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Mitochondrial oxygen consumption is a unique indicator of stallion spermatozoal health and varies with cryopreservation media

Christa R. Darr [a], Gino A. Cortopassi [b], Sandipan Datta [b], Dickson D. Varner [c,d], Stuart A. Meyers [a,*]

[a] Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, California, USA
[b] Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California, USA
[c] Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, USA
[d] Veterinary Medical Teaching Hospital, Texas A&M University, College Station, Texas, USA

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ABSTRACT

Mitochondrial oxygen consumption is a sensitive indicator of spermatozoal health in the context of cryopreservation. We investigated oxygen consumption of equine sperm mitochondria during incubation in four commercially available sperm cryopreservation extenders: modified INRA 96, BotuCrio, EZ Freezin–"LE" and "MFR5", in addition to several other parameters including motility, reactive oxygen species (ROS) production and viability. All experimental endpoints, with the exception of average path velocity, were affected significantly by freezing extender type after freezing and thawing. Sperm in INRA 96 had the lowest average progressive motility after thawing (24 ± 4.8%, $P < 0.05$). Sperm in EZ Freezin–"LE" had the highest post thaw viability (79 ± 3.1%, $P < 0.05$) and lowest post thaw ROS production (13 ± 2.4%), but sperm in BotuCrio had the highest maximal oxygen consumption levels, while also demonstrating similar ROS production and viability. This difference would not have been detected using conventional sperm analytical methods. In addition, sperm in BotuCrio had the highest average total motility (49 ± 7.4%), progressive motility (41 ± 6.4%), and velocity (VAP, 90 ± 3.6 μm/s) indicating that this medium preserved mitochondrial function optimally after cryopreservation. Mitochondrial oxygen consumption was positively correlated with traditional measures of sperm function including motility and viability ($r = 0.62$ and $r = 0.49$, respectively, $P < 0.05$), thus making it a sensitive method for determining cryopreservation success and mitochondrial function in stallion sperm.

1. Introduction

It is well-known that the process of cryopreservation causes damage to sperm in three specific ways: cold shock, oxidative stress through reactive oxygen species (ROS) production, and osmotic shock [1–4]. Cellular functions shown to decrease after freezing include sperm viability, mitochondrial membrane potential, and motility. Osmotic shock is one of the most harmful insults to sperm function. Sperm undergoing osmoregulation, even without changes in temperature, have prominent changes in plasma membrane integrity and motility and can only withstand deviations of approximately 100 mOsm/kg from isosmolal conditions (approximately 300 mOsm/kg for sperm)

* Corresponding author. Tel.: 530-752-9511; fax: 530-752-7690.
E-mail address: smeyers@ucdavis.edu (S.A. Meyers).

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without significant effects on viability and motility [5]. This appears to be a major problem in the process of sperm cryopreservation in stallions because most commercial media are at least 300 mOsm/kg greater than isosmolal levels for sperm (ranging 600–1200 mOsm/kg). In addition to the plasmalemmal and mitochondrial damage that occurs with osmotic shock, it has been demonstrated that superoxide production also increases in hyperosmolal solutions, further indicating the damage that sperm must endure before and during freezing and thawing [4,6].

Sperm motility generally decreases by 50% after a single freeze-thaw cycle [7]. The process of freezing additionally causes cryodamage morphologically, including marked swelling of the mitochondrial-rich sperm midpiece associated with the uncoupling of oxidative metabolism, increased ROS production, and increased apoptotic-like changes [8]. Mitochondrial oxidative function is absolutely essential for sperm motility [9] and could be an important determinant of spermatozoal viability and overall robustness. Because equine sperm motility depends on mitochondrial energy metabolism, mitochondrial damage during the cryopreservation process may be a major cause of decreased post thaw motility and sperm function. However, until recently, mitochondrial activity has been difficult to measure in high throughput for sperm.

