

The effect of caffeine, thimerosal and thapsigargin on stallion sperm  
hyperactivation

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## Abstract

Ejaculated mammalian sperm become fertilization-competent after undergoing physiological changes in the female's reproductive tract, namely capacitation and hyperactivation. The latter is characterized by a change in sperm motility from symmetrical flagellar bends to vigorous, asymmetrical, high-amplitude flagellar bends, with the pattern of motility being species-specific. The main trigger responsible for the initiation of hyperactivated motility is an increase in intracytoplasmic  $\text{Ca}^{2+}$  arising potentially from both intracellular and/or extracellular sources. Therefore, pharmacological reagents that increase intracytoplasmic  $\text{Ca}^{2+}$  have been used to induce hyperactivation in sperm from a variety of species. Recently, hyperactivation has been characterized for stallion sperm using procaine treatment, a reagent hypothesized to increase membrane permeability to extracellular  $\text{Ca}^{2+}$ . Since hyperactivation of stallion sperm is crucial for supporting *in vitro* fertilization (IVF), this study examined the effect of exposing equine sperm to different concentrations of caffeine, thimerosal and thapsigargin, three reagents demonstrated to induce hyperactivation in other species. Sperm from three ejaculates from each of three stallions were exposed to concentrations gradients of each of these reagents to examine their effect of sperm hyperactivation using a computerized sperm motility analyzer (CASA) to measure motion parameters associated with this pattern of motility. In all experiments, positive controls included treatment with procaine. All three reagents tested failed to induce hyperactivation in the stallion, suggesting physiological differences in the response of stallion sperm to these reagents as compared to other species.

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## Introduction

Hyperactivation is part of the continuum of physiological changes that sperm must undergo to become fertilization-competent. The initial forward motility displayed by ejaculated sperm becomes irregular, vigorous and asymmetrical, causing the sperm to depart from its normal path of a straight line and to travel in a species-specific pattern. This change in motility provides sperm with the ability to travel through the viscous environment of the oviductal lumen and to penetrate the cumulus cells and zona pellucida surrounding the oocyte (Suarez et al., 1992; Stauss et al., 1995; *reviewed by* Suarez, 2003).

The most crucial signal to the induction of hyperactivation appears to be an increase in intracytoplasmic  $\text{Ca}^{2+}$  concentrations, both from intracellular and extracellular sources (Ho and Suarez, 2003; Marquez et al., 2007). Therefore, hyperactivation may be induced *in vitro* by treating sperm with pharmacological agents postulated to increase intracytoplasmic  $\text{Ca}^{2+}$  concentrations. Several such agents have been used to study sperm hyperactivation in laboratory and domestic species, including procaine (Marquez and Suarez, 2004; McPartlin et al., 2009), caffeine (Ho and Suarez, 2001), thimerosal (Ho and Suarez, 2001; Marquez et al., 2007), and thapsigargin (Ho and Suarez, 2001, 2003).

Recently, procaine treatment has been used to characterize hyperactivated motility in stallion sperm; moreover, induction of hyperactivation in capacitated sperm supported high rates of homologous *in vitro* fertilization (IVF; McPartlin et al., 2009). Because only two foals have ever been produced using IVF (Palmer et al., 1991), further characterization of the conditions that support capacitation and hyperactivation in the stallion bears special importance. Therefore, the objectives of this study were to analyze the effect of

pharmacological agents reported to induce hyperactivation in sperm from other species in their ability to support this pattern of motility in stallion sperm.

### **Literature Review**

Mammalian spermatozoa are not fertilization-competent immediately after ejaculation, but achieve this ability after undergoing the processes of capacitation and hyperactivation (*reviewed by* Yanagimachi, 1994 and Suarez, 2003). Capacitation comprises the molecular changes that prepare the sperm for the acrosome reaction, an exocytotic event that allows sperm to penetrate the outer vestments of the oocyte (*reviewed by* Yanagimachi, 1994). Hyperactivation refers to a change in the motility pattern of the sperm, from regular, symmetrical flagellar bends to high-amplitude, asymmetrical flagellar bends (Suarez and Dai, 1992; Ho et al., 2002; *reviewed by* Suarez, 2008). This change in motility is important for fertility as it helps sperm navigate through the twisting lumen and viscous environment of the oviduct (Suarez et al., 1991). Hyperactivation is also thought to help the sperm penetrate through the cumulus oophorus, the layer of granulosa cells directly surrounding the oocyte, as well as the zona pellucida overlying the membrane of the oocyte (Suarez et al., 1992; Suarez and Dai, 1992; *reviewed by* Suarez, 2003). It has also been hypothesized that hyperactivation plays a role in allowing sperm to detach from the oviductal epithelium of the isthmus, a location believed to act as a sperm reservoir, so that they can proceed upstream, towards the site of fertilization (Dermott and Suarez, 1992).

