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Mathematical prediction of freezing times of bovine semen in straws placed in static vapor over liquid nitrogen ☆

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A B S T R A C T

A widespread practice in cryopreservation is to freeze spermatozoa by suspending the straws in stagnant nitrogen vapor over liquid nitrogen (N_2V/LN_2) for variable periods of time before plunging into liquid nitrogen (−196 °C) for indefinite storage. A mathematical heat transfer model was developed to predict freezing times (phase change was considered) required for bull semen and extender packaged in 0.5 ml plastic straws and suspended in static liquid nitrogen vapor. Thermophysical properties (i.e. thermal conductivity, specific heat, density, initial freezing temperature) of bovine semen and extender as a function of temperature were determined considering the water change of phase. The non-stationary heat transfer partial differential equations with variable properties (nonlinear mathematical problem) were numerically solved considering in series thermal resistances (semen suspension–straw) and the temperature profiles were obtained for both semen suspension and plastic straw.

It was observed both the external heat transfer coefficient in stagnant nitrogen vapor and its temperature (controlled by the distance from the surface of liquid nitrogen to the straw) affected freezing times. The accuracy of the model to estimate freezing times of the straws was further confirmed by comparing with experimental literature data. Results of this study will be useful to select "safe" holding times of bull semen in plastic straws placed N_2V/LN_2 to ensure that complete freezing of the sample has occurred in the nitrogen vapor and avoid cryodamage when plunging in LN_2. Freezing times predicted by the numerical model can be applied to optimize freezing protocols of bull semen in straws.

Introduction

Successful cryopreservation of bovine spermatozoa is a fundamental step in the conservation of valuable genetics, the improvement of genetic progress and the application of assisted reproductive technologies [52,4]. Long-term storage of semen allows for transport and utilization over considerable distances, and also provides a window of opportunity for animal testing prior to gamete utilization. Semen cryopreservation is a standard practice that permits efficient utilization and propagation of animals and it ultimately leads to overall genetic progress [42].

In addition, genome cryobanking and germplasm repositories established with the objective of preserving agricultural biodiversity and indigenous species in the event of catastrophic loss have now become national and international initiatives [63]. Cryopreservation of reproductive cells is accomplished basically in two ways: using conventional, slow cooling methods or by applying ultra fast, high cooling rates (vitrification) in the presence of cryoprotectant concentrations that are compatible with reproductive cell survival. Although vitrification [12] is showing much promise in the preservation of oocytes and embryos, the cryopreservation of semen is still mostly done by slow cooling mainly because it allows to preserve the relatively large volumes of diluted ejaculate (from 0.25 to 0.5 ml) necessary for artificial insemination with acceptable quality of post-thaw survival parameters in domestic species [6,7,16,36,41,42]. Automated, programmable freezers are routinely used to accomplish controlled, slow cooling of bovine semen packed in polypropylene straws [84].

Freezing procedure during cryopreservation is a critical factor on sperm viability, because cooling rates that are too high or too low can be detrimental for the cells. Rapid cooling causes a shortage in the period of water efflux, resulting in excessive intracellular ice formation and consequent cell death [43,44]. On the contrary,

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slow cooling often injures the cells due to mechanical and/or osmotic effects of external medium [36,48]. To determine the optimum cooling rates, the method using the programmable freezer is the most precise, but due to the expense of these systems, a widespread practice is to freeze the sample by suspending the straws in nitrogen vapor (N_2V) over liquid nitrogen (LN_2) for variable periods of time before plunging into LN_2 (−196 °C) for indefinite storage [27]. Insulated, Styrofoam® boxes containing LN_2 have been used successfully for sperm cryopreservation [21]. When using a Styrofoam® box, the rack containing the samples is placed into the N_2V/LN_2 at a height of 3–4 cm above LN_2 for 7–8 min and the straws are then plunged in LN_2 [64]. Alternatively, Chemineaux et al. [18] suggested that 0.5 ml straws should be frozen 4 cm above LN_2 for 5 min. However, other freezing heights and times have been reported with acceptable results in terms of cell viability post-thaw [40].

The temperature gradient formed in the vapor phase of N_2V/LN_2 depends on several factors: distance to liquid nitrogen surface, amount of liquid nitrogen relative to air space, dimension of the container, etc. All of these factors can contribute to variability in the temperature range at which the putative freezing of the sample occurs and all should be taken into account when assuming complete freezing of the sample has occurred during holding time in the nitrogen vapor. It is noteworthy that Ritar et al. [68] studied freezing of caprine semen by holding straws in LN_2 (4 cm above liquid nitrogen) for a few seconds followed by plunging into LN_2. They reported that straws exposed to vapor for only 10 s did not cool sufficiently before plunging, and cell viability was seriously impaired.

In previous reports [73,74] Sansinena et al. performed a numerical simulation of cooling rates during vitrification (i.e. ice formation was avoided) by solving the non-stationary heat transfer partial differential equations for plastic (French) straws and "minimal volume" vitrification devices; a wide range of heat transfer coefficient values likely to represent experimental conditions were used in the simulations.

