Review

Towards storage of cells and gametes in dry form

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We review published data on cell/gamete lyophilization. Most studies have utilized the same established protocols for cryopreservation (storage in liquid nitrogen) as for cell lyophilization (dehydration of frozen samples by water sublimation). Surveying natural lyoprotectants, we suggest trehalose and late embryogenesis abundant (LEA) proteins as ideal candidates for the reversible desiccation of mammalian cells/gametes. We find that despite the numerous water subtraction techniques, scientists have relied almost exclusively on lyophilization. There is thus room for improvement in both medium formulation and water subtraction strategies for dry cell/gamete storage. We believe the development of dry processing protocols for use in biobanks of cells/gametes, at reduced cost and with minimal carbon footprint, is within our grasp.

Introduction

The demand for long-term eukaryotic cell storage methods emerged in the first half of the 20th century once experimentalists began successfully exploiting spermatozoa and cells for medicine, research, and animal breeding. Initially, the options for storage were freezing, vitrification, and freeze drying (i.e., lyophilization) [1–3]. For the difficulties to maintain structural integrity in dry cells, lyophilization was partially dropped, whereas freezing was developed further; helped also by technological advances in mechanical freezer development, coupled with the availability of liquid nitrogen (LN). LN was first liquefied by two Polish physicists, Wroblewski and Olszewski in 1883 [4], and was widely used after World War II, becoming a key element in early cryobiology. The route to successful cryopreservation was far from straight and owes a great deal to serendipity. The first successful cryopreservation of spermatozoa [5] was accomplished with a bottle of sucrose – the common cryoprotectant in vogue at that time – contaminated with glycerol. The accidental discovery of glycerol and its analogs as the most powerful cryoprotectants paved the way for deep-freezing cells in LN. The science of cryobiology started in 1940 and laid down all physical/chemical requirements enabling a cell to withstand reversibly the long-term exposure at subzero temperatures. A common feature of all cryopreservation protocols is the addition of a suitable permeable cryoprotectants, for example, glycerol or ethylene glycol, to the freezing medium. The cryoprotectant binds to intracellular water, thus reducing intracellular damage. Then the sample undergoes controlled cooling in order to induce progressive dehydration prior to being plunged into LN. The dehydration is essential, because it avoids/reduces intracellular ice injury. The search for avoiding intracellular ice formation resulted in the evolution of the freezing paradigms, which is now named vitrification. Vitrification relies on the ability of highly concentrated solutions of cryoprotectants to supercool to low temperatures, forming a glassy matrix without the formation of ice [6]. Since the first report, there has been massive investment in vitrification research, which has now become the first cryostorage choice for human oocytes and embryos [7,8]. Overall, the current freezing protocols are straightforward and efficient, with a good recovery rate at thawing [9], but they are not devoid of problems.

Liquid nitrogen storage is expensive, requiring continuous monitoring of its levels and supply [9]; it is inconvenient, necessitating dedicated facilities and equipment, especially for many developing countries; it is potentially dangerous for the operator and can become contaminated by viruses or other pathogens [10]; and finally, it makes the shipping of samples difficult (Table 1). Aside from these technical and practical inconveniences, LN storage also poses an environmental concern, given that its industrial production and the maintenance of its storage centers have a high carbon footprint. Given the increasing demand of biobanks for various purposes, including medicine, research, pharmaceutical endeavors, and biodiversity preservation, there is a pressing need to develop alternative storage options. In this review we critically analyze the data published on dry preservation of mammalian cells and gametes; surveying all organisms undergoing desiccation in their life cycle; listing the naturally available lyoprotectant molecules; and finally, we speculate about their potential use for dry storage of cells and gametes.

Freeze-dried spermatozoa

Following the successful lyophilization of spermatozoa [5], in the 1950s, some attempts were made to lyophilize red blood cells and platelets [11,12]. Although drying red blood cells proved unsuccessful, the lyophilized platelets nevertheless preserved the morphology and function of the

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Table 1. Pros and cons of cryoconservation versus lyophilization storage

Method	Cryoconservation	Lyophilization
Ease/practicality	+	+++++
Specialized equipment	++++	+++++
Cost for long-term storage	+++++	+
Hazard	+++++	+
Pathogen contamination	+++++	+

cells [13]. Why were nucleated cells or cell particles such as platelets not the first targets for lyophilization trials? We speculate that there were at least two reasons; one practical and one biological. In practical terms, freeze-dried blood would simplify the storage and transport of blood banks enormously. Biologically, red blood cells and platelets are devoid of nuclei, and it is likely that early 'lyobiologists' feared that the nuclear structure would be incompatible with dry storage.