Capacitation and hyperactivation are often linked together in the continuum of sperm changes required for fertilization, especially since both events depend upon calcium,

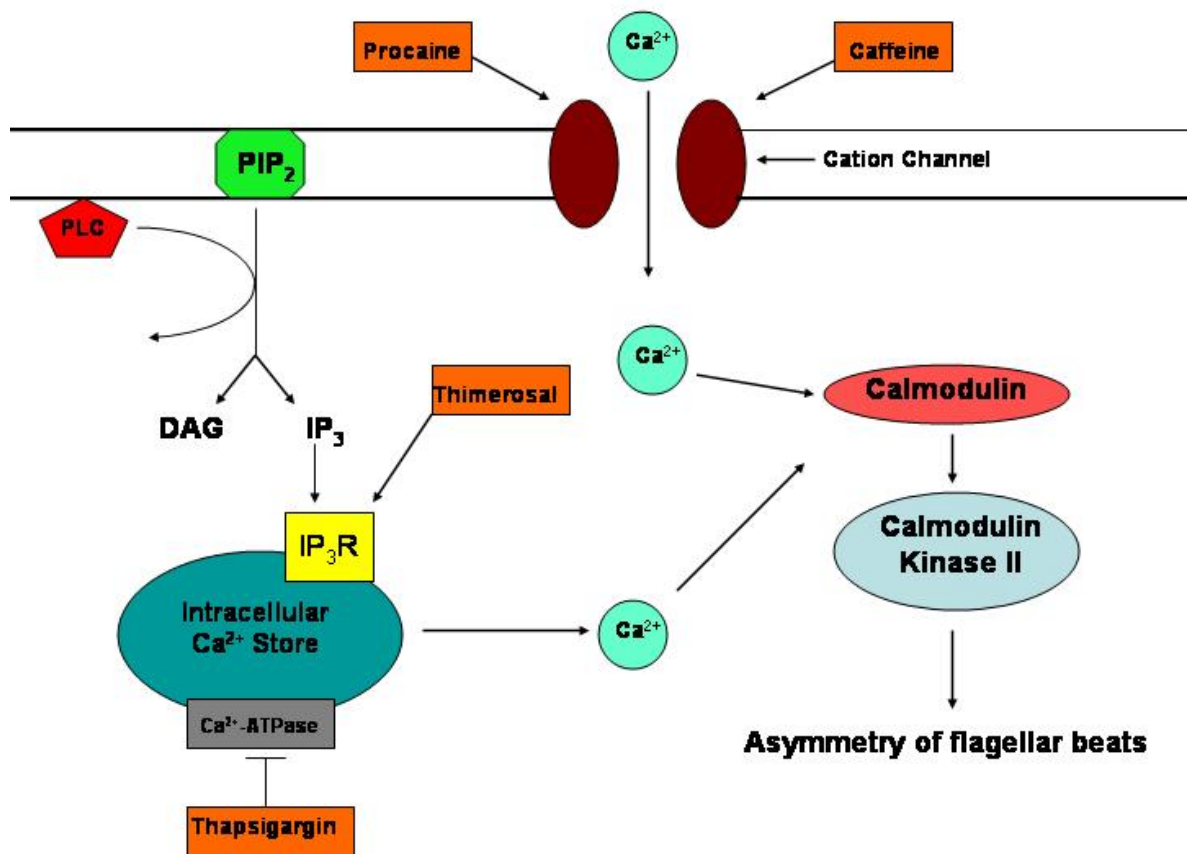
bicarbonate and cAMP synthesis (Boatman and Robbins, 1991; Hess et al., 2005; Xie et al., 2006; *reviewed by* Suarez, 2008). However, the molecular pathways regulating these processes are independent of each other, as evidenced by the fact that hyperactivation can be induced with pharmacological agents in uncapacitated guinea pig and bull sperm (Muijca et al., 1994; Marquez and Suarez, 2004). Moreover, bull sperm incubated under capacitating conditions that support acrosomal exocytosis do not spontaneously hyperactivate (Marquez and Suarez, 2004), further supporting the notion that the two processes are not coupled to each other.

