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Osmotic tolerance and intracellular ion concentrations of bovine sperm are affected by cryopreservation

A.-K. Blässe[a], H. Oldenhof[b,*], M. Ekhlasi-Hundrieser[a,1], W.F. Wolkers[c], H. Sieme[b], H. Bollwein[a,2]

[a] *Clinic for Cattle, University of Veterinary Medicine Hannover, Hannover, Germany*
[b] *Clinic for Horses - Unit for Reproductive Medicine, University of Veterinary Medicine Hannover, Hannover, Germany*
[c] *Institute of Multiphase Processes, Leibniz Universität Hannover, Hannover, Germany*

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Abstract

In this study, the effects of cryopreservation on osmoregulation and ion homeostasis in bovine sperm were studied. We determined: (1) the osmotic tolerance limits and cell volume response upon exposure to anisotonic conditions, (2) the intracellular pH and potassium concentration, and (3) expression and localization of proteins encoding for potassium and chloride ion channels. A flow cytometric approach was used for simultaneous assessment of cell volume and viability of propidium iodide stained sperm in anisotonic media. Osmotic tolerance was found to be decreased after cryopreservation, especially in the 120 to 60 mOsm/kg osmotic range. The critical osmolality at which half of the sperm population survived increased from 55 to 89 mOsm/kg. The osmotic cell volume response for viable sperm was similar before and after cryopreservation, with an osmotic inactive volume of about 70%. The intracellular pH, determined by recording changes in carboxyfluorescein fluorescence of sperm in media with different pH before and after addition of digitonin, decreased from 6.28 in diluted sperm to 6.16 after cryopreservation. The intracellular potassium concentration, determined using the potassium ionophore nigericin and incubation in media with various potassium concentrations, increased from 154 mM to 183 mM before and after cryopreservation, respectively. The levels of the chloride and potassium ion channel proteins chloride channel 3 protein (CLC-3) and two pore domain potassium channel 2 protein (TASK-2), as detected using Western blot analysis, were not affected by cryopreservation. Immunolocalization studies showed that CLC-3 is present in the acrosome and midpiece as well as in the upper and lower tail. In conclusion, cryopreserved sperm exhibit reduced tolerance to hypotonic stress, a decreased intracellular pH, and increased intracellular potassium level.

Keywords: Bull spermatozoa; Cell volume; Chloride channels; Cytosolic K^+ concentration; Cytosolic pH; Membrane integrity; Potassium channels

1. Introduction

Artificial insemination in dairy cattle breeding is predominantly performed using cryopreserved bovine sperm. During cryopreservation, sperm are exposed to cooling, ice crystal formation, oxidative stress, and osmotic stress [1,2]. These stresses may cause cell damage and result in reduced viability after thawing. Sperm are exposed to

* Corresponding author. Tel.: +49 (0)511 953 8530; fax: +49 (0)511 953 82 8530.
E-mail address: harriette.oldenhof@tiho-hannover.de (H. Oldenhof).
[1] Present address: Werlhof-Institute, Hannover, Germany.
[2] Present address: Clinic of Reproductive Medicine, University of Zurich, Zürich, Switzerland.

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hypertonic stress both during the addition of cryoprotective agents and during subsequent freezing, whereas during thawing cells are exposed to hypotonic conditions [1,3]. Exposure of sperm to changes in the extracellular solute concentration leads to transport of water and solutes, including ions into or out of the cell, until reaching equilibrium between the extra- and intracellular environment. Sperm have osmotic tolerance limits above and below which cell death occurs [4]. Osmoregulation involves water transport via passive diffusion through the phospholipid bilayer or through water channel proteins, such as aquaporins. Ion homeostasis is achieved by active transport involving different ion channel proteins [5]. In sperm, the ion channel proteins two pore domain potassium channel 2 protein (TASK-2), volume-dependent potassium channel protein (Kv1.5) and slow voltage-gated potassium channel protein (minK) have been identified to play a role in potassium homeostasis [6–8], while chloride channel 3 protein (CLC-3) and nucleotide-sensitive chloride channel protein (ICln) have been implicated [9,10].