However, when freezing takes place thermophysical properties that are involved in the heat transfer partial differential equations undergo abrupt changes with temperature due to ice formation; this represents a highly non-linear mathematical problem that must be solved numerically. The main challenge when trying to numerically solve phase change problems is related to the lack of convergence due to the behavior of the thermophysical properties, especially when using the apparent specific heat where the sensible heat is merged with the latent heat to produce a specific heat curve with a large peak around the freezing point [61]. This function is considered a quasi delta-Dirac function with temperature depending on the amount of water in the sample. Several techniques were applied to deal with this problem, the most efficient and widely applied method is the implementation of the enthalpy variable, which can be obtained through the integration of the specific heat with temperature [22,23,51,61] and the Kirchhoff function, which is the integral of the thermal conductivity [75,77]. However the enthalpy and/or Kirchhoff formulation is unable to solve the heat transfer with phase change problem when two or more materials with different enthalpy values are in intimate contact. In this kind of problems the energy transfer through a series of thermal resistances, (i.e. semen suspension in series with the plastic wall of the straw) leads to an unsolved formulation because the mathematical boundary condition has different values of enthalpy and Kirchhoff function at the interphase of both materials [61]. In the present work an alternative formulation of the apparent specific heat is described in order to numerically solve a thermal resistance in series problem with change of phase in order to calculate freezing times of bovine semen contained in plastic straw when placed in nitrogen vapor over liquid nitrogen.

The objective of present study was: (a) to develop a mathematical model to predict actual freezing times required for bull spermatozoa/extender packaged in polypropylene straw (0.5 ml) suspended in static N_2V over LN_2 (N_2V/LN_2); (b) to determine the actual thermophysical properties (i.e. thermal conductivity, specific heat, density, initial freezing temperature) of bull semen/extender as functions of temperature considering the water change of phase (ice formation); (c) to introduce these properties into the numerical finite element program used to solve the non-stationary heat transfer partial differential equations for the semen suspension in plastic straw considering thermal resistances in series; (d) to predict freezing times for different nitrogen vapor temperatures and heat transfer coefficients; and (e) to compare the obtained predictions with data from the literature in order to determine "safe" holding times of plastic straw in static nitrogen to ensure that complete freezing of the sample has occurred in the nitrogen vapor, before plunging in the liquid nitrogen.

Materials and methods

Semen samples

Several French polypropylene straws of a commercially available, red Angus bull, were obtained for the characterization of physical/chemical properties of diluted bovine semen. All straws belonged to one single bull and were packed from the same ejaculate; sperm concentration in 0.5 ml straw was adjusted to 30×10^6 and diluted in commercial bull semen extender (Andromed®, Minitube, Germany). Extender composition (as reported by manufacturer) consisted of phospholipids, TRIS buffer, citric acid, sugars, glycerol, ultrapure water and antibiotics.

Mathematical modeling of the heat transfer considering phase change transition

The system (straw and semen + extender) can be described as two concentric finite cylinders of different materials: the fluid and the straw. Dimensions of the polypropylene straw were 130 mm length, 2.6 mm o.d., 1.9 mm i.d. and 0.35 mm wall thickness. The differential equations that represent the heat transfer in the fluid and the plastic support considering radial and axial coordinates are:

$$\rho_s(T)Cp_s(T)\frac{\partial T}{\partial t}r = \frac{\partial}{\partial r}\left(k_s(T)r\frac{\partial T}{\partial r}\right) + \frac{\partial}{\partial z}\left(k_s(T)r\frac{\partial T}{\partial r}\right) \quad \text{in } \Omega_s \quad t > 0 \quad (1)$$

$$\rho_p Cp_p \frac{\partial T}{\partial t}r = \frac{\partial}{\partial r}\left(k_p r\frac{\partial T}{\partial r}\right) + \frac{\partial}{\partial z}\left(k_p r\frac{\partial T}{\partial z}\right) \quad \text{in } \Omega_p \quad t > 0 \quad (2)$$

where T is temperature, ρ corresponds to the density, Cp specific heat, k thermal conductivity and the subscripts s and p correspond to the mixture of semen + extender and plastic material, respectively. It can be noticed that in the semen suspension the thermal properties are temperature dependent since there is a phase change transition of water into ice, however in the plastic support the thermophysical properties (k_p, ρ_p, Cp_p) are considered constant.

The surfaces exposed to N_2V/LN_2 are the bottom circle and the lateral plastic cylinder; the top circle was considered isolated ($q = 0$) since the semen is in contact with air inside the straw (Fig. 1). The warmest point in the system can be identified in Fig. 1.

The equation that represents the boundary convective condition is:

$$-k_2(\nabla T \cdot n_2) = h \cdot (T - T_v) \quad \text{in } \delta\Omega \quad t > 0 \quad (3)$$

where h is the surface heat transfer coefficient, T_v is the temperature of the N_2V/LN_2 and $\delta\Omega$ represents the surface of the straw

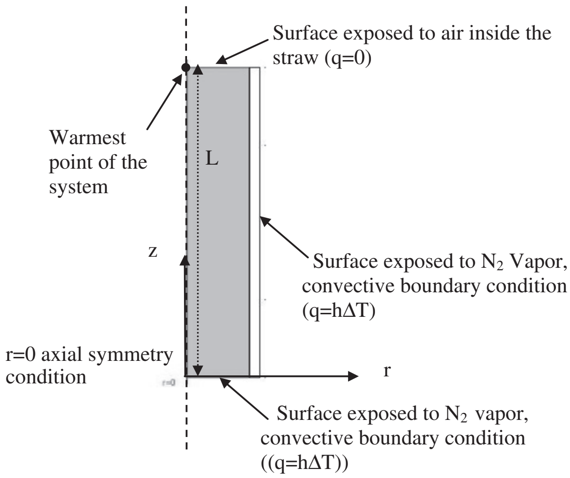

Fig. 1. Geometry of the sample and boundary conditions.

exposed to the nitrogen vapor. The initial condition was considered uniform in both material domains being:

$$T = T_0 \quad \text{at } t = 0 \tag{4}$$

Eqs. (1 and 2) and the boundary condition were numerically solved using the finite element method in COMSOL Multiphysics 3.2 software [24,25]. The domain was discretized in triangular elements in order to obtain accurate numerical approximations. The program calculates the time–temperature curves at all the nodes that constitute the mesh; in particular the hot point in the domain was determined in order to evaluate the slowest freezing rate (worst condition).