The absolute requirement for  $\text{Ca}^{2+}$  in the initiation of hyperactivated motility has been demonstrated in studies using Triton X-100-treated (demembrated) sperm (Ho et al., 2002). Reactivation of demembrated sperm motility was achieved in a medium low in  $\text{Na}^+$  and high in  $\text{K}^+$ , with the addition of ATP and  $\sim 50$  nM  $\text{Ca}^{2+}$ ; further increasing the concentration of  $\text{Ca}^{2+}$  in the medium to  $\sim 400$  nM induced sperm hyperactivation, thus suggesting that  $\text{Ca}^{2+}$  is acting directly upon the sperm's cytoskeletal elements to regulate this pattern of motility. Similar results were obtained using demembrated sea urchin sperm; since these lack the dense outer fibers and the fibrous sheath present in the flagellum of mammalian sperm, these results further suggested that  $\text{Ca}^{2+}$  is exerting a direct action upon the axoneme (Brokaw and Nagayama, 1985). Later studies in sea urchin sperm also showed that the hyperactivation-related changes in the flagellar bending patterns arise from the selective action of  $\text{Ca}^{2+}$  upon dynein arms (Bannai et al., 2000). Further work with demembrated murine and bovine sperm identified the target of the  $\text{Ca}^{2+}$  signal to be calmodulin, which in turn activates calmodulin kinase II to trigger hyperactivation (Ignotz and Suarez, 2005; Schlingmann et al., 2007).



The increase in intracytoplasmic  $\text{Ca}^{2+}$  levels required for hyperactivation potentially originates from both extracellular and intracellular sources (Marquez et al., 2007). This is evidenced by studies showing that pharmacological agents both increasing the permeability of the sperm membrane to extracellular  $\text{Ca}^{2+}$ , as well as inducing  $\text{Ca}^{2+}$  release from intracellular stores are able to support sperm hyperactivation *in vitro* (Ho and Suarez, 2001, 2003; Marquez and Suarez, 2004; Carlson et al., 2005; Marquez et al., 2007). For instance, procaine and caffeine have been used to define hyperactivation in bovine and murine sperm (Marquez and Suarez, 2004; Carlson et al., 2005). Both these agents are believed to hyperactivate sperm by inducing an influx of extracellular  $\text{Ca}^{2+}$  (Fig. 1); additionally, procaine may also increase the amount of intracellular ATP required to maintain motility (Mujica et al., 1994). Other studies demonstrate that agents affecting the emptying of intracellular stores also induce sperm hyperactivation (Ho and Suarez, 2001). The main 1,4,5-triphosphate ( $\text{IP}_3$ )-gated intracellular  $\text{Ca}^{2+}$  store in the sperm is the redundant nuclear envelope (RNE), located near the site of attachment between the head and flagellum (Ho and Suarez, 2003). The RNE was determined to be  $\text{IP}_3$ -gated through labeling with anti- $\text{IP}_3$  receptor antibodies, which localized to a central region in the neck. The acrosome, another  $\text{IP}_3$ -gated  $\text{Ca}^{2+}$  store, served as a positive control in this experiment and was also labeled by the anti- $\text{IP}_3$  receptor antibodies. Calreticulin, a high-capacity  $\text{Ca}^{2+}$ -binding protein that is often abundant in  $\text{Ca}^{2+}$  stores, was found to co-localize in the anti- $\text{IP}_3$  receptor antibody labeled region delineating the RNE (Ho and Suarez, 2003). Therefore, thimerosal, an  $\text{IP}_3$  agonist (Fig. 1), has also been used to induce bovine sperm hyperactivation (Ho and Suarez, 2001; Marquez et al., 2007). Moreover, in sperm from knockout mice for the main extracellular  $\text{Ca}^{2+}$  channels CatSper 1 and 2, treatment with thimerosal was able to

overcome the inability of these sperm to hyperactive (Marquez et al., 2007), further suggesting that  $\text{Ca}^{2+}$  from intracellular stores can support sperm hyperactivation. Similarly, thapsigargin, an agent that inhibits  $\text{Ca}^{2+}$ -ATPase (Fig. 1), a cation pump that replenishes intracellular  $\text{Ca}^{2+}$  stores, is also able to support hyperactivation in bovine sperm (Ho and Suarez, 2001). Altogether, these studies support the notion that the contribution of  $\text{Ca}^{2+}$  from both intracellular stores and the extracellular medium might be important for triggering sperm hyperactivation.



