Journal of Equine Veterinary Science 59 (2017) 57–63

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Addition of Antioxidants Myoinositol, Ferulic Acid, and Melatonin and Their Effects on Sperm Motility, Membrane Integrity, and Reactive Oxygen Species Production in Cooled Equine Semen

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A R T I C L E I N F O

Article history:
Received 7 May 2017
Received in revised form 14 September 2017
Accepted 16 September 2017
Available online 22 September 2017

Keywords:
Equine semen
Antioxidant
Ferulic acid
Melatonin
Myoinositol

A B S T R A C T

The present study aimed to evaluate the influence of myoinositol (MYO), ferulic acid (FA), and melatonin (MEL) in equine cooled semen. Ejaculates were collected and distributed into the following four treatments: MYO, FA, MEL, and control. A skim milk–based extender was used. Samples were cooled at 5°C and evaluated at 0, 4, and 8 hours after storage for motility, plasma and acrosomal membranes integrity, mitochondrial potential, and production of reactive oxygen species (ROS). Motility characteristics were not affected by treatment, except for the amplitude of lateral head displacement, which was higher in MYO (8.3 ± 0.2) compared with the control group (7.8 ± 0.2). No difference was observed among treatments for intact plasma membrane (%). However, the percentage of cells with intact plasma and acrosomal membranes and high mitochondrial potential was greater in the MEL (78.1 ± 2.0) and FA groups (78.8 ± 1.7) compared with the control group (73.8 ± 2.0). The high mitochondrial potential (%) was also greater in groups treated with MEL (80.1 ± 1.9) and FA (81.0 ± 1.5) compared with the control group (76.6 ± 2.0). In addition, percentage of cells with intact acrosome membrane was greater in MEL group (99.7 ± 0.1) compared with all other treatments. ROS production was not affected by treatments. In conclusion, FA and MEL provided the best protection to mitochondria, acrosome, and plasma membranes, suggesting that the addition of these antioxidants to equine semen extender can improve sperm quality.
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1. Introduction

The use of cooled-shipped equine semen for artificial insemination is a routine practice in the equine industry [1]. Cooling procedure reduces sperm metabolic activity and microbial growth, maintaining sperm viability for longer periods compared with fresh semen [2]. The development of semen extenders and cooling protocols has improved equine semen fertility. However, the longevity of equine sperm is still limited when compared with pigs and ruminants [3].

Animal welfare/ethical statement: Approved by ethics committee on the use of animals, faculty of veterinary medicine and animal science, University of São Paulo.

Conflict of interest statement: The authors have declared no conflict of interests.

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0737-0806/$ – see front matter © 2017 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.jevs.2017.09.006

Researchers have suggested that the decrease in equine sperm quality after storage at 5°C may be due to oxidative damage caused by an increase in reactive oxygen species (ROS) during the cooling procedure [4]. ROS and reactive nitrogen species (RNS) are present in all organisms living in aerobic environment [5]. At physiological levels, both ROS and RNS are required for normal sperm function such as hyperactivation, capacitation, and acrosome reaction [6]. However, the imbalance between the production of these molecules and the ability of biological systems to readily detoxify the medium (or repair the damages caused) is known as oxidative stress [7].

Oxidative stress has been associated with reduced sperm motility [8], reduced plasma membrane fluidity and integrity [9], changes in mitochondrial function [10], chromatin alterations [11], damage to the enzymatic antioxidant system [12], and acrosomal membrane injuries [13]. As a result, a reduction in sperm function is observed and sperm cells may become unable to fertilize the oocyte and to produce a viable pregnancy [7,14].

A wide range of scavengers and antioxidant enzymes have been used aiming to block and/or to prevent oxidative stress in different cell systems. These antioxidants may act directly removing reactive species (RS), and preventing the spread of lipid peroxidation in cell membranes [15].

Inositol is a hexahydroxycyclohexane [16] that can be arranged in nine stereoisomers [17]. In this regard, myoinositol (MYO) is the most important stereoisomer in the nature [17] and its biochemical derivatives are widely distributed in mammalian tissues and cells exerting important biological functions [16]. Some authors have demonstrated the antioxidant activity of inositol and its derivatives, especially at the mitochondrial level and mainly against superoxide anion, hydrogen peroxide, and hydroxyl radicals [18,19].