Experimental measurement of thermophysical properties by differential scanning calorimetry (DSC)

Moisture content of the semen/extender was determined gravimetrically (at 90 °C for 24 h, forced convection oven).

Specific heat and latent heat of ice melting of the mixture of semen + extender were measured by using a Differential Scanning Calorimeter (DSC) (TA Instruments, New Castle, DE, USA) model Q100 controlled by a TA 5000 module with a quench cooling system under a nitrogen atmosphere at 20 ml/min. Samples of semen–extender suspension were enclosed in sealed aluminum pans. An empty pan was used as a reference sample. Pans were heated at 2 °C/min from −150 to 20 °C, with isothermal periods at the initial and final temperatures. In order to measure the specific heat three scans were made: one for the sample, one for a standard (sapphire), and one for the empty sample pan. In these scans the reference holder contained an empty pan. Distilled water was also scanned using the same program to verify equipment calibration. The specific heat was calculated following the ASTM E1269 procedure and McNaughton and Mortimer [53] recommendations.

The latent heat of melting (ΔH_m) was determined as indicated by Roos [71]. The ΔH_m was calculated by integrating the peak of the melting curve; this value was used to estimate the unfrozen water fraction in the material. The temperature integration limits of the peak were chosen when a clear separation between curve and base line was detected. Unfrozen water (xb), was considered as the difference between total water content and the amount of frozen water in the deep frozen material. The fraction of frozen water was obtained from the ratio between the latent heat of melting determined for the material and the heat of melting of pure water.

Mathematical modeling of thermophysical properties of bovine semen

Apparent specific heat

Experimental data of the apparent specific heat, where the sensible heat is merged with the latent heat, produced a curve with a large peak around the freezing point. However for mathematical modeling purposes the specific heat capacity function has to rise smoothly to a peak over a finite range of temperature [58]. Therefore based on experimental data a Cp vs. temperature curve was constructed using a Gaussian and Heaviside function [24,25,58] maintaining the experimental values of the initial freezing point, the range of temperature change, the latent heat of melting. The equation used in the numerical program to represent the specific heat as a function of temperature is as follows:

$$\mathrm{Cp}(T) = \mathrm{Cp}_{\mathrm{ff}} + \frac{\Delta H_{\mathrm{m}}}{\mathrm{Ts}} \cdot f(H_{\mathrm{ea}}) + D \cdot \Delta H_{\mathrm{m}} \tag{5}$$

where $\mathrm{Cp}_{\mathrm{ff}}$ is the specific heat of the fully frozen state, Ts is the peak temperature point (equivalent to the μ media in the Gaussian curve) and D is a Gaussian curve defined as

$$D(T) = \frac{e^{\left(-\frac{(T-T_s)^2}{\mathrm{d}T^2}\right)}}{\sqrt{\pi\,\mathrm{d}T^2}}$$

where dT is the half width of transition, that is the temperature difference from melt within which 84% of the latent heat occurs [58]; $f(H_{\mathrm{ea}})$ is the Heaviside function which is a built in function in COMSOL Matlab environment that has continuous second order derivatives. This function enables the numerical finite element software [23,24] to successfully deal with the abrupt change in the apparent specific heat of the sample with temperature.

Initial freezing temperature

The initial freezing temperature Tf was experimentally determined from the freezing curves. Tf was measured by using thermocouples inserted in the bovine semen + extender samples contained in the plastic straw by applying the tangent method [34].

Thermal conductivity

Thermal conductivity of the semen + extender was calculated using the Choi and Okos [19] equation as follows:

$$k(T) = \sum x_i^{\mathrm{v}} \cdot k_i(T) \tag{6}$$

where k is the global conductivity, given in W/m °C, k_i is the thermal conductivity (W/m °C) of the component i (where i corresponds to the different components: water, ice (if the temperature is lower than the initial freezing temperature Tf), carbohydrate, fat, etc.), x_i^{v} corresponds to the volumetric fraction of each component.

Ice content as a function temperature

The ice content as a function temperature (at $T <$ Tf) was estimated using the equation proposed by Miles et al. [54]:

$$\mathrm{xh} = (x_{\mathrm{wo}} - \mathrm{xb})\left(1 - \frac{\mathrm{Tf}}{T}\right) \tag{7}$$

where xh is the mass fraction of ice, Tf and T are given in °C, x_{wo} is the total mass fraction of water in the sample. The value of xb was experimentally measured using DSC measurements.

Heat transfer coefficient (h)

As mentioned before, cryopreservation of spermatozoa is based on freezing of semen in plastic straws (i.e. 0.5 ml French straws) immersed in static N_2V and at a specific distance above LN_2, usually contained in a Styrofoam® box. A vertical temperature gradient (inside the Styrofoam® box) from the surface of LN_2 to the straw may influence natural convection in N_2V, thus making any prediction of heat transfer coefficients very difficult. Since 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; and a range of h 5–20 W/m^2 K was adopted [20,66]. This range of heat transfer coefficients was used in the numerical finite element program to calculate freezing times for different nitrogen vapor temperatures (T_v).