Ion channel proteins play an important role in regulating the intracellular pH and ion concentrations in response to various stimuli. Upon capacitation, for example, the intracellular pH and the permeability of the plasma membrane for potassium ions increases, which leads to alkalization and a decrease in the intracellular potassium concentration. This in turn results in a hyperpolarization of the plasma membrane. These changes have been implicated to regulate the increase in intracellular calcium and release of calcium from the acrosome [11]. In sperm, the intracellular pH is regulated via a specific Na^+/H^+ exchanger that is driven by the Na^+/K^+-ATPase generated sodium gradient [12]. The potassium gradient and the resting membrane potential are predominantly maintained via Na^+/K^+-ATPase activity and low permeability of the plasma membrane for potassium [13,14].

In this study, the effects of cryopreservation on osmoregulation and ion homeostasis in bovine sperm have been investigated. We have determined: (1) the osmotic tolerance limits and cell volume response upon exposure to anisotonic conditions, (2) the intracellular pH and potassium concentration, and (3) expression and localization of proteins encoding for potassium and chloride ion channels.

2. Materials and methods

2.1. Semen collection, dilution, and cryopreservation

Semen was collected from six Holstein Friesian bulls that were held at the Clinic for Cattle of the University of Veterinary Medicine, Hannover, Germany. Semen was collected using an artificial vagina (Model Hanover, Minitüb, Tiefenbach, Germany), and a dummy or bull. The volume of the ejaculate, sperm concentration, motility, and morphology were evaluated, and raw semen was diluted to 60×10^6 cells per mL by direct addition of Andromed extender supplemented with or without glycerol (Minitüb). The extender was prewarmed at 37 °C before use. Ejaculates that had more than 900×10^6 sperm per mL, with more than 70% progressively motile sperm and less than 20% morphologically abnormal sperm were used for experiments.

For standard cryopreservation, semen was diluted and cryopreserved using Andromed freezing extender containing about 7% glycerol (Minitüb). In parallel, for assessment of the intracellular pH and potassium concentration without interfering commercial extender components, sperm was diluted and cryopreserved using skim milk extender [15]. Skim milk extender exists of: 0.15% skim milk, 25 g/L glucose monohydrate, 1.5 g/L lactose monohydrate, 1.5 g/L raffinose pentahydrate, 0.4 g/L potassium citrate monoydrate, 0.3 g/L sodium citrate dihydrate, 4.76 g HEPES, 500 mg/L penicillin, and 500 mg/L gentamicin. After dilution, samples were equilibrated for 24 h at 4 °C, after which they were packed into 0.25-mL straws and cooled down to −150 °C with 60 °C per min using a programmed rate freezer (IceCube 14S–B, Minitüb). Straws were stored in liquid nitrogen for at least 24 h and thawed by incubation for 30 sec in a water bath of 37 °C.

2.2. Flow cytometric assessment of sperm volume and plasma membrane integrity in (an)isotonic media

Simultaneous measurement of sperm volume and viability was done using a flow cytometer (Cell Lab Quanta SC, Beckmann Coulter, Krefeld, Germany) as previously described [16]. These measurements were done using twelve ejaculates from six bulls. Osmotic tolerance was assessed as impermeability of the sperm plasma membrane for propidium iodide (PI) upon dilution into (an)isotonic saline solutions. HEPES buffered saline solution of 300 mOsm/kg (HBS; 137 mM NaCl, 20 mM HEPES, 10 mM glucose, 2.5 mM KOH, pH 7.4) was used as isotonic medium. Hypotonic solutions were prepared by dilution of HBS with water, hypertonic solutions by addition of NaCl. For assessment of osmotic tolerance limits, before and after cryopreservation, sperm diluted in freezing extender was used. For assessment of the volume response upon exposure to (an)isotonic media prior to cryopreserva-