**Figure 1.** Mechanism controlling induction of hyperactivation in sperm with sites of action for procaine, caffeine, thimerosal and thapsigargin indicated.

The pattern of hyperactivated motility is species-specific; equine sperm will exhibit a vigorous, circular pattern when swimming in an aqueous medium as observed visually

under phase-contrast microscopy. More objectively, sperm hyperactivation can be assessed by analyzing specific motion parameters via a computer assisted semen analyzer (CASA). Thus by tracing the actual path of the sperm, hyperactivated stallion sperm exhibit a star-shaped pattern (McPartlin et al., 2009). The motility parameters analyzed by the CASA system include: (1) Percent of motile sperm (MOT, %), or the percentage of sperm that are moving; (2) percent of progressive sperm (PRO, %), or the percent of sperm moving in a straight line path; (3) average path velocity (VAP,  $\mu\text{m}/\text{sec}$ ), or the time-averaged velocity of the sperm head along its average path; (4) straight line velocity (VSL,  $\mu\text{m}/\text{sec}$ ), or the time-averaged velocity of the sperm head along the straight line between its first and last detected position; (5) curvilinear velocity (VCL,  $\mu\text{m}/\text{sec}$ ), or the time-averaged velocity of the sperm head along its actual curvilinear path; (6) amplitude of lateral head displacement (ALH,  $\mu\text{m}/\text{sec}$ ), or the magnitude of lateral displacement of a sperm head about its average path; (7) beat cross frequency (BCF, Hz), or the frequency with which the sperm head moves back and forth in its track across the cell path; (8) straightness (STR, %), or the departure of the cell path from a straight line (VSL/VAP); and, (9) linearity (LIN, %) which is another measurement of the departure of the cell path from a straight line (VSL/VCL). Specific measures of the different motion parameters assessed by means of CASA have been used to define hyperactivated motility in different species (Muijca et al., 1994; Baumber and Meyers, 2006; Marquez et al., 2007). In these, a decrease in VSL, STR and LIN values and an increase in VCL and ALH are considered hallmarks for hyperactivated sperm (*reviewed by* Suarez, 2008). Therefore, equine sperm that depart from moving along their typical straight line path and instead move in a more circular pattern are considered to be hyperactivated.

Table 1. Definition of motion parameters measured by Computerized Sperm Motility Analysis (CASA).

<u>CASA Parameters</u>	<u>Definition</u>
Motility (MOT, %)	Percent of sperm moving
Progressive Motility (PRO, %)	Percent of sperm moving in straight line path
Average Path Velocity (VAP, $\mu\text{m}/\text{sec}$ )	Average velocity of sperm head average path
Straight-line Velocity (VSL, $\mu\text{m}/\text{sec}$ )	Average velocity of sperm head straight-line path
Curvilinear Velocity (VCL, $\mu\text{m}/\text{sec}$ )	Average velocity of sperm head curvilinear path
Amp. Lat. Head Disp. (ALH, $\mu\text{m}/\text{sec}$ )	Magnitude of lateral displacement of a sperm head
Beat Cross Frequency (BCF, Hz)	Frequency which sperm head moves back and forth
Straightness (STR, %)	Departure of the cell path from a straight line
Linearity (LIN, %)	Departure of the cell path from a straight line

The motions that define procaine-induced hyperactivation in the stallion have been recently reported (McPartlin et al., 2009). Stallion sperm do not spontaneously hyperactivate when incubated in capacitating conditions; therefore procaine treatment has been used to define this pattern of motility in this species. In agreement to what has been reported in other species, hyperactivation of procaine-treated stallion sperm is defined by an increase in VCL and ALH and a decrease in VSL, LIN and STR (McPartlin et al., 2009). Moreover, by coupling sperm capacitation and procaine-induced hyperactivation, our laboratory has reported very high rates of homologous IVF (McPartlin et al., 2008, 2009), further emphasizing the requirement of both this events for successful fertilization. This is a crucial finding considering that only two foals have ever been produced using this technology (Palmer et al., 1991) and a better understanding of the conditions that support this process in stallion sperm may further lead to the improvement of assisted reproduction technologies in this species. Therefore, the objectives of this study were to use CASA to analyze the ability of agents other than procaine to support sperm hyperactivation, and presumably IVF, in the horse.