Results and discussion

Experimental results

The specific heat of the bovine semen + extender (between −150 and 20 °C) was obtained using the experimental data obtained by DSC. The latent heat of melting was ΔH_m = 264.95 kJ/kg and the unfreezable water resulted in a 4.88% (wet basis). Eq. (5) that represents Cp as a function of temperature considering the experimental data from the DSC measurements, the range of temperature phase change, the latent heat (ΔH_m) and the specific heat values of the fully frozen and unfrozen state is shown in Fig. 2.

The initial freezing temperature experimentally determined was Tf = −2.8 °C.

In order to apply Choi and Okos [19] predictive equations for the thermophysical properties of the semen sample the following composition of the dry matter (mass fractions, wet basis) was adopted: carbohydrates = 0.098, fat = 0.031, and protein = 0.027 The moisture content of the semen + extender was experimentally measured and found to be 84.4%.

Thermal conductivity, density and ice fraction

The thermal conductivity and the density of the semen + extender sample as functions of temperature calculated by using the parallel model reported by Choi and Okos [19] are shown in Fig. 3a and b, respectively. These data were used as input in the numerical finite element program. Fig. 4 shows the ice fraction as a function of temperature (calculated using Eq. (7)). The ice fraction vs. temperature is useful information since it can help to establish different freezing times or freezing rates, based on the temperature that has to be reached to get a certain ice fraction.

Survey of literature data on experimental freezing conditions in static N_2V (0.5 ml plastic straws) and "safe" freezing times of straw at different distances above liquid nitrogen level

Most results for spermatozoa cryopreservation are based on freezing of semen + extender in plastic straws (i.e. 0.5 ml French straws), precooled and stabilized at 5–6 °C, which are then suspended in static N_2V at specific distances (and hence temperatures) above LN_2, before plunging into the liquid phase. Cooling rates are expected to vary with the distance from the surface of LN_2 to straw [33]; therefore, by controlling the distance above LN_2 one can control the temperature of the cooling vapor. The LN_2 is placed usually in a Styrofoam® box at a height which may vary from different sources. In a few cases a nitrogen storage tank was used instead of the Styrofoam® box [28]. In order to minimize the fluctuations in cooling rate when freezing in N_2V/LN_2, it is important to make sure that conditions of freezing are controlled appropriately. To estimate the optimal height above LN_2 (where the samples are to be held), researchers used to empirically hold several sperm samples at different levels; the higher the sample is above the surface the longer the freezing time should be [47].

In 1996, Faure [33] noted that freezing curves produced by static N_2V generally vary greatly from sample to sample within a freeze, and particularly from freeze to freeze. Also, due to the low heat capacity of N_2V, a small amount of heat will warm the vapor considerably, and static equilibrium is then easily disturbed. Yi et al. [85] reported the effect on vapor temperature on the sample distance from LN_2; they reported that at 5, 11 and 17 cm

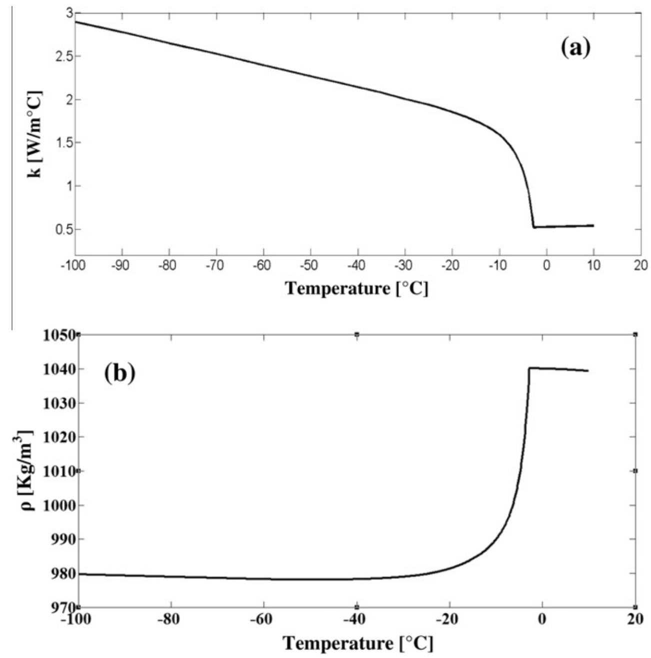

Fig. 3. (a) Predicted thermal conductivity and (b) density of semen + extender sample as a function of temperature.

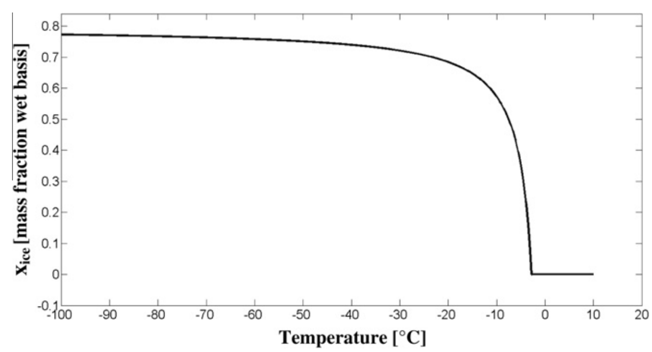

Fig. 4. Ice mass fraction vs. temperature of semen + extender sample (Eq. (7)).

