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Desiccation tolerance in bovine sperm: A study of the effect of intracellular sugars and the supplemental roles of an antioxidant and a chelator ☆

Ranjan Sitaula [a,c], Heidi Elmoazzen [c], Mehmet Toner [c], Sankha Bhowmick [a,b,c,*]

[a] Biomedical Engineering and Biotechnology Program, University of Massachusetts Dartmouth, N. Dartmouth, MA, USA
[b] Department of Mechanical Engineering, University of Massachusetts Dartmouth, N. Dartmouth, MA, USA
[c] Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School and Shriners Hospital for Children, Boston, MA, USA

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ABSTRACT

Desiccation preservation holds promise as a simplified alternative to cryopreservation for the long term storage of cells. We report a study on the protective effects of intracellular and extracellular sugars during bovine sperm desiccation and the supplemental effects of the addition of an antioxidant (catalase) or a chelator (desferal). The goal of the study was to preserve mammalian sperm in a partially or completely desiccated state. Sperm loaded intracellularly with two different types of sugars, trehalose or sucrose, were dried with and without catalase and desferal and evaluated for motility and membrane integrity immediately after rehydration. Intracellular sugars were loaded using ATP induced poration. Drying was performed in desiccator boxes maintained at 11% relative humidity (RH). Results indicated that sperm exhibited improved desiccation tolerance if they were loaded with either intracellular trehalose or sucrose. Survival was further enhanced by the addition of 1 mM desferal to the desiccation buffer. Though sperm motility after drying to low dry basis water fractions (DBWF) did not show significant improvement under any of the tested conditions, there was an increase in the sperm membrane integrity that could be retained after partial desiccation through the use of intracellular sugars and desferal.

Introduction

Preservation of sperm for extended time periods has widespread applications in the fields of medicine, scientific research and in the dairy industry [24,25,44]. Cryopreservation, which has been the standard technique for the long-term preservation of spermatozoa, poses logistical and handling difficulties due to the necessity of storage in liquid nitrogen [24,37]. Desiccation preservation offers an attractive alternative to the cryopreservation technique. The method, which mimics a naturally occurring phenomenon called anhydrobiosis, uses disaccharides, such as trehalose and sucrose, to protect biologics and cells in a dehydrated state [7–10]. The potential for molecular immobilization without disruption or conformational changes to the structure of the biological system is the key rationale for using sugars in preservation of biological systems [29]. As water is removed, sugars form a viscous matrix that reduces molecular mobility [10,19]. Anhydrobiotic engineering of mammalian cells requires that sugars be present on both sides of the plasma membrane in order to afford significant desiccation tolerance [16,1,6,45,11,15]. Therefore, an appropriate quantity of sugar must be loaded through the sperm plasma membrane. Various techniques have been used to load sugars into the cells [16,1,6,45,11]. These methods include thermal poration [23], electroporation [42], ATP poration [15] and the use of α-hemolysin to create transient non selective pores in cell membranes [16,1].

Though desiccation preservation offers an attractive alternative to cryopreservation, there has been limited success in achieving dry storage of sperm using intracellular sugars [24,37]. Genetic integrity has been successfully preserved, but motility has not [4,34,30]. Although reconstituted freeze-dried spermatozoa can be used to fertilize mammalian oocytes by using intracytoplasmic sperm injection (ICSI) [25,26,46], little success has been achieved in preserving motility and membrane integrity after recovery from dehydrated states. After the successful recovery of fowl sperm post desiccation by Polge et al. [39], there has been a limited success in recovering motile sperm [40,36,47,28]. Previous studies have added glycerol to egg yolk–citrate buffer and then performed various freeze-drying protocols. Post rehydration motility has consistently been lost for sperm dried to moisture content fell below 10% with no storage survival. Studies without cytotoxic glycerol in the freeze-drying media have also shown similar trends [18].

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* Corresponding author. Address: 285 Old Westport Road, Room # Textile 210, N. Dartmouth, MA 02747, USA.
E-mail address: sbhowmick@umassd.edu (S. Bhowmick).

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The role played by metabolic arrestors like antioxidants and chelators at low water contents is largely unexplored in the literature. A major factor contributing to the disruption of cellular function in sperm during preservation at near ambient conditions is the formation of Reactive Oxygen Species (ROS) [4,14,43], which include small peroxides, super oxide anions and hydroxyl free radicals [43,3,2,13,27]. Studies have shown that there is a higher production of these free radicals at higher storage temperatures than in the cryopreserved state [43]. These molecules combine with cellular components and transform into oxygen based damage products. Plasma membranes in sperm have been found to be highly sensitive to peroxidation. Lipid peroxidation causes sperm plasma membranes to be leaky and that leads to a complete loss of motility and membrane integrity [13].

