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Biopreservation: The Impact of Freezing and Cold Storage on Sample Quality

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Executive Summary

A widely discussed topic in biological applications is the impact of the sample freezing process and the final holding and storage temperatures on the stability of biological samples. In order to preserve biological material in its optimal condition, a slow freezing process with storage temperatures held to the absolute practical minimum is essential. While these guidelines are generally recognized, the rationale behind this process is rarely elaborated. The goal of this whitepaper is to provide background on the scientific basis of these manipulations and its impact on the viability of the biological material.

Introduction

The sample storage process, often referred to as biopreservation, consists of four stages: preparation, cooling, storage and thawing. These stages are intimately linked via the "journey of deepening hypothermic stress experienced by a cell known as the Hypothermic Continuum" wherein events occurring during one stage impact adjoining stages, both up and down stream[1]. The ultimate goal of biopreservation is to place a sample into a state of suspended animation allowing for its retrieval in a fully functional condition. While highly effective for a variety of sample types including cells, tissues, bacteria, viruses and isolated subcellular components (organelles, DNA, RNA, proteins), the process may have negative consequences. These fall within two categories: 1) physiochemo-osmometric and 2) biochemical/molecular[2,3]. Ultimately, the results of these actions are seen following thawing when downstream analysis reveals sample quality degradation. In cell or tissue samples, this is seen as a reduction in yield or viability, function, reproductive capacity and in the induction of delayed apoptosis and necrosis. In bacteria, viruses, sub-cellular organelles, DNA, RNA or protein samples, decreases in quantity, quality, purity, etc. are often the result of sub optimal biopreservation.

Physical Impact of Freezing

All biopreservation entails reducing the temperature of a sample from normothermic temperatures (typically 37 °C) to a final storage temperature[1,3]. Immediately prior to the cooling phase, samples are placed into a medium containing a cryoprotective agent (DMSO, glycerol, ethylene glycol, sugars, etc.) and maintained at approximately 4 °C for several minutes (<30 mins) to allow for equilibration. Sample cooling is accomplished at a controlled rate of approximately -1 °C/min in order to obtain optimal preservation of mammalian cells[4]. For bacteria, viruses and isolated sub-cellular components (organelles, DNA, RNA and proteins), controlled rate cooling is less of a concern. Controlled rate cooling is utilized to minimize volume excursions and allow for water efflux from a cell into the extracellular environment[4-7]. This results in cellular dehydration and freeze concentration of intracellular solutes (salts, ions, cryoprotective agents, etc.) and reduces the probability of ice formation inside a cell. Cellular dehydration continues until the temperature decreases below the glass transition temperature (Tg), the point at which the sample transitions to a glassy state (Tg of pure water is approximately -135 °C)[8].

While cellular dehydration is highly beneficial in reducing the probability of intracellular ice formation, when samples are stored at temperatures above Tg, dehydration continues throughout the storage interval resulting in increased damage. As samples continue to dehydrate, structural damage to membranes, organelles, proteins and nucleic acids occurs[1]. This damage can be detrimental to samples in several ways including activation of delayed molecular-based repair or degradation pathways (apoptosis, necrosis, unfolded protein response (UPR), DNA repair, etc.) or if the damage is too severe it may result in sample loss immediately post-thaw[9].

Molecular Impact of Freezing

In addition to the physical impacts of freezing on samples there is also impact at the molecular level which is a result of several factors including 1) a decrease in available energy and metabolic activity, 2) uncoupling of biochemical pathways and 3) activation of stress response pathways[9,10]. During the storage process, the reduction in temperature results in a slowing of metabolism and biochemical pathways as a result of decreases in kinetic energy levels (a decrease of 10 °C equals a 3 % decrease in available thermal energy)[1]. For mammalian cells, a simple measure which represents an average of the total change in metabolism is Q_{10} equating to a decrease of approximately 50 % in metabolism (O_2 consumption) for every 10 °C drop in temperature (Q_{10} of 2)[11,12].

In essence, low temperature storage slows, but does not stop, all cellular biochemical reactions regardless of the temperature, unless below Tg. As biochemical processes and pathways are temperature dependent, even at -20 °C and -80 °C, biochemical reactions still occur resulting in continued damage to samples whether they are cells, sub-cellular components, DNA, RNA, protein, bacteria or viruses. These reactions are often unregulated and incomplete resulting in the formation and accumulation of toxic intermediate compounds, such as free radicals, anaerobic metabolism byproducts, waste products which cannot be cleared due to temperature suppression of the necessary salvage pathways or reactions. These byproducts can result in either direct damage or the activation of molecular-stress responses following thawing[10].

