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Original Research

Effects of Pentoxifylline, Caffeine, and Taurine on Post-Thaw Motility and Longevity of Equine Frozen Semen

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ABSTRACT

Frozen semen provides several advantages to the breeder relative to fresh or cooled semen. However, some stallions are undesirable candidates for semen freezing due to poor post-thaw motility or longevity caused by membrane damage, osmotic stress, and oxidative stress during cryopreservation. The objective of this study was to determine the effect of post-thaw addition of pentoxifylline, caffeine, or taurine on sperm motility and longevity in equine frozen semen. Pentoxifylline, caffeine, or taurine was incorporated immediately into thawing frozen semen from nine warmblood stallions. Spermatozoa motility and longevity parameters were recorded and analyzed for each additive and for an untreated control. Of the three additives, only pentoxifylline improved total and progressive semen motility relative to that of untreated control. Pentoxifylline also increased semen curvilinear velocity, average path velocity, and straight line velocity relative to those of caffeine, taurine, or control. Semen treated with pentoxifylline also showed greater longevity relative to that of caffeine- or taurine-treated or untreated semen. Taurine improved linearity in comparison to that of semen treated with pentoxifylline, caffeine, or control but did not improve other parameters. Pentoxifylline may be useful in enhancing the quality of equine frozen semen and therefore improving its fertility. Additional studies are warranted that examine the effect of these additives on the conception rate. Pentoxifylline can be used to increase motility and longevity of equine frozen semen and theoretically increase probability of conception and overall breeding success rates.

1. Introduction

Frozen semen continues to increase in importance and popularity in the artificial insemination of horses. However, conception rates with thawed frozen semen are lower than those with fresh or cooled semen [1]. Post-thaw motility and longevity are reduced due to membrane damage caused by osmotic and oxidative stress during freezing and thawing events [2], and much recent research has investigated the molecular damage experienced by equine spermatozoa as a result of these different stresses [3]. During cryopreservation, ice crystals form in the extracellular solution, creating a high solute concentration in the unfrozen extender [3]. The hypertonic extracellular environment causes intracellular dehydration as water leaves the cell to balance the osmotic gradient, causing cell shrinkage. The opposite phenomenon occurs during thawing, when the spermatozoa are exposed to a hypotonic environment and cells swell from water influx as intracellular and extracellular solute concentrations equilibrate [3]. Extreme modulations in cellular volume can damage the

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plasma membrane as well as other cellular components. Freezing and thawing also increases reactive oxygen species (ROS) generation in plasma membranes and mitochondria. ROS-mediated lipid peroxidation (LPO) and oxidative stress can cause cryodamage and further contribute to reduced motility and lifespan of post-thaw semen [4]. Thus, protocols are needed which reliably enhance motility and survivability of frozen semen after thawing. In this study, we explored three post-thaw additives postulated to improve these parameters in equine semen.

Phosphodiesterase inhibitors have been used as cryprotectants and have been shown to increase motility and longevity of spermatozoa [5,6]. Caffeine and pentoxifylline are methylxanthine derivative phosphodiesterase inhibitors. These compounds positively affect sperm function by upregulating glycolysis via a buildup of cyclic adenosine monophosphate (cAMP) through inhibition of the enzyme cAMP phosphodiesterase [7]. Elevated cAMP levels increase the rate of glycolysis, which generates adenosine triphosphate (ATP), used to power sperm movement [5]. Pentoxifylline and caffeine have been shown to improve motility and longevity of fresh and frozen semen in a variety of mammalian species [8–14]. Pentoxifylline has been used successfully to enhance post-thaw parameters of equine frozen semen [13]; however, caffeine has not been studied as a post-thaw additive to frozen semen in horses. Because these two additives have similar properties and mechanisms of action, pentoxifylline can be used as a positive control to investigate the effect of caffeine on post-thaw frozen equine semen.

Beta amino acids have roles as antioxidants; they are found naturally in semen and elsewhere in the body to protect cells from damage from ROS [15,16]. The beta amino acid taurine has been shown to stimulate spermatozoa motility and to assist in the acrosome reaction in cooled equine semen but has not been investigated in frozen equine semen upon thawing [6]. Taurine serves as an antioxidant found naturally at low concentrations in mammalian semen [15,17]. It acts as a scavenger that protects spermatozoa from ROS damage, theoretically increasing motility and longevity [16,18,19]. Taurine has been used with mixed results in attempts to enhance mammalian semen [6,16,20–23].