The goal of the current study was to investigate the protective effects of intracellular and extracellular sugars during bovine sperm desiccation, and the supplemental effects of the addition of an antioxidant (catalase) and a chelator (desferal, an iron chelator). The addition of sugars was based on the hypothesis that their presence in an optimal concentration would minimize the deleterious effects of dehydration stresses and osmotic shock during drying. Antioxidants and chelators were hypothesized to help reduce the oxidative stresses, thereby minimizing membrane and mitochondrial damage during desiccation. While antioxidants counteract the formation of peroxide, chelators inhibit the formation of free metal ions, thereby minimizing the production of hydroxyl radicals [43]. Trehalose and sucrose were selected as protectants because of the protective effects they have been shown to have in other desiccation preservation studies of biologics and mammalian cells [16,1,6,45,11,15]. Catalase and desferal were chosen as antioxidants because their use as ROS scavengers has been reported often in the sperm literature [3,2].

Materials and methods

Semen shipment

Fresh bovine semen samples from Holstein bulls (aged between 2 and 3 years) were shipped every week from ABS Global, WI, via United Parcel Service (UPS) next day service. The sperm were diluted in a 1:2 ratio with Egg Yolk Tris extender (EYT) and placed in a 15 ml conical tube. EYT was composed of 20% egg yolk (by volume), 2.42 weight% tris (hydroxymethyl aminomethane), 1.38 weight% citric acid monohydrate, and 1.0 weight% fructose. During shipment, the tube was packed in a foam brick refrigerant which maintained the temperature at 4 °C. The sample was stored in a refrigerator at 4 °C upon arrival. Progressive motility of sperm was checked immediately on arrival and was typically in the range of 80–85% and the membrane integrity was over 90%. The concentration of sperm in the semen sample was about 1000 million/ml. To ensure that there was no loss in cell function over time, all the experiments were performed within 4 days of sperm delivery. During this stored period, the motility and membrane integrity did not fall below 65% and 80%, respectively.

Desiccation buffer

For the drying experiments, all samples were suspended in Tyrode based buffer [31] (composition in Table 1) with and without the additives. Except for one control set, which consisted of sperm suspended in plain Tyrode buffer (250 mosm), all the desiccation buffers were isotonic Tyrode buffers (325 mosm) with 0.2 M extracellular trehalose or sucrose. For experiments with desferal and catalase, the additives were added to the desiccation buffer. Concentrations of 1 mM desferal and 0.05 mg/ml catalse were used. Thus, the different buffer compositions included (i) Tyrode buffer (ii) Tyrode + extracelluar sugars, (iii) Tyrode + extracelluar sugar + desferal (iv) Tyrode + extracelluar sugar + catalase (v) Tyrode + extracellular sugar + desferal + catalase.

Table 1
Composition of Tyrode buffer.

Composition	g/l
NaCl	5.69
KCl	0.23
$CaCl_2 \cdot 2H_2O$	0.29
$MgCl_2 \cdot 6H_2O$	0.08
$NaHCO_3$	2.09
NaH_2PO_4	0.04
Glucose	0.9
HEPES	2.3

Loading intracellular sugar

An ATP poration method was used to load sugars into sperm. This poration technique works on the principle that the addition of a tetra-ionic form of ATP (ATP^{4-}) induces ion fluxes across the membrane and causes the formation of non-selective pores in cells with purinergic $P2X_7$ receptors [15]. It has been reported in the literature that bovine sperm membranes contain purinergic receptors [33,35]. The poration buffer was a Tyrode based isotonic buffer with 0.2 M trehalose (or sucrose) and 5 mM ATP disodium salt (Sigma, catalogue # A7699) with an osmolality of 325 mosm. The poration protocol consisted of suspending bovine sperm in room temperature (25 °C) poration buffer at a concentration of approximately 50 million sperm/ml. The sample was then incubated at 37 °C for 40 min to allow for sugar uptake. To achieve pore closure, the sample was diluted by a factor of 10 in a dilution buffer which was composed of Tyrode buffer containing 0.2 M sugar (trehalose or sucrose, depending upon the sugar loaded) [15].

Measurement of intracellular sugar

Measurement of intracellular sugars using a High Performance Liquid Chromatography (HPLC) analysis was performed in an Agilent 1100 series system as described by Eroglu and coworkers [16]. Sperm loaded with trehalose or sucrose were washed thrice in Phosphate Buffered Saline (PBS) to remove extracellular sugar. After the final wash and subsequent centrifugation, the cells were pelleted at the bottom of the eppendorf tubes and were resuspended in HPLC grade water. The samples were then frozen and stored at −20 °C until further analysis. For further analysis, the samples were heated at 95–100 °C for about 20 min. Pressure was periodically released by opening the caps of the eppendorf tubes. At the end of heating, the suspension was allowed to cool to room temperature and it was then centrifuged at 4 °C to pellet the cell debris. The supernatant was aspirated and transferred into a 2 ml HPLC glass vial. The solution was then subjected to HPLC measurement to calculate the sugar concentration. Samples were held at 4 °C in a thermostatted autosampler compartment and the sugars were separated using an ion exchange column held at 35 °C. The area of the sample peaks detected with an electrochemical detector was used to calculate the intracellular concentrations of sugars. Quantification was done by comparison to calibration curves for trehalose and sucrose that were generated using similarly prepared standard solutions of known concentrations.

Drying method

For all drying experiments, 20 µl samples were placed on O – ring glass slides (Fisher Scientific, Cat. # 22–339–408) inside 10 mm etched circles. All drying experiments were conducted at