Ferulic acid (FA) is an organic component from the cell walls of plants (which arises from the metabolism of tyrosine and phenylalanine) being considered one of the main constituents of fruits, vegetables, and seeds [20,21]. Antioxidant activity is the specific biological function of FA, and this ability of eliminating reactive forms of oxygen and free radicals have been confirmed by several studies [22,23].

Melatonin (MEL) is the main hormone produced and secreted by the pineal gland [24], which participates in several physiological functions, including immune system, circadian rhythm, and regulation of reproduction in seasonal species [25]. In addition, strong evidences have pointed to MEL as an important scavenger of both oxygen and nitrogen reactive molecules [26].

Although several antioxidants have been used to reduce sperm damages caused by the RS, the compounds MYO and FA have never been studied in equine semen. Similarly, MEL addition was studied only by a few researchers [10]. Therefore, this study aimed to evaluate the effects of these three antioxidants (MYO, FA, and MEL) in sperm characteristics during the cooling process of equine semen up to 8 hours of storage.

2. Material and Methods

2.1. Animals

Four ejaculates (from each animal) were collected from four fertile stallions ($n = 16$), aged between 4 and 8 years, stabled at the School of Veterinary Medicine and Animal Science, University of Sao Paulo (USP), Pirassununga, SP, Brazil. Semen collections were performed using an artificial vagina.

2.2. Sample Preparation and Dilution

After semen collection, the gelatinous fraction was appropriately separated from the ejaculate using a nylon filter. Sperm motility and vigor (predilution) were assessed visually using a phase contrast microscope equipped with a warming stage (37°C) at a magnification of 100×. To determine sperm concentration before dilution, an aliquot of semen was diluted (1:100) in formaldehyde-saline solution, and cell count was performed using a Neubauer chamber under phase-contrast microscopy (magnification, 400×).

Skim milk medium (composition: 4.9 g glucose, 2.4 g skim milk powder, 0.15 g $NaHCO_3$, 100,000 IU penicillin G, 0.007 g streptomycin, dd.H_2O—q.s.q. 100 mL) [27] was prepared in a single batch and divided into 30 mL aliquots and stored at −80°C.

The stock solutions of antioxidants were previously prepared (**MYO**: 0.5 M in deionized water—cod. I7508, Sigma, St. Louis, MO, USA; **FA**: 25 mM in dimethyl sulfoxide (DMSO)—cod. W-518301, Sigma; **MEL**: 1 mM in DMSO—cod. M5250, Sigma) and stored at 5°C for up to 3 days. Just before semen collection, antioxidants were added to the skim milk–based extender in a volume required to achieve a final concentration of 30 mM of MYO, 165 μM of FA, and 1 μM of MEL. The concentration used for MEL was the one that showed better results in a previous study by Silva et al [10]. To determine the amount of MYO and FA, both of which have never been used in equine semen before, preliminary tests were carried out. Based on studies performed in human semen [19,28], different concentrations were tested and motility was evaluated using the standard microscope. The concentration that showed better results was used in this study.

Semen aliquots were then distributed among the following four treatments: (1) MYO, (2) FA, (3) MEL, and (4) control (CON, skim milk medium without any treatment), in a volume required to achieve a final concentration of 25×10^6 sperm/mL, in a total fixed volume of 40 mL per treatment (extender and semen). To avoid a possible ordering effect on the results, for each semen collection, a new order of dilution and analysis was respected in a manner that all treatments passed once for each ordinal position of dilution and analysis (first–fourth).

After dilution, a 2-mL aliquot from each treatment was separated into a microcentrifuge tube for analysis related to time 0 hours (5 minutes after the addition of extender). The remainder were distributed into eight tubes, one for each treatment (MYO, FA, MEL, and CON) and time (4 and 8 hours), which were bottled in a total volume of 15 mL, removing as much air as possible for posterior cooling.

2.3. Cooling Procedure

To initiate cooling, the diluted semen bottles were allocated in the cooling device BotuFLEX (Botupharma, Botucatu, SP, Brazil). For each cooling period, an

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