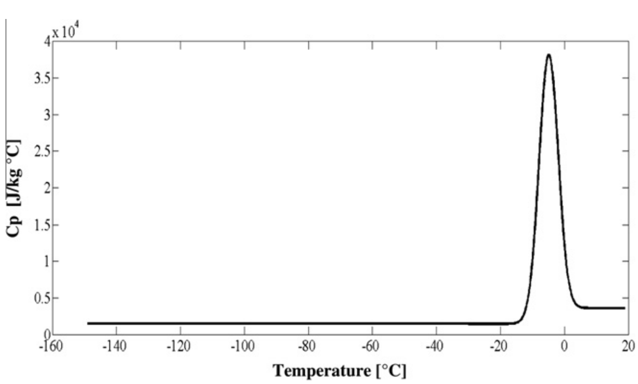

Fig. 2. Apparent specific heat of semen + extender sample at different temperatures using Eq. (5).

above the surface of LN_2 the vapor temperatures were −141, −95 and −42 °C, respectively.

Table 1 compiles available literature data on freezing conditions for straws suspended in static N_2V: vapor temperature, holding time of straw and height from the level of LN_2 to plastic straw. The reported heights above LN_2 surface varied between 1 and 15 cm, so one would expect variations in the vapor temperature and thus in freezing rates. Not all authors measured the actual nitrogen vapor temperature; however, reported values were: −70 to 80, −100, −120, −140 and −160 °C. In the majority of cases, experimental holding times of straws in N_2 vapor ranged between 5 and 20 min.

There are various possibilities to define the freezing time of semen + extender packaged in 0.5 ml straw and frozen in N_2V. Unfortunately, few authors reported the sample temperature after freezing in N_2V immediately before plunging in LN_2. In most cases, only the height over LN_2 and holding time were informed. In a few cases the sample temperature at the end of freezing in vapor was measured. Robbins et al. [69] working with bovine spermatozoa reported a straw temperature of −80 °C before plunging in LN_2.

Although only few authors reported both the height from LN_2 level and vapor temperature, an attempt was made to correlate these data. As shown in Fig. 5, an acceptable straight-line correlation (r^2 = 0.888) was obtained. It is noteworthy that several experimental factors may have influenced the vapor temperature measurement used in Fig. 5; namely the amount of LN_2 relative to air space, dimension of the container, etc., all of which surely contributed to some variability in the measured temperatures. As expected, the higher the sample is above the surface of LN_2, the higher is the vapor temperature [47].

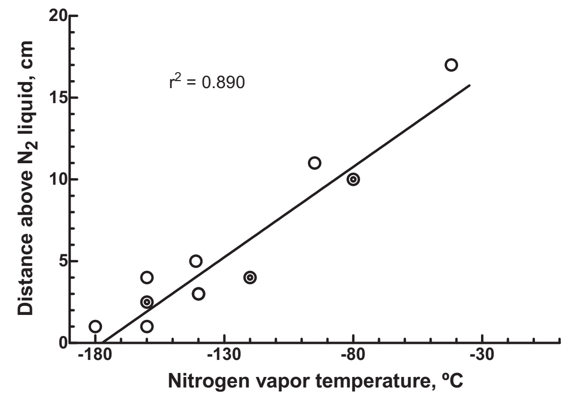

Fig. 5. Correlation between distance above N_2 liquid level and vapor temperature (data obtained from several authors [1,5,13,30,31,35,44,49,59,56,79,81]).

Table 1
Survey of literature data on freezing conditions for semen packaged in 0.5 ml plastic straws suspended in N_2V/LN_2 contained (in most cases) in Styrofoam® box.

Height above level of N_2 liquid (cm)	Holding time in static vapor (min)	Nitrogen vapor temperature (°C)	Reference
n.r.	5, 10, 15	−80, −90, −100	[83]
6	n.a.	n.r.	[52]
2.5	10, 15	−160	[57]
15	30	n.r.	[60]
4	15	n.r.	[9]
5	10, 20	n.r.	[38]
15	15	n.r.	[29]
n.r.	10	n.r.	[82]
4	10	n.r.	[76]
n.r.	15	−120	[26]
7	10	n.r.	[15]
3	10	−140	[81]
5	n.r.	n.r.	[28]
4	10	n.r.	[78]
4	⩾0.5	n.r.	[68]
4	8	−160	[1]
n.r.	20	n.r.	[3]
10	15	−80	[31]
4	15	n.r.	[8]
n.r.	5	−80	[10]
4	10	n.r.	[65]
2.5	15	n.r.	[11]
n.r.	15	−120	[39]
5	20	n.r.	[14]
3–4	7–8	n.r.	[32]
4	5	n.r.	[18]
n.r.	9	−110	[50]
3	15	n.r.	[37]
About 1		−160	[44]
15	15	n.r.	[56]
1	7–10	−180	[79]
4.5	12	n.r.	[64]
4.5	10	n.r.	[57]
4.5	10	n.r.	[70]
3	5	n.r.	[46]
3	10	n.r.	[62]
4	10	n.r.	[80]
5	6	n.r.	[45]

n.r.: not reported.
n.a.: not available.

