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Controlled ice nucleation—Is it really needed for large-volume sperm cryopreservation?

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ABSTRACT

Controlled ice nucleation (CIN) is an integral stage of slow freezing process when relatively large volumes (usually 1 mL or larger) of biological samples in suspension are involved. Without it, a sample will supercool to way below its melting point before ice crystals start forming, resulting in multiple damaging processes. In this study, we tested the hypothesis that when freezing large volumes by the directional freezing technique, a CIN stage is not needed. Semen samples collected from ten bulls were frozen in 2.5-mL HollowTubes in a split-sample manner with and without a CIN stage. Thawed samples were evaluated for viability, acrosome integrity, rate of normal morphology, and, using computer-aided sperm analysis system, for a wide range of motility parameters that were also evaluated after 3 hours of incubation at 37 °C. Analysis of the results found no difference between freezing with and without CIN stage in any and all of the 29 parameters compared ($P > 0.1$ for all). This similarity was maintained through 3 hours of incubation at 37 °C. Possibly, because of its structure, the directional freezing device promotes continuous ice nucleation so a specific CIN stage is no longer needed, thus reducing costs, energy use, and carbon footprint.

1. Introduction

To maintain viability and functionality in biological systems when cryopreserved, samples are suspended in cryoprotective media that are composed of water with various additives. During the cooling process, the water in the system turns progressively into ice all the way to the point when the unfrozen solution becomes concentrated and viscous enough to vitrify. Through the initial stages of chilling down to sub-zero temperatures, the water in the system has the tendency to chill to beyond its melting temperature without forming ice. This process is known as supercooling or undercooling. For freezing to occur, water molecules should group together in a stable cluster above a critical size [1,2]. Once these clusters form, other water molecules will attach themselves to them as the sample cools to form ever-growing ice crystals. Two possible mechanisms may lead to the formation of ice crystallization centers. Water molecules may attain the needed configuration by attaching themselves around a solid or liquid substrate in the system. Alternatively, water molecules may arrange themselves through random density fluctuations into a stable nucleating centers around which ice crystals may grow. These two processes are known as heterogeneous or facilitated ice nucleation and homogeneous ice nucleation, respectively [3]. Impurities in the solution and on the internal surface of the container can act as nucleation centers. These impurities are the dominant, or, possibly, the only nucleation centers when freezing relatively large volumes (~1 mL or more) at near the melting point. As temperatures go down, homogeneous ice nucleation gains dominance [4]. Nucleation becomes increasingly likely as the temperature decreases because the

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number of molecules needed to form a stable crystal nucleus decreases with decreasing temperature [1].

When cryopreserving biological samples by slow freezing, the process of supercooling may be hazardous [5]. It is not unconceivable that samples will supercool by 10 °C or more before ice crystals form, resulting in a large temperature gap between the melting temperature and the surrounding temperature. Once ice nucleation centers form, three damaging processes may take place. First, because of the large temperature gap, ice crystals will grow in a very fast and uncontrollable fashion, causing mechanical damage to the cells in the suspension [6]. Second, the abrupt ice formation will result in a massive release of the latent heat of fusion, leading to momentary increase in the temperature to close to the melting temperature, followed by prolonged isothermal period while the surrounding temperature continues to drop. The result will be faster-than-desired cooling rate down to close to the surrounding temperature [7,8]. The temporary increase in temperature may also cause recrystallization, which is a damaging process by itself. Third, in the absence of ice formation the volume of the cells in the suspension remains unchanged. Water is withdrawn from the cells in response to shifts in the osmotic pressure caused by the removal of water from the extracellular milieu in the form of ice. As long as ice crystals do not form, the cells remain in their initial volume. Once ice starts forming and does so in faster-than-desired fashion, intracellular ice crystals may form and kill the cells.

The importance of controlled ice nucleation (CIN) and crystal growth is manifested by the fact that even at low subzero temperatures there is only little damage to cells in a suspension as long as there is no freezing [9]. To prevent the damaging processes described above from happening, a CIN step, sometimes referred to as "seeding", is normally included early in the freezing process [10,11]. By intentionally creating ice crystals at relatively high subzero temperatures, the damaging effects of supercooling can be greatly minimized. Controlled ice nucleation can be done by a wide range of methods including the addition of ice crystals to the system, manually cooling a spot on the container to generate ice crystals inside, include chemicals known as ice crystals catalysts, application of high voltage to an electrode in the sample, mechanically inducing ice nucleation by shaking, tapping, or applying ultrasound to the sample, cooling the sample abruptly to a very low temperature, or by exposing the sample to elevated pressure, cooling it, and then decreasing the pressure [3]. All these techniques allow us to control when and sometimes also where ice crystallization will start. When freezing cells in suspension, it also allows us to maintain large spaces between the forming ice crystals in which the cells may accumulate to the point when these unfrozen channels vitrify.

The directional freezing technique was adapted to the cryopreservation of biological samples in the 1980s [12]. It has later progressed to the point that freezing equipment on the basis of this technique was developed, the so called multithermal gradient (MTG) devices [13]. These devices, suitable for freezing large volume samples (nowadays normally up to 8 mL of sperm in suspension or ovarian tissue slices or whole organs in even larger volumes), are constructed from two blocks—a warm block (usually around +5 °C) and a cold block (usually set at −50 °C) with a defined gap between them. This structure results in a linear temperature gradient through which the sample is continuously moved so that heat is transferred from the frozen portion of the sample both to the surrounding highly thermal-conductive metal and to the portion of the sample still in the high-temperature warm block. Ice crystals will thus grow in the opposite direction to the direction in which the sample is moved. In the early generations of the MTG devices, ice nucleation was induced in samples in glass tubes by touching the bottom of the tubes with cotton wool soaked with liquid nitrogen (unpublished observation). Later on, a seeding stage was incorporated into the freezing program. Suspended sperm samples are now pushed from the warm block over the gap and into the cold block till about 2 mm of the tube with the sample are in the cold block. The machine then stops for 30–60 seconds, depending on the program, so ice can form in that part of the sample. After this CIN stage, the sample is pushed over the temperature gradient in a continuous manner so that the ice crystals that have formed can grow in an opposite direction to the direction of movement in a controlled way. In essence one can say that after the initial CIN stage, as the sample is pushed into the cold block, ice nucleation continues throughout the freezing process. If continuous ice nucleation takes place during the directional freezing process [14,15], our thinking was, maybe the initial seeding stage is not needed at all. The study described here was designed with the aim of testing this hypothesis.

2. Material and methods

Unless otherwise stated all materials were purchased from Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany.

2.1. Semen collection and handling

Semen was collected from ten Holstein–Friesian bulls (age 15–39 months), one ejaculate each, at a commercial insemination center. The bulls were jumped on a teasing bull and semen was collected by artificial vagina as part of the regular semen collection process of the center. The insemination center's technician evaluated all samples on collection for motility and concentration. So as to avoid prolonged exposure to glycerol during transportation, evaluation, and chilling, ejaculates were diluted to a concentration of $\sim 200 \times 10^6$ spermatozoa/mL in glycerol-free cauda epididymal plasma-2 bovine extender [16] that contains: 15.1 -mM NaCl, 7 -mM KCl, 3 -mM $CaCl_2 \cdot 2H_2O$, 4 -mM $MgCl_2 \cdot 6H_2O$, 11.9 -mM $NaHCO_3$, 8 -mM NaH_2PO_4, 19.8 -mM KH_2PO_4, 55 -mM fructose, 5.5 -mM sorbitol, 2% (w/v) bovine serum albumin, 133.7 -mM Tris, 10% (v/v) egg yolk, 46.8 -mM citric acid, and 1 -mg/mL gentamycin. Diluted samples were place in a prewarmed (37 °C) transportation incubator (Minitube, Tiefenbach, Germany) and transported to the laboratory, an hour and a half away, for further evaluation and freezing.

2.2. Sperm evaluation

Samples were evaluated for motility, viability, acrosome integrity, and morphology on arrival (fresh) and after