Journal of Equine Veterinary Science 58 (2017) 51–57

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

The Effects of an Oxygen Scavenger and Coconut Water on Equine Sperm Cryopreservation

Kelly T. London [a], Bruce W. Christensen [a,*], Camilla J. Scott [a], Katie Klooster [b], Philip H. Kass [a], Ghislaine A. Dujovne [a], Stuart A. Meyers [b]

[a] Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA
[b] Department of Anatomy, Cell Biology, and Physiology, School of Veterinary Medicine, University of California, Davis, CA

ARTICLE INFO

Article history:
Received 17 February 2017
Received in revised form 14 August 2017
Accepted 14 August 2017
Available online 24 August 2017

Keywords:
Coconut water
Egg yolk
Sperm cryopreservation
Stallion sperm
Oxyrase

ABSTRACT

Alternative sources of lipoproteins in semen extenders could replace animal by-products. We hypothesized that: (1) post-thaw semen parameters and fertility would not be different in coconut water (CW)–treated samples compared with egg yolk (EY)–treated samples and (2) the use of an oxygen scavenger (Oxyrase) would improve post-thaw sperm motility and membrane integrity and decrease lipid peroxidation. Experiment 1: three ejaculates each from five stallions were split into four treatments: EY, CW, egg yolk with Oxyrase, and coconut water with Oxyrase. Computer-assisted sperm analysis measured progressive and total motility, velocity, and linearity. Membrane integrity, apoptosis, and lipid peroxidation were evaluated using propidium iodide, annexin, and BODIPY fluorescent probes, respectively. Samples were cryopreserved, stored in liquid nitrogen, and then thawed to 37°C and analyzed again. Experiment 2: one ejaculate was divided into two aliquots and cryopreserved using either CW or EY. In a crossover design, 12 mares were bred on two consecutive cycles with either EY or CW. Pregnancy evaluations were at 14-day gestation. No differences were detected in sperm parameters between CW and EY ($P > .05$). Oxyrase did not improve sperm motility parameters in post-thaw samples, nor did it show protective effects for viability or against membrane damage ($P > .05$). More mares became pregnant using CW than EY (11/12 vs. 6/12, respectively; $P = .013$). Use of CW is a viable alternative to animal-based products in the cryopreservation of stallion semen.

1. Introduction

Both physiological and logistical obstacles limit the widespread use of cryopreserved semen. On the logistical side, international laws governing the export and import of products containing animal-derived ingredients (such as milk or egg yolk [EY]) vary from country to country and limit the sharing of genetics through cryopreserved stallion semen. Milk and EY are thought to help preserve sperm viability by interacting with lipid-binding proteins (termed Binder of Sperm [BSP] proteins) present in seminal plasma and preventing the BSP proteins from inducing the removal of cholesterol and phospholipids from the sperm membranes [1]. In EY, it is thought to be low-density lipoproteins and in milk casein micelle interactions that bind up the BSP proteins. Development of semen extenders for the cryopreservation of stallion semen that contain alternate sources of lipoproteins to disengage BSP proteins would open up these markets to the importation of cryopreserved stallion semen and would reduce concerns of infectious disease transmission.

Animal welfare/ethical statement: This project was approved on 4/3/14 through the Institutional Animal Care and Use Committee at the University of California, Davis; protocol #18102.

Conflict of interest statement: The authors declare no conflict of interest.

* Corresponding author at: Bruce W. Christensen, Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, 1089 Veterinary Medicine Drive, Davis, CA 95616.
E-mail address: theriodvm@gmail.com (B.W. Christensen).

0737-0806/$ – see front matter © 2017 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2017.08.014

Promising results have been reported in stallions and other species using plant-based sources such as soy or coconut [2–11].

Binder of Sperm proteins are just one of the many obstacles to sperm in successfully navigating the freeze/thaw process. Volume excursions, mechanical stresses, centrifugation, osmotic damage, and cryopreservative toxicity all present challenges [12]. Freezing subjects the sperm to osmotic fluctuation and membrane changes that expose the sperm to oxidative stress [13,14], which is associated with the production of reactive oxygen species (ROS). These ROS are paradoxically produced by the mitochondria and known to cause damage to the mitochondria [15]. As the source of ATP production, Ca^{2+} homeostasis, and other important cell functions, mitochondrial damage is a major problem to cell function and survival. Compared to fresh sperm, exposure to cool temperatures causes an increase in cellular production of ROS, resulting in increased susceptibility to DNA, membrane, and mitochondrial oxidative damage [16,17].