At a temperature of −40 °C about 95% of the water in the semen + extender mixture in the straw is frozen (Fig. 4); however, in order to include a margin of safety, a lower −69 °C was adopted for the calculation of freezing times in this study. For this reason, we arbitrarily define here a "safe" freezing interval as the time required to reduce the temperature from its initial value (+6 °C) to −69 °C at the warmest point of the semen/extender packaged in straw. At this temperature not only all freezable water has been transformed into ice, but also a reasonable safety margin is included to avoid the possibility of plunging the straw in LN_2 before all water was transformed in ice (which may cause cell cryodamage and impair sperm viability). Therefore, a range of N_2V temperatures were selected for the numerical simulations, fixing at −69 °C the final temperature to be achieved in the freezing process.

Fig. 6 shows a predicted freezing curve at the warmest point of straw at selected N_2V temperature and external heat transfer coefficient using the model developed in the present work. Table 2 shows predicted "safe" freezing times for bovine semen + extender in 0.5 ml plastic straws suspended in N_2V/LN_2, obtained by applying the mathematical model for different values of the heat transfer coefficient and vapor temperatures (−70 to −160 °C); time is given in minutes rounded to first decimal figure. As observed, predicted "safe" freezing times (Table 2) ranged between 1.0 min (for h = 20 W/K m^2 and T_v = −160 °C) to about 20 min (for h = 5 W/K m^2 and T_v = −70 °C).

It is interesting to compare these values with literature straw holding times (Table 1) in nitrogen vapor. Thirty-five different researchers reported holding times of straws (in static N_2V) ranging between 1 and 20 min. These values can be compared with those predicted by our mathematical model for different external heat transfer coefficients and vapor temperatures (Table 2). This allows us to predict that complete freezing of the samples likely occurred while holding the straw in the N_2V, before plunging in LN_2. This aspect will be further discussed in the paper.

In 1990, Ritar et al. [68] studied freezing of caprine semen by holding straws in N_2V 4 cm above LN_2 following by plunging into liquid nitrogen. They reported that straws exposed to vapor for only 10 s did not cool sufficiently before plunging, and cell viability was seriously impaired. This observation is in complete agreement with predictions shown in Table 2, since the minimum predicted freezing time (at −160 °C, h = 20 W/m^2 K) is 60 s.

Fig. 7a shows the effect of vapor temperature on "safe" freezing times (time to reach −69 °C in the warmest point of straw)

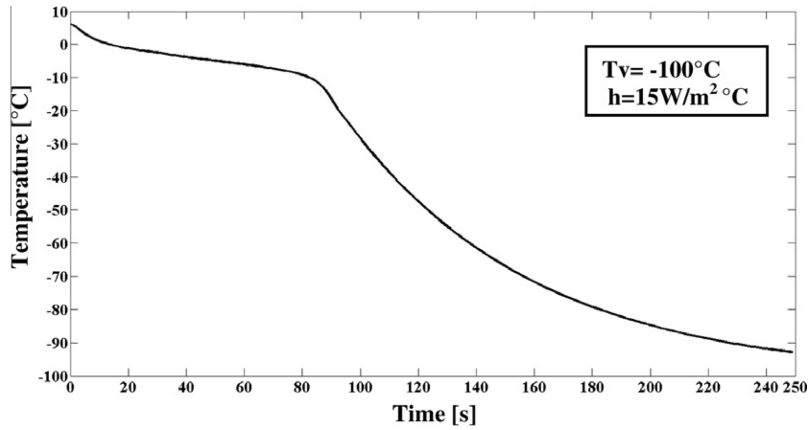

Fig. 6. Temperature at the warmest point of straw vs. cooling time at selected N_2V temperature and external heat transfer coefficient.

Table 2
"Safe" freezing times calculated as minutes to reach a temperature value of −69 °C at the warmest point of the plastic straw containing semen + extender.

h (W/m² K)	T_v (°C)					
	−70	−80	−90	−100	−130	−160
5	19.1	11.2	8.9	7.6	5.3	4.1
10	9.7	5.7	4.5	3.8	2.7	2.1
15	6.5	3.8	3.1	2.6	1.8	1.4
20	4.9	2.9	2.3	2.0	1.4	1.0

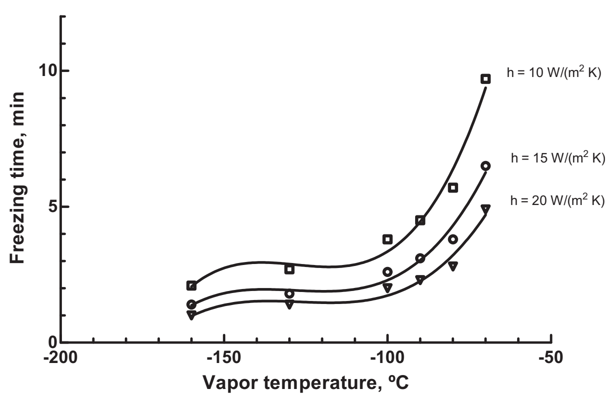

Fig. 7a. Effect of vapor temperature on "safe" freezing times of straw suspended in N_2V/LN_2 at selected values of external heat transfer coefficient (h).

calculated with the numerical model at selected values of the external heat transfer coefficient (h). As observed, freezing times of straw decreased with lowering temperature of N_2V. Since higher vapor temperatures are associated with increasing heights above the level of LN_2, these results illustrate how cooling rates would be expected to vary with the distance from straw to the surface of LN_2 [85]. This aspect will be further discussed in the paper.