Because mitochondria are a sensitive target for damage by freezing and because mitochondria are absolutely essential for spermatozoal function and viability, we decided to test whether the central parameter of mitochondrial function (mitochondrial oxygen consumption, MITOX), was correlated with other parameters of sperm viability (VIA), motility (MOT), and oxidative status (ROS). We also wanted to compare the four commercially available freeze extenders in their ability to preserve mitochondrial function before and after freezing. With this system, we were able to determine the effects of osmotic shock (before freezing) and the effects of cold shock (after freezing) on all parameters in each media. For these experiments, we used the BD oxygen biosensor system (BD Biosciences, Bedford, MA, USA), which allows for high-throughput analysis without the need for a closed chamber to measure oxygen consumption, as required for the commonly used but low throughput Clark electrode method [10,11].

2. Materials and methods

2.1. Semen collection

Stallions were maintained on a diet of mixed grass hay and grain, with fresh water ad libitum and daily exercise according to Institutional Animal Care and Use Committee protocols at the University of California. Single ejaculates from light breed resident stallions at the UC Davis Center for Equine Health and UC Davis Animal Science Horse Barn ($n = 5$) were used in this research and obtained through collection with a Missouri artificial vagina (Breeder's Choice, MI, USA) or Colorado artificial vagina (Animal Reproduction Systems, Chino, CA, USA) with an in-line nylon gel filter. Before mounting of the breeding phantom and semen collection, the stallion's penis was washed with warm water and sterile cotton and dried thoroughly. Ejaculates were processed immediately after collection.

2.2. Chemicals and reagents

All chemicals were purchased from Sigma–Aldrich (Saint Louis, MO, USA) unless otherwise indicated. EZ-Freezin "LE" and EZ-Freezin "MFR5" were purchased from Animal Reproduction Systems (Chino, CA, USA). INRA 96 was purchased from Breeder's Choice (Rochester Hills, MI, USA) and BotuCrio was purchased from BET Labs (Lexington, KY, USA).

2.3. Semen processing, freezing, and thawing

Gel-free sperm concentration was determined with a NucleoCounter SP-100 (ChemoMetec A/S, Allerød, Denmark), and neat semen was diluted 1:1 with pre-warmed (37 °C) INRA 96 extender (Breeder's Choice). No more than 10 mL of extended semen was placed in 15-mL conical-bottom tubes with 30 μL of SemSep equine cushion fluid (MOFA, Verona, WI, USA) placed under the sperm for centrifugation. Semen was centrifuged at $400 \times g$ for 20 minutes, the supernatant was aspirated, and the sperm pellet was then resuspended in each of four freezing extenders: (1) modified INRA 96 with added egg yolk (3%; v:v) and glycerol (2.5%; v:v; INRA), (2) BotuCrio, (BOTU), (3) E-Z Freezin "LE" (LE), and (4) E-Z Freezin "MFR5" (MFR). The freezing extenders were stored according to the manufacturer's instructions and thawed appropriately immediately before resuspension of sperm. The INRA was prepared by adding fresh egg yolk (commercially supplied) to the INRA 96 extender, then centrifuging at $10{,}000 \times g$ for 15 minutes. The supernatant was then filtered through a 0.45-μm filter, and glycerol was added. Sperm concentration in each media was adjusted to approximately 200×10^6 sperm/mL, as determined with a Nucleocounter SP-100. Aliquots of fresh sperm in each of the four freezing extenders were immediately analyzed for motility, ROS production, viability, and oxygen consumption. Sperm diluted in the four freezing media was loaded into 0.5-mL straws, sealed with polyvinyl chloride sealing powder, and subjected to a single standardized freezing protocol modified from Salazar et al. [12]. Briefly, loaded straws were placed in a controlled rate freezer (Planer Kryo 10 Series, Middlesex, UK) at room temperature and frozen according to the following freezing curve: −2.0 °C/min from 25 °C to 22 °C, −0.3 °C/min from 22 °C to 10 °C, −0.2 °C/min from 10 °C to 4 °C, −10.0 °C/min from 4 °C to −10 °C, and −17.0 °C/min from −10 °C to −110 °C. After completion of the freezing program, straws were plunged into liquid nitrogen and placed into canes with goblets for liquid nitrogen storage at −196 °C. For analysis of frozen sperm, five straws for each stallion in each freezing media were thawed in a 37 °C water bath for 30 seconds. Aliquots were then prepared for analysis of post thaw motility, ROS production, viability, and oxygen consumption.