Plasma membranes are high in polyunsaturated fatty acids and are very sensitive to peroxidation by ROS, which are produced when exposed to oxygen. Peroxidation causes the membranes to become porous and leads to loss of sperm motility [18]. A possible explanation for this is close proximity of mitochondria (the chief source of ROS [15]) to the axoneme. Additionally, since mitochondria lack protamines, which protect against oxidation, they are more likely to experience damage from ROS.

Because of the negative role of oxygen in the formation of ROS and the involvement of ROS and oxygen radicals in reducing sperm quality in cooled or frozen/thawed sperm, we hypothesize that sperm survival would be improved if the cooling or freezing procedures were done in anaerobic environments [19]. Oxyrase is a commercial preparation of *Escherichia coli* membrane fragments that contain an extract of bacterial electron transport systems. In the presence of a suitable hydrogen donor substrate (e.g., lactate), Oxyrase can decrease O_2 in solutions to very low levels [20] and has been shown to provide beneficial cryopreservation effects in murine and equine sperm [19,21].

The present study compares the post-thaw motility and fertility of stallion semen cryopreserved using EY or coconut water (CW). We also investigate the effect of adding Oxyrase to both EY and CW extended cryopreserved sperm on post-thaw motility and membrane oxidation.

2. Materials and Methods

2.1. Reagents

EquiPlus parts A and B stallion semen extender were obtained from Minitube of America (Verona, WI). INRA96 semen extender was purchased from IMV Technologies (L'Aigle, France). Chicken eggs were purchased from a local grocery store. Coconut water was obtained using commercially available C2O Pure Coconut Water (C2O Pure Coconut Water, LLC, Seal Beach, CA). EC-Oxyrase (product no. EC-0100) was obtained from Oxyrase Corp. (Mansfield, OH) and is supplied as a sterile frozen stock solution at 30 units/mL (U/mL) in 20 mM phosphate buffer at a neutral pH and will be termed "Oxyrase" henceforth throughout this manuscript. One unit of Oxyrase activity will reduce dissolved oxygen (in 1 mL of air saturated 40 mM phosphate buffer, pH 8.4, at 37°C) at the rate of 1% per second. All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Animals

Light breed horse stallions (n = 5, age range 4–16 years) were housed at the Center for Equine Health and Animal Science Horse Barn at the University of California, Davis. Stallions were maintained according to the Institutional Animal Care and Use Committee protocol approved by the University of California. Stallions were all previously trained for semen collection from a phantom into a Missouri model artificial vagina equipped with an inline nylon mesh filter to separate the gel fraction of the ejaculate. Semen was collected three times from each stallion for a total of 15 ejaculates. After an initial "clean out" collection, semen was collected from each stallion on 3 separate days, each collection separated by approximately 1 week.

2.3. Experiment 1: Semen Preparation and Analysis

Motility evaluation was determined using 50 μL of neat semen which was diluted with EquiPlus part A at a 1:5 ratio and kept at 37°C for 10 minutes prior to baseline progressive motility evaluation using a computer-assisted sperm analysis (CASA) system (SpermVision, MOFA Global, Verona, WI). The Sperm Vision system acquires 30 frames per field at a rate of 60 Hz with an optimal light threshold of 95, minimum cell identification area of 16 μm^2, and maximum cell identification area of 60 μm^2. Cells were considered immotile if they had an average orientation change of $<9.5°$. Cells were considered locally motile if the straight-line distance was <6.0 microns. All other cells were considered progressively motile [22–24]. Using another subsample of neat semen, concentration was determined using a NucleoCounter (Chemometec, Lillerød, Denmark). Prior to addition of semen, Oxyrase was added to extender of the appropriate treatments at a concentration of 8.68% and incubated at 24°C for 30 minutes to allow for the removal of oxygen. Ejaculates were extended at 37°C 1:1 in EquiPlus part A (Minitube USA, Delevan, WI) and divided into four treatment groups: EY, egg yolk with Oxyrase (EYO), CW, and coconut water with Oxyrase (CWO). Two additional portions of the ejaculate (1 mL each) were retained and diluted to 1×10^6 sperm/mL for the BODIPY 581/591 C11 [4,4-difluro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-dia-za-s-indacene-3-undecanoic acid] fluorescent assay preparation and prefreeze analysis of the Annexin V and propidium iodide (PI) fluorescent assays (see below). Each of the four treatment groups was centrifuged and resuspended to 400×10^6 cells/mL in either EquiPlus part A or EquiPlus part A with Oxyrase. Samples were then diluted to 200×10^6 cells/mL with the cooled EquiPlus part B formulation appropriate to the treatment group (CW or EY, with or without Oxyrase). Samples were then loaded into 0.5 mL clear polyethylene straws (IMV technologies, Maple Grove, MN) at 200×10^6 cells/mL and cryopreserved using a controlled rate freezer (Planar PLC, Middlesex, UK) with an initial cooling