At the end of 1998, the cell and gamete storage paradigm shifted, when Wakayama and Yanagimachi demonstrated that lyophilized mouse spermatozoa, stored at room temperature for 3 months, were able to generate normal offspring [14]. The spermatozoa were nonviable and motionless at rehydration, but the handicap of the lost motility was easily overcome by their direct injection into the oocytes. These findings demonstrated for the first time that nuclear and cellular viability are not equivalent. The lyophilized spermatozoa were essentially dead, yet were still able to interact with the programming machinery of the oocytes, giving rise to normal pups. This discovery was an important milestone; the fertilizing ability of lyophilized spermatozoa has since been confirmed in other species including horses [15], dogs [16], rats [17], cattle [18], pigs [19], rabbits [20], marsupials [21], and humans [22].

Other experiments, performed after the publication of Wakayama and Yanagimachi's data, demonstrated that somatic cells subjected to thermal stress (55°C and 75°C) for 30 min, and obviously killed by the treatment, were capable of generating normal lambs upon transfer into enucleated sheep oocytes [23]. Hence, it was established that cell viability and nuclear viability are likewise not equivalent in somatic cells. Soon after, sheep somatic cells – granulosa cells and lymphocytes – lyophilized with the addition of trehalose in the freezing medium in Israel, were shipped by ordinary mail to Italy. The lyophilized cells were kept on a shelf in a cardboard box and used 5 years later for nuclear transfer. Surprisingly, the reconstructed embryos developed to the blastocyst stage [24]. Subsequent work reproduced these results in mice [25] and pigs [26]. The current list of successful nuclear transfers of lyophilized cells includes sheep, pigs, mice, and cattle (Japanese Brown Cattle; Matzukawa, personal communication). Thus, multiple research teams have shown that lyophilized cells can be successfully reprogrammed after nuclear transfer and can develop until the blastocyst stage. The next step would be to demonstrate that these cloned embryos can develop into normal offspring, and experiments to that end are currently ongoing.

The above results prompted scientists to look beyond LN for the storage of cells. Two independent research groups reported the successful dry storage of human mesenchymal stem cells and cord blood cells. In both cases, the cells maintained the capacity to form colonies under appropriated conditions *in vitro* [27,28]. The findings are significant in showing that dry storage of cells and gametes is a viable option.

We are clearly in the early stages of dry storage of cells and gametes, and there is a large margin for improvement. For one, if we exclude the addition of trehalose, the freezing protocol we used in our original study [24], but also in those leading to successful dry storage of spermatozoa [14], is even more primitive that those used for drying yeast. The cells and spermatozoa were suspended in small aliquots (200 μl) of standard buffered medium, snap-frozen in LN, and water-extracted with a conventional lyophilizer. Yeast cells are instead lyophilized efficiently using a combination of sugars and yeast extracts, which probably exert a bulk effect analogous to the egg yolk in sperm freezing medium. None of these ingredients have ever been used for cell lyophilization.

It appears likely that improvements in the composition of lyophilization media will improve the dry storage of cells and gametes overall, but there is also room for improvement in the drying procedures. Is lyophilization the only way, or are alternative options also worthy of exploration?

Selection of ideal lyoprotectants

The discovery of the protective action of glycerol ensured the success of deep-freezing gametes and cells [4]. Since then, other cryoprotectants have been identified and optimal cell type–cryoprotectant combinations have been established. We must do the same for dry cell and gamete storage, starting with the individuation and selection of the best lyoprotectants.

This is particularly important for eukaryotic cells and gametes. Mammals are sensitive to water loss, and dehydration can be life-threatening; therefore, a coordinated physiological pathway keeps water loss to a minimum. Eukaryotic cells sense desiccation and respond by increasing stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 mitogen-activated protein kinases (MAPKs [29]); a general protective pathway activated by a wide range of stimuli. However, even if the cells sense water loss, the cellular response protects cells from an increase in ionic concentration and osmotic stress, and not water loss [29].

The sugar trehalose was the first lyoprotectant; its effectiveness was shown by Crowe *et al*. in platelet-drying studies, following the observation that many anhydrobiotes accumulate the sugar when drying [13]. The protective effects of trehalose and the underlying mechanisms have been described in many authoritative reviews [30]. The induced expression of trehalose in cultured cells confers a partial desiccation tolerance in fibroblasts [31,32]. However, whether the cells can resume growing following rehydration is unclear. Trehalose has also been used as a cryoprotectant, for oocyte freezing [33], but it is during drying that trehalose is most helpful. In a recent study, *Caenorhabditis elegans* was engineered to express the sugar. Only trehalose-expressing dauer larvae survived dehydration, whereas controls did not [34]. Even in our