mixed populations of animal frozen and vitrified oocytes and embryos in their cryogenic dewars.

Traditional storage management recommendations for frozen cells indicate material should be maintained at or below the cytoplasmic glass transition temperature (-130ºC) and that storage at higher temperatures for prolonged periods of time (i.e., months) could result in reduction or loss of cell viability [2,4]. In field conditions, cryogenic dewars are typically filled with liquid nitrogen to full capacity. However, due to static evaporation loss, canisters may remain partially suspended in liquid nitrogen vapor. In addition, samples are exposed to higher temperatures at neck of containers at removal. Noteworthy, storage recommendations have originally been formulated for

331 cells cryopreserved under equilibrium freezing conditions and not in
332 consideration of the thermodynamics of vitrified materials, which exhibit the
333 risk of irreversible devitrification and cryodamage due to immediate freezing
334 and crystallization under subzero temperatures [5].

335 In our study, differential scanning calorimetry analysis of a commonly
336 used vitrification solution showed a glass transition temperature range of -126
337 to -121ºC, after which the solution enters a rubbery state until reaching a
338 devitrification onset, peak and end of -109, -102 and -97ºC, respectively.
339 Because the effects of storage of reproductive cells under rubbery conditions
340 has not yet been determined they should, as a precautionary measure, be
341 handled and stored at temperatures below the glass transition for the medium
342 in which they are cryopreserved. Results of this study indicate cells should be
343 stored at temperatures of -121ºC or lower, to avoid entering the rubbery state
344 followed by devitrification.

345 Measurement of temperatures in the vapor phase showed they can be
346 as high as -50ºC, with temperatures in the neck of storage dewars reaching
347 even higher values. Temperature in the vapor phase are highly susceptible to
348 variations due to atmospheric conditions, perturbation of the temperature
349 profiles by vapor mixing due to removal, raising or lowering of canisters within
350 the dewar and other factors [32]. The temperature distribution of the nitrogen
351 vapor inside the dewar is clearly non-uniform due to the natural convection
352 occurring when there is no external disturbance, therefore as expected lower
353 temperatures are measured at the bottom near the liquid N_2 level and there is
354 a temperature increase as we approach the neck of the dewar. However, it is
355 important to point out that after raising and lowering of the canisters the new

N_2 vapor temperature distribution in the axial direction exhibits higher temperatures compared with the N_2 temperature profile in a full container.

Results of the present study indicate storage of vitrified oocytes and embryos in the liquid nitrogen vapor phase (as opposed to completely immersed in liquid nitrogen) shows the potential risk of devitrification. Furthermore, results from the mathematical modeling of the devitrification risk indicate that, for two external temperatures (-50 and -70ºC) and two heat transfer coefficients for nitrogen vapor, a commonly used vitrification support such as the OPS could reach devitrification temperatures between 104 to 30 seconds. It must be pointed out that the indication of findings in terms of seconds is only done to emphasize that, for the modeled temperatures, rubbery state and devitrification could happen very quickly. They are not meant to be “time-based guidelines”; variables such as loading volume, media composition and others are likely to have an impact in the overall performance of the vitrification device.

Finally, this study has implications, not only in the storage conditions of the samples, but also in the management of the material upon warming. Due to the reduced time-handling period before samples reach critical rubbery and devitrification values, caution should be exercised when handling canisters and individual canes. Based on our results, we recommend vitrified samples should only be raised to the vapor phase and neck of dewar in one quick, fluid motion and only upon their immediate transfer to warming solutions.

ACKNOWLEDGEMENTS

Authors have no financial relationships to disclose. This research was funded by Facultad de Ciencias Agrarias, Universidad Católica Argentina, Ciudad Autónoma de Buenos Aires, Argentina y Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA-CONICET), Universidad Nacional de La Plata, Buenos Aires, Argentina.

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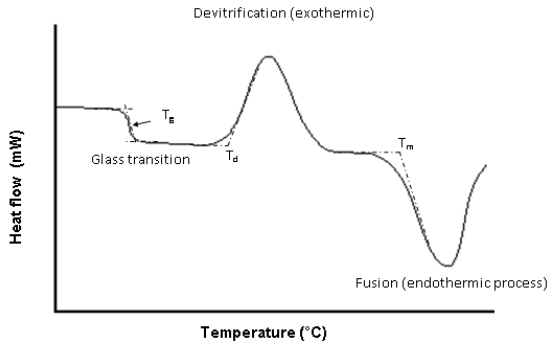

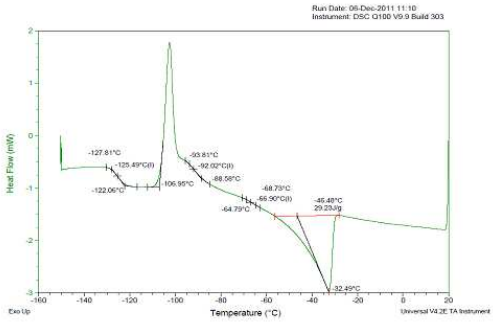

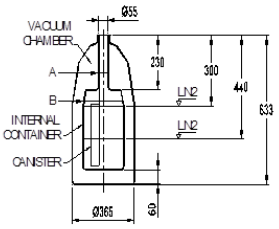

Temperature (°C) in vapor phase of full and half-full cryogenic dewar in relation to critical rubbery and devitrification range