## **Materials and Methods**

***Chemicals and Reagents.*** All chemicals and reagents used in this study were purchased from Sigma Chemical Company (St. Louis, MO).

***Media Preparation.*** The media used in this study was modified Whitten's (MW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 5.5 mM glucose (anhydrous), 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt and 1.0 mM pyruvic acid). Modified Whitten's served as both the non-capacitating medium as well as the transport medium used to bring ejaculate samples to the laboratory. Capacitating medium was the non-capacitating MW base medium with the addition of 25 mM NaHCO<sub>3</sub> and 7 mg/mL BSA. Both media were brought to a final pH of 7.25 with HCl.

***Semen Collection and Preparation.*** Semen from three different stallions was collected with an artificial vagina, filtered to remove the gel fraction and 3 mL were added to 6mL of pre-warmed MW medium for a total volume of 9 mL. The semen was transported to the laboratory (~10 minutes) at 37°C and upon arrival was centrifuged in a 15-mL conical tube at 100g for 1 minute (at 37°C) in order to remove dead sperm and particulate matter from the sample. The supernatant was then transferred to a 14-mL round-bottom tube and centrifuged at 600g for 5 minutes at 37°C and the sperm pellet obtained was resuspended in 1.5 mL non-capacitating MW. The concentration of sperm was then determined via hemacytometer and sperm were then resuspended to a final concentration of  $10 \times 10^6$  sperm/mL in capacitating MW. Samples were incubated in 500  $\mu$ L aliquots in 5-mL round-bottom polyvinyl alcohol-coated tubes for 30 minutes at 37°C to allow for sperm to

acclimatize to the medium before the addition of the corresponding reagents used to test hyperactivation. While analysis of the sperm sample was performed, tubes were kept constantly at 37°C in a water bath.

***Testing of Hyperactivation-Inducing Reagents*** . For each experiment, CASA measurements were taken for sperm incubated under capacitating conditions (negative control), capacitating conditions plus 5 mM procaine (positive control to induce hyperactivation; McPartlin et al., 2009) as well as two of the three reagents tested. The pharmacological reagents and concentrations tested included: Caffeine at 2, 4, 6, 8, 10 and 12 mM; thimerosal at 20, 40, 60, 80, 100 and 120 µM; and, thapsigargin at 10, 20, 30, 40 and 50 µM. Stock solutions of these reagents in MW were prepared either on the day of the experiment (caffeine, 24 mM; thimerosal, 200 µM) or ahead of time and frozen at -20°C in 10 µl aliquots (thapsigargin, 1000 µM). The concentration gradients tested in our experiments were chosen based on previous studies with bovine sperm (Ho and Suarez, 2001).

***Measurements using CASA***. Two µL of sperm solution (test reagent plus sperm) was pipetted into the well of a four-chambered slide, inserted into the CASA system, and 11 fields were analyzed. Each sample condition was analyzed within one minute and 30 seconds (timed with stopwatch) after the addition of the test reagent to the sperm. Care was taken to minimize the time it took to perform the CASA analysis in order to avoid introducing time of exposure to the reagents as a variable, as some of these reagents would have an immediate effect on sperm motility.

***Experimental Design and Statistical Analysis.*** Three ejaculates from each of three stallions were used for each condition tested, providing nine points of data for each treatment to be analyzed. Averaged motion measures obtained via CASA were analyzed by two-way repeated measures ANOVA using SigmaStat software (San Jose, CA). When significant differences were detected, the Student-Newman-Keuls method was applied to assess all pairwise multiple comparisons ( $P < 0.05$ ).

## Results

### *Effect of Caffeine on Hyperactivation*

Our laboratory has recently defined the motion parameters associated with hyperactivation in stallion sperm using procaine (McPartlin et al., 2009). Therefore, in this study, procaine was used as a positive control for induced hyperactivation. In this experiment, procaine-treated sperm displayed all the motion measures indicative of hyperactivation (Table 2) which included decreases in VSL, STR and LