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Brief Communication

An explanation for why it is difficult to form slush nitrogen from liquid nitrogen used previously for this purpose ☆

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ABSTRACT

Slush nitrogen (SN) is used to avoid the Leidenfrost effect, which is problematic when using liquid nitrogen (LN). Slush nitrogen's usefulness has been demonstrated by its requirement for the successful cryopreservation of insect embryos. To convert LN to SN, typically, the pressure above a Dewar of LN is reduced, using a vacuum pump in a sealed system until conversion occurs. It has been observed that LN from a fresh tank will readily produce SN; however, repeated use of the same LN results in the inability to form SN in subsequent trials. The current experiments were designed to identify the cause of this phenomenon. The hypothesis is that gaseous oxygen from the surrounding, ambient air condenses and mixes with the LN to form a mixture with a lower freezing point and; therefore, prevents the formation of SN. The hypothesis was tested and found to be true.

In regards to cooling rates, there are two general approaches to cryopreserving cells. The first is called slow-equilibrium freezing. An example of a slow-equilibrium freezing protocol is when cells are cooled from 0 °C to −40 °C at <3 °C/min and then plunged into liquid nitrogen (LN) for storage. The disadvantages of slow-equilibrium freezing for some cell types are their sensitivity to the pressures of extracellular ice formation (EIF) [1,3] and chilling injury [2,9]. These issues cause failure in the form of cellular death. Another approach to cryopreservation, the focus here, is termed vitrification. Vitrification reduces problems caused by EIF and chill injury. Vitrification attempts to create an amorphous solid state, extracellularly and intracellularly. Typically, cells are rapidly cooled from room temperature to −196 °C by plunging the container containing the cells into LN. A countering effect on achieving speed when cooling with LN is the formation of a vapor layer around the plunged specimen. The warmer specimen vaporizes the LN and the vapor insulates the specimen; an event referred to as the Leidenfrost effect [6]. The Leidenfrost effect is most commonly described as the phenomenon in which a liquid, in near contact with a mass significantly hotter than the liquid's boiling point, produces an insulating vapor layer which keeps that liquid from boiling rapidly. In the case of rapid freezing, the insulating vapor layer also prevents the solid mass from rapid cooling, a phenomenon that must be avoided in cryopreservation. Reduction in the Leidenfrost effect can be achieved using a semi-solid form of nitrogen called slush nitrogen (SN). In 1960, Luyet and Kroener [7] found the cooling rate with SN was substantially faster to LN. Slush nitrogen has been used to cryopreserve *Drosophila melanogaster* embryos [8] and mammalian embryos [5].

Over a 2.5 yr period, our lab has had predictable, but unexplainable, difficulties in making SN. Slush nitrogen could be readily produced with a fresh batch of LN. When the LN had been used several times prior to making SN, it became difficult to impossible to get the LN into a semisolid state. We discarded a theory that LN had "memory", like the Mpemba effect of water [4]. Efforts to find an explanation for this observation included scrutiny to our homemade vacuum chamber and other components used for making SN. Even after holding LN in the vacuum chamber for nearly four times as long as required to succeed with a fresh tank of LN, SN failed to form when using LN that had been previously taken through the freezing cycle several times. Next, the possibility of contamination with other dissolved gasses was explored.

Earth's atmosphere consists of approximately 79% nitrogen, 20% oxygen and a small concentration of other gasses. It seemed possible that oxygen, the second most abundant gas, might have condensed into the slush nitrogen. Liquid nitrogen has a lower boiling point (−196 °C) than liquid oxygen (LO, −183 °C) and ves-

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sels containing liquid nitrogen can condense oxygen from the surrounding, ambient air. Our hypothesis is that a mixture of LN and LO is unable to form a slush under the conditions of the experiments. Freezing point depression and colligative properties are well-known principals of physical chemistry. Simply, the freezing point of a pure substance is decreased when a solute is introduced. When a pure substance freezes, the molecules are oriented into a highly ordered matrix by intermolecular forces. If this order is disrupted by a solute, more energy must be removed from the system for a solid to be formed. This is what we believe to be happening when liquid oxygen is dissolved in liquid nitrogen. The colloidal properties of oxygen and nitrogen mixes have been previously studied [10]. In this study, aliquots taken from a fresh tank of LN and from LN that had been previously taken through several cycles of slush formation and were subsequently "non-slushable" were analyzed for oxygen content.

The percentage of oxygen in LN was quantified using a Vernier Labquest (serial #1003893) hand-held monitor and Vernier oxygen gas sensor (02-BTA). A 250 mL Nalgene™ bottle, which had been pre-chilled to LN temperatures, was filled with 100 mL aliquots of LN. Immediately following the addition of the 100 mL aliquots; a sequence of deflated latex balloons (Gayla Industries, stock #30901) were placed around the opening of the bottle. The latex balloons captured the gaseous molecules from the boiling liquid. Typically, five balloons were required to capture the gas phase of a 100 mL liquid volume. After filling each balloon with gas, a hemostat was used to close the balloons until their attachment to the oxygen sensor. The diameter of the oxygen sensor (2.8 cm) allowed for trouble-free attachment of the balloons. The balloons' elasticity ensured positive pressure against the wall of the oxygen sensor. Once the balloon was attached to the oxygen sensor, a final room oxygen measurement was recorded and then the hemostat was relaxed allowing the contents of the balloon to escape through small holes on the oxygen sensor's housing. This method provided a steady stream of gaseous molecules from the balloons for accurate oxygen concentration measurements. Oxygen measurements were recorded for each balloon every 60 s. A typical balloon expelled its contents through the oxygen sensor in less than 240 s.

Oxygen concentrations were estimated from the means of the 60 s and 120 s interval measurements for each balloon within a treatment. These time points were chosen because a constant stream of gas was present then, which was necessary for accurate measurements. It was observed that when the balloons became flaccid, the oxygen sensor showed higher oxygen readings, which was expected.

Four 100 mL aliquots of LN from a typical laboratory tank (34 L Taylor-Wharton), which held LN that had been repeatedly used to make SN, enough so the LN would no longer form SN, were assayed for LO. The first and second aliquots were examined by capturing the gas of the boiling LN in series from balloons labeled one through five. Oxygen concentrations were then measured in the same order. The third and forth 100 mL aliquots were performed by placing the Nalgene™ bottle in a cardboard box (length 32 cm, height 35 cm and width 26.5 cm) that had been purged with argon. The height of the argon was determined by slowly lowering a flame from a Bic™ lighter into the box until the flame was extinguished. This approach ensured the Nalgene™ bottle was isolated from atmospheric oxygen. Boiled LN was captured and measured for oxygen concentration in a series identical to the first and second aliquots.

We measured oxygen concentration from unenriched LN obtained from a full 18 L LN laboratory tank. It was noted that this LN tank, prior to it being filled with fresh LN, was acclimated to room temperature. From this, two 100 mL aliquots of LN were measured for oxygen concentration in corresponding order of fill.

LN was enriched with oxygen by two methods. Both methods involved placing the opening of a PVC tube (approx. 3 mm O.D.) from an oxygen source to 7 cm below the surface of a container of LN. A fine stream of oxygen bubbles served to introduce the LO into the LN. In the first method, gaseous oxygen was bubbled into 1 L of LN for 8 min and 100 mL of this was assayed for oxygen. The second method streamed oxygen gas for 12 min into 2.1 L of LN, of this, 1.6 L was used in a failed attempt to make SN, and then 100 mL of this was assayed for oxygen concentration.

Visual comparisons were made between 1.6 L of unenriched LN and oxygen enriched LN during SN production. Unenriched LN or LN enriched with oxygen (obtained from the second method described above for enriching fresh LN with oxygen) was placed into a Dewar (15 cm I.D., 17 cm O.D., 33 cm height) and then placed into a vacuum chamber. A Hitachi vacuum pump (model

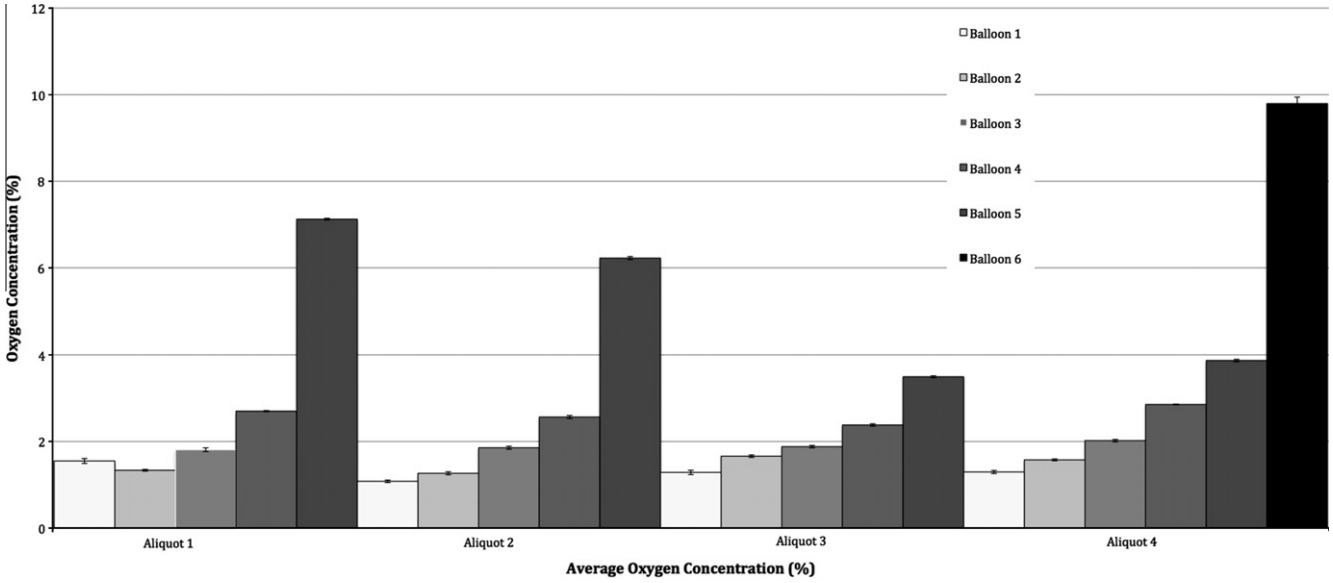

Fig. 1. Average percent oxygen concentration from the suspected O_2 contaminated LN tank for four aliquots consisting of five–six balloons each. Aliquots 1 and 2 are from the balloons filled and measured in sequence for O_2 from balloons one through five. Aliquots 3 and 4 are from shielding the Nalgene bottle with argon gas and assayed same as Aliquots 1 and 2. Error bars represent standard deviation for oxygen measurements at 60 s and 120 s.