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

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ABSTRACT

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.

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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 [55,4]. 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 [44].

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 [66]. 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 [13] 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 [6,7,17,38,43,44]. Automated, programmable freezers are routinely used to accomplish controlled, slow cooling of bovine semen packed in polypropylene straws [88].

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 [45,46]. On the

contrary, slow cooling often injures the cells due to mechanical and/or osmotic effects of external medium [38,50]. 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 [28]. Insulated, Styrofoam® boxes containing LN_2 have been used successfully for sperm cryopreservation [22]. 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 [67]. Alternatively, Chemineaux et al. [19] 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 [42].

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. [71] 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 [76,77] 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 [64]. 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 [23,24,53,64] and the Kirchhoff function, which is the integral of the thermal conductivity [78,81]. 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 [64]. 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:

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

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

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:

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

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