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Original Research

Effects of Pentoxifylline on Equine Epididymal Sperm

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ABSTRACT

This study evaluated whether pentoxifylline (PTX) present in the flushing extender influenced the function of equine epididymal spermatozoa after recovery and after thawing. For this experiment, 58 testicles from 29 Brazilian Jumping Horses were used. Cauda epididymides of each stallion were separated and flushed with a skim milk extender, with or without 7.18 mM PTX and then subjected to the freezing process. Samples flushed with the extender containing PTX showed a significant increase in total motility, progressive motility, straight line velocity, curvilinear velocity, and percentage of rapid sperm immediately after the recovery of epididymal sperm and after 15 minutes of incubation at 37°C ($P < .05$). However, the presence of PTX in the flushing extender did not affect the post-thaw motility parameters or plasma membrane integrity ($P > .05$). The results of this study showed that the PTX present in the flushing extender improved motility parameters of recently recovered epididymal sperm and had no deleterious effects on plasma membrane integrity and freezability of equine epididymal sperm.

1. Introduction

Epididymal spermatozoa remain quiescent in the cauda epididymidis [1], and most spermatic cells are immotile after harvesting. Biochemical studies mainly in rodents and in bovine epididymis have shown that the development of motility from the caput to the cauda epididymidis is associated with increased levels of intraspermatic pH, adenosine monophosphate (cAMP), and calcium ions, mediators responsible for the activation of sperm kinetics [2,3].

The presence of specific substances in extenders used to flush the epididymal duct may stimulate sperm motility and allow better use of recovered spermatic cells. Pentoxifylline (PTX), a methylxanthine derivative, has been shown to enhance motility of human, ram, and equine ejaculated spermatozoa in vitro [4-6]. PTX inhibits the activity of the enzyme phosphodiesterase, thereby elevating the concentrations of intracellular cAMP [7] and increasing tyrosine phosphorylation in the tail [8]. It has also been reported that the addition of PTX to cryopreserved equine semen after thawing increases the percentages of motile and progressively motile sperm [6,9-11] and its longevity [6]. Furthermore, PTX was related to the preservation of plasma membrane integrity of human spermatozoa [12]. However, the effect of PTX on the motility and viability of stallion epididymal spermatozoa has not been reported.

The recovery of spermatozoa from cauda epididymides and their cryopreservation represent a great technological advance, as it is the last possibility for preserving genetic material from dead or deceased valuable stallions.

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Table 1
Methodologies of CASA for equine sperm analyzer

Characteristic	Adjusted to
Number of frames	30
Minimum contrast	60 pixels
Minimum cell size	3 pixels
Contrast to static cells	30 pixels
Straightness	80%
Average path velocity cutoff	30.0 μm/s
Minimum VAP to progressive cells	70.0 μm/s
VSL cutoff to slow cells	20.0 μm/s
Static head size	0.62-2,98
Static head intensity	0.24-1.19
Static head elongation	100-0
Magnification	1.95×
Temperature	37°C

VAP, average path velocity.

Therefore, the aim of this study was to evaluate the effect of PTX on equine epididymal spermatozoa after recovery and after thawing.

2. Materials and Methods

Twenty-nine Brazilian Jumping Horses aged 3 to 5 years were used. Initially, three ejaculates from each stallion were collected with an interval of 2 days to eliminate cells from cauda epididymides and to stabilize the sperm parameters.

After this procedure, the stallions underwent bilateral orchiectomy. A standing castration was performed using 10% xylazine hydrochloride, 0.5 mg/kg IV, combined with 0.05 mg/kg 1% hydrochloride acepromazine IV for sedation. Local anesthesia was performed by intratesticular injection of 10 mL of 2% lidocaine hydrochloride with epinephrine.

Immediately after castration, the cauda epididymides was isolated from the testis. The connective tissue was carefully dissected to allow the passage of the flushing extender, and the cauda epididymis was straightened. A 10-μL pipette tip was attached to a 10-mL syringe. Sperm harvesting was performed by a retrograde flushing technique.

For the control samples (Control), the cauda epididymides of one testicle of each stallion was flushed using 40 mL of a skim milk-based extender (Botu-Semen; Botupharma, Botucatu, Brazil). Then, the contralateral cauda epididymides was flushed using 40 mL of a skim milk-based extender containing 7.18 mM PTX (Botu-Turbo; Botupharma), corresponding to the treated group (PTX). The flushing fluid and sperm from each epididymidis of each animal was recovered in a 200-mL beaker and incubated at 37°C for 15 minutes.

For the cryopreservation procedure for epididymal sperm, samples were centrifuged at 600 × *g* for 10 minutes. The supernatant was removed, and the pellet was resuspended in a freezing egg yolk-based extender (Botu-Crio; Botupharma) at a final concentration of 160 million total spermatozoa per milliliter. Semen samples were packed into 0.5-mL straws, maintained at 5°C for 20 min in a commercial refrigerator (Minitub; Porto Alegre, Brazil), and subsequently frozen in liquid nitrogen vapor, 6 cm above the surface of liquid nitrogen, for 20 min. The straws were immersed in liquid nitrogen and stored at −196°C until analysis. After thawing at 46°C for 20 seconds [13], the sperm were transferred to a 1.5-mL plastic tube and maintained in a dry block at 37°C for 15 minutes for semen evaluation.

Sperm recovered from the cauda epididymides with the skim milk-based extender (Control) and with the skim milk-based extender containing PTX were analyzed immediately after sperm recovery, after incubation at 37°C for 15 min, after centrifugation following dilution in the freezing extender, and post-thaw. Five fields per sample were selected for evaluation of motility parameters by computer-assisted sperm analysis (CASA) (HTM-IVOS 12; Hamilton Thorne Research). The CASA setup is described in Table 1. The plasma membrane integrity was evaluated at ×400 magnification using the fluorescent probes carboxyfluorescein diacetate and propidium iodide as described by Harrison and Vickers [14].

Data were analyzed by ANOVA followed by the Tukey test to compare the treatments. The significance level was set at .05 (SAS Institute Inc., Cary, NC).

3. Results

Mean number of spermatozoa recovered from the cauda epididymis by retrograde flushing technique was $14.38 \pm 9.50 \times 10^9$ spermatozoa per epididymis.

In recovered spermatozoa, there were no differences ($P > .05$) in plasma membrane integrity in samples exposed or not exposed to PTX, although PTX groups yielded higher motility parameters (Table 2).

Table 2
Mean values and standard deviations of the sperm parameters TM, PM, VSL, VCL, BCF, and RAP evaluated immediately after recovery for Control and PTX samples

Sample[a]	TM (%)	PM (%)	VSL (μm/s)	VCL (μm/s)	BCF (Hz)	RAP (%)
Control	17.1 ± 14.0[a]	5.5 ± 6.0[a]	60.3 ± 11.3[a]	150.5 ± 25.0[a]	26.2 ± 5.8[a]	9.4 ± 8.9[a]
PTX	53.3 ± 21.9[b]	20.6 ± 11.3[b]	77.0 ± 11.4[b]	194.9 ± 30.2[b]	28.8 ± 3.2[b]	43.9 ± 22.2[b]

BCF, beat cross frequency; PM, progressive motility; RAP, rapid cells; TM, total motility; VCL, curvilinear velocity; VSL, straight-line velocity.
Different letters in a column indicate differences ($P < .05$).
[a] Control sperm were recovered from the cauda epididymides with the flushing extender without pentoxifylline; PTX sperm were recovered from the cauda epididymides with the flushing extender containing 7.18 mM of pentoxifylline.