Fig. 7b shows the effect of external heat transfer coefficient on "safe" freezing times of straw frozen at selected N_2V temperatures. As observed, h is a relevant parameter controlling freezing rates since the increase in h resulted in a dramatic reduction in freezing time.

In order to simplify the calculation of "safe" freezing times an equation was obtained using a mathematical regression of Table 2 by a forward stepwise method in SYSTAT vs. 12. The equation is as follows:

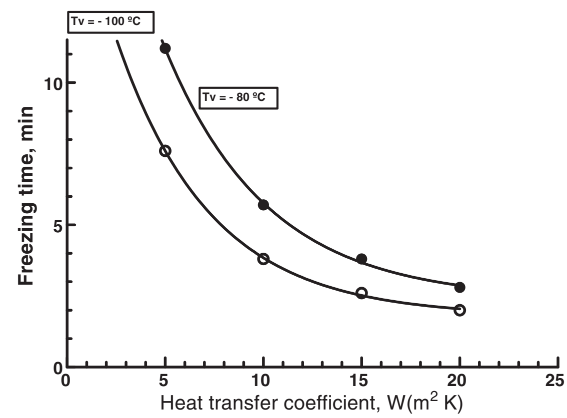

Fig. 7b. Effect of external heat transfer coefficient (h) on "safe" freezing times of straw suspended in N_2V/LN_2, at selected vapor temperatures. "Safe" freezing time is defined as the time to reach −69 °C in the warmest point of straw suspended in static liquid N_2V.

$$\begin{aligned}\mathrm{Time}^{(\mathrm{safe})} &= 923.322 - 24.676(T_v) + 0.24458(T_v)^2 - 1.0611 \\ &\times 10^{-3}(T_v)^3 - 25.865 \times 10^{-4}(h)^3 + 8.052 \\ &\times 10^{-4}(h)^2(T_v) - 8.53 \times 10^{-5}(h)(T_v)^2 + 1.71645 \\ &\times 10^{-6}(T_v)^4 \end{aligned} \quad (8)$$

with R^2 = 0.98714, and where time is the "safe" freezing time (min) and T_v (K) and h (W/m² K).

Fig. 8 compares "safe" freezing times$^{(\mathrm{safe})}$ estimated by the finite element method with "safe" freezing times estimated by the polynomial equation (Eq. (8)). A good correlation was observed because data are distributed over the 45° straight line.

Roth et al. [72] used a thin thermocouple probe to measure the cooling rates of sperm packaged in 0.5 ml plastic straw immersed in N_2V (vapor temperature was not reported). The straw temperature reached −70 °C after 3.0 min and calculation with the present numerical model (for h = 10 W/(m² K) and T_v = −130 °C) resulted in a predicted freezing time of 2.72, which is in good agreement with the experimental value.

Reid et al. [67] reported that the freezing rate for 0.5 ml straws placed over −80 °C liquid N_2V was 8.8 °C/min. This freezing rate can be matched by applying the numerical model as follows. Fig. 7b indicates that for N_2V at −80 °C and considering a heat transfer coefficient, h = 7.0 W/m² K, the time to reduce straw temperature from +6 °C to −69 °C is 8.5 min, leading to a freezing time of 8.8 °C/min. Kobayashi et al. [46] and Robeck et al. [70] reported

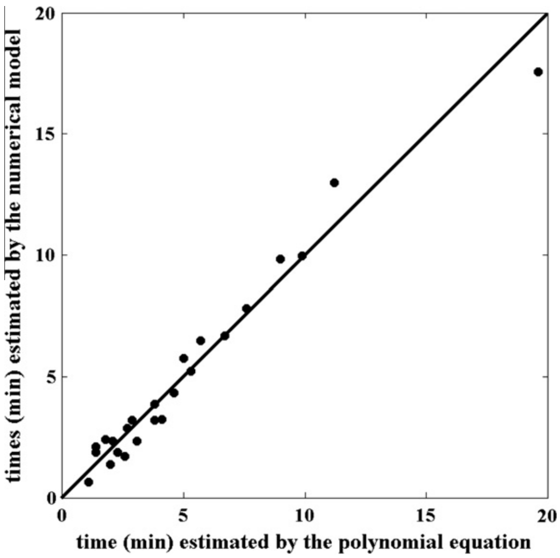

Fig. 8. Comparison of "safe" freezing times$^{(safe)}$ of 0.5 ml plastic straw (containing semen + extender) estimated by the finite element method and by the corresponding polynomial (Eq. (8)).

that freezing rates of straw placed at 3 and 4.5 cm above liquid nitrogen were 12 °C/min and 27 °C/min, respectively. These freezing rates may be matched by the numerical model using h = 10–15 W/(m^2 K) at vapor temperatures, T_v, between −80 °C and −130 °C (Table 2).

As mentioned before, at −40 °C practically all water in straw has been frozen; thus, an alternative way to define freezing time is the period required to bring the temperature at the warmest point of straw to −40 °C. In other words, it represents the minimum freezing time to ensure that practically all water has been frozen before plunging in liquid nitrogen. The numerical model was used to calculate this "minimum" freezing time at various vapor temperatures and heat transfer coefficients.

Fig. 9 shows a plot of the time necessary to reach −40 °C vs. the heat transfer coefficient at various vapor temperatures. The strong effect of heat transfer coefficient on time is again noteworthy; at the highest vapor temperature the effect was more pronounced that at the lowest ones.