Further complicating the equation is that many of the stressors experienced by a sample, if not immediately lethal, can initiate molecular death or biochemical degradation cascades upon thawing. These factors include metabolic

uncoupling, free radical production, alterations in cell membrane structure and fluidity, disregulation of cellular ion balance, release of calcium from intracellular stores, osmotic fluxes, and cryoprotective agent exposure. For example, free radical production and accumulation during storage can directly damage DNA, protein and mitochondria integrity in cells and isolated samples[9,13,14]. In addition to physical impacts, free radical accumulation can also activate the UPR and apoptotic pathways resulting in further sample degradation[15]. In many cases, the accumulation of these sub-lethal stressors during biopreservation results in activation of apoptosis followed by a shift to secondary necrosis due to a lack of energy and continual "build up" of stressors upon thawing. The manifestation of this sub-lethal damage may not be evident immediately after thawing, but may take several hours to days to be detected[2,3].

Impact of Sample Storage Conditions

As described above, damage during storage continues at both the physical and molecular level. Physically, samples continue to undergo freezing induced dehydration and solute concentration resulting in continued and deepening damage. Biochemically, reactions continue, although at a highly reduced rate. Based on a Q_{10} of 2, O_2 consumption continues at an estimated rate of 6 % at 4 °C, 1.6 % at -20 °C, 0.5 % at -40 °C, 0.05 % at -70 °C, and 0.02 % at -80 °C compared to 37 °C (100 %). Theoretically, this continues until Tg is reached at which point all biochemical reactions halt. While highly reduced, over long term storage this biochemical activity is significant. For instance, from an energy and/or O_2 consumption calculation perspective, storage at -80 °C for 40 days is equivalent to 1 day at 37 °C (1 year at -80 °C is equivalent to approximately 9 days at 37 °C). Importantly, temperature based suppression of biochemical activity is not universal as individual pathways, processes and reactions respond differently[16]. As such, while highly suppressed, biochemical processes continue but in an uncoupled and unregulated manner thereby resulting in continual sample degradation. These stress events become amplified in samples as storage temperatures and intervals increase.

Specific responses of individual pathways, processes and reactions to extended storage are not well understood, however it is well accepted that storage temperature has a significant impact on the time samples can be held before significant compromise is noted[17,18]. For instance, in mammalian cells reports of hold times at a given temperature range from less than a week at 4 °C; to less than a month at -20 °C; to 6-12 months at -70 °C and -80 °C. Storage in liquid nitrogen at -196 °C effectively prevents all thermally driven chemical reactions, and only activities driven by ionizing radiation are operable at this temperature. It is estimated that thousands of years would elapse before a measurable effect on cryopreserved cultures could be noted[19,20]. For bacteria, viruses, DNA, RNA and protein samples, storage intervals of 1 – 2 years (or longer) at -80 °C have been reported to be successful. It is important to note that these hold times are for reference only and should be determined for individual samples by examining appropriate parameters specific to the intended end use following thawing. Further, these intervals are based on stable holding at the final temperature. Repeated freeze thawing of any sample stored at any temperature will result in accelerated sample degradation.

Summary

The combination of continued physical, biochemical and molecular responses of samples to the freezing process greatly impacts their quality. Observations of reduced yield, viability, function, and reproductive capacity are often found in cell, bacteria and virus samples whereas decreases in quantity, quality and reactivity are common in DNA, RNA and protein samples. Assuming samples are thawed according to established protocols, this deterioration in quality is a direct result of the stress and degradation during freezing and storage. As a result of the ongoing damaging events that samples experience during storage, the longer a sample is stored and the higher the storage temperatures, the greater the degree of sample degradation. For long term storage of samples, retention at the lowest feasible temperature is preferred. Optimal storage is realized in liquid nitrogen, where, at -196 °C all biochemical reactions cease.

Given the complexity of events which occur during the routine process of freezing, it is important to be aware of the impact that sample preparation, cooling, storage temperature, storage time, and warming have on overall sample quality. The benefits of freezing and storage at a given temperature needs to be weighed against the ultimate storage goal (time, end use, sample complexity, etc.) for an individual sample.

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