The objective of this study was to determine the effect of post-thaw addition of pentoxifylline, caffeine, or taurine on sperm motility and longevity in equine frozen semen. Pentoxifylline has been explored previously [13], while caffeine, a similar additive, has not been studied in depth with equine frozen semen [24]. Taurine has been used with mixed results in a number of species, although it has never been applied to equine frozen semen. Therefore, this study was able to demonstrate the effect of pentoxifylline, caffeine, and taurine on the motility and longevity of equine frozen semen.

2. Materials and methods

Frozen semen from nine warmblood stallions representing a range of known post-thaw motilities was provided by a single commercial cryopreservation facility, and semen from each stallion was assigned to one of four treatment groups: caffeine, pentoxifylline, taurine (Sigma-Aldrich, St. Louis, MO) or an untreated control. Freezing methodology is proprietary and unknown to the researchers, but conditions were identical for all stallions, and the freezing extender was lactose-based. Kenney's extender, made from D-glucose, non-fat dry milk and sterile water (Hamilton Research, Inc., South Hamilton, MA), a common universal extender, allowed the additives to be made to appropriate concentration within separate aliquots. Concentrations for caffeine (2 mM), pentoxifylline (3.5 mM), and taurine (25 mM) were determined based on previous research [6,10,12] and preliminary study. In the preliminary study, four concentrations of caffeine and taurine and three concentrations of pentoxifylline were examined, and those yielding the greatest motility and longevity were selected for this study. During the preliminary trial, semen was added to a control of Kenney's extender and compared to a range of concentrations for each of the three compounds. Caffeine was tested from 1 mM to 5 mM (i.e., 1, 2, 3.5, and 5 mM), Pentoxifylline was tested from 1 mM to 7 mM (1, 3.5, and 7 mM), and Taurine was tested from 25 mM to 100 mM (10, 25, 50, and 100 mM).

For each stallion, two 0.5-mL straws of frozen semen from the same ejaculate were thawed in a 37°C water bath for 30 sec and then transferred to a warmed test tube. Straw contents were mixed, and 220 μL was added to a second test tube containing 2 mL of Kenney's extender plus treatment or control. The final concentration was approximately 25 M spermatazoa/mL. Samples were evaluated at 0, 10, 20, and 30 min and again every 30 min until total motility (TM) was equal to or below 10%. Samples were maintained in a 37°C water bath between evaluations. All treatments for a given stallion ejaculate were performed on the same day to avoid treatment by day effect.

Computer-assisted sperm analysis (CASA; Hamilton-Thorne, Beverly, MA) was used to analyze the sperm samples over a 150-min period. Parameters included TM, progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), and linearity (LIN; derived from VSL/VCL) [25]. Longevity was defined as time, to the nearest 30 min, when sperm TM decreased to 10% or less. Minimum number of frames was set to 40, with a frame rate of 60 frames/s. Minimum

Table 1
CASA parameters TM, PM, VCL, VAP, VSL, and LIN of frozen semen

Additive	TM (%)	PM (%)	VCL (μm/s)
Control	36.19 ± 2.84 B	19.78 ± 2.73 B	116.42 ± 5.09 B
Pentoxifylline	42.83 ± 2.84 A	26.76 ± 2.73 A	132.72 ± 5.09 A
Caffeine	39.89 ± 2.84 AB	21.89 ± 2.73 B	119.03 ± 5.09 B
Taurine	34.70 ± 2.84 B	20.94 ± 2.74 B	113.21 ± 5.11 B
Additive	**VAP (μm/s)**	**VSL (μm/s)**	**LIN (%)**
Control	63.65 ± 4.11 B	54.71 ± 4.32 B	46.71 ± 2.13 B
Pentoxifylline	75.45 ± 4.11 A	65.08 ± 4.32 A	48.69 ± 2.13 B
Caffeine	66.71 ± 4.11 B	57.45 ± 4.32 B	46.83 ± 2.13 B
Taurine	66.71 ± 4.12 B	58.45 ± 4.33 B	51.19 ± 2.14 A

Table shows least square means ± SEM based on mixed models for CASA parameters TM, PM, VCL, VAP, VSL, and LIN of frozen semen diluted post-thaw with pentoxifylline, caffeine, or taurine over a 150-min period. Different letters within columns for each additive indicate a statistically significant difference, with $P < .05$.