Estimation of "safe" freezing times of straw at different distances above LN_2 level

Combining the data shown in Fig. 5 with those shown in Table 2, it is possible to estimate the freezing times for different distances

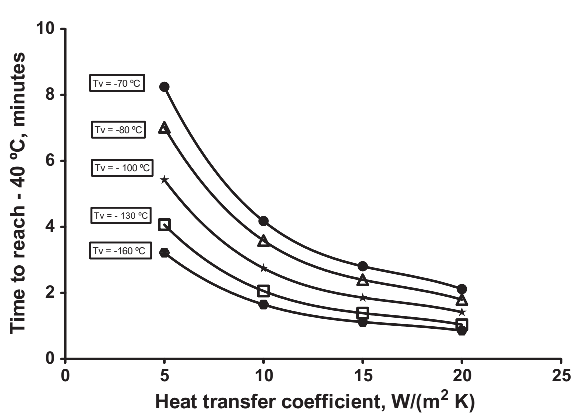

Fig. 9. Combined effect of heat transfer coefficient (h) and N_2V temperatures (T_v) on time to reach −40 °C in the warmest point of straw.

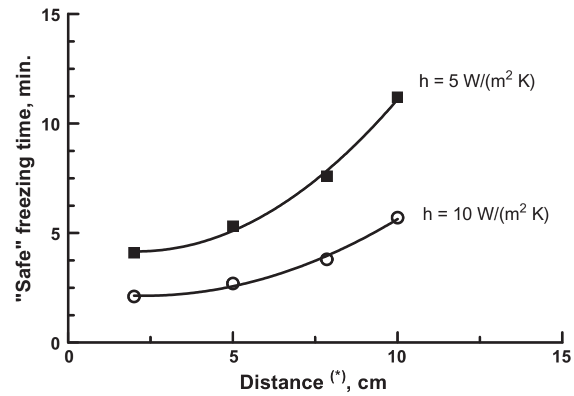

Fig. 10. Estimation of "safe" freezing times for 0.5 ml plastic straw located in N_2V/LN_2 at various distances from the surface of liquid nitrogen. *Distance from the straw to the surface of liquid nitrogen.

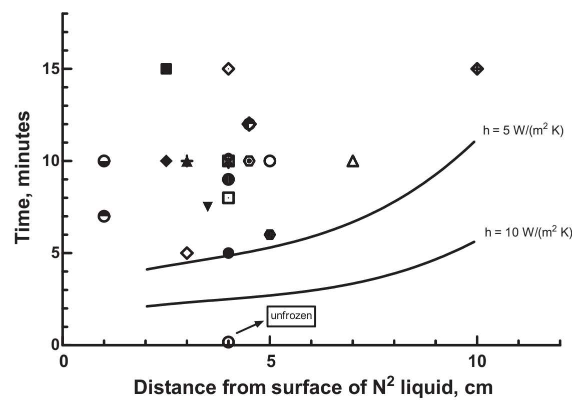

Fig. 11. Comparison of literature experimental freezing conditions (time-distance) of 0.5 ml straws in N_2V (symbols) with predicted safe freezing time (lines) at various distances above surface of N_2 level, for two selected heat transfer coefficients. *Symbols*: From data reported by several authors [1,2,8,9,11,15,17,18,31,32,37,38,55–57,62,64,65,68,70,76,78–81]

from the LN_2 level, as shown by Fig. 10 for selected values of the heat transfer coefficient. An estimation of "safe" freezing times for straw placed at different distances over LN_2 may be now made. The results, for distances between about 2 and 10 cm, are shown in Fig. 10, indicating that the higher the sample is above the surface of LN_2, the longer the freezing time should be.

Fig. 11 allows a direct comparison of several literature combinations of holding time–sample distance, with safe freezing times predicted by the model. As observed in over 23 experimental points, only one does not fulfill conditions needed for freezing (marked "unfrozen" in Fig. 11). This point was reported by Ritar et al. [68], who stated that straw exposed to vapor for only 10 s did not cool sufficiently before plunging and cell viability was seriously impaired. This observation is in complete agreement with present predictions.

Conclusions

A mathematical heat transfer model was developed to predict freezing times (phase change was considered) required for bovine semen + extender packaged in 0.5 ml plastic straw and suspended in static N_2V. Thermophysical properties (i.e. thermal conductivity, apparent specific heat, ice fraction) of bovine semen + extender as a function of temperature were determined considering the water change of phase. The non-stationary heat transfer partial differential equations with variable properties (nonlinear mathematical problem) were numerically solved using a new formulation of the apparent specific heat in order to cope with the complex problem of phase change transition in the bovine semen suspension in series with the plastic straw. The temperature profiles were obtained for both the semen suspension and the plastic straw during the freezing process in N_2V/LN_2.

Predictions performed with the model confirmed that experimental holding times of straws in vapor as reported by a large number of researchers seemed to fall in the "safe range"; i.e. the sample packaged in 0.5 ml plastic strews was likely to be frozen before plunging in LN_2.

The accuracy of the numerical method to predict freezing times of straw was tested with actual literature data. It was observed that both the external heat transfer coefficient in static N_2V and its temperature (controlled by the distance from the surface of LN_2 to the straw) affected freezing times. It is hoped that freezing times predicted by the numerical model will be useful to optimize freezing protocols of semen straws in N_2V/LN_2. Finally, future experimental measurements of heat transfer coefficients in N_2V/LN_2 would greatly contribute to obtain more accurate predictions using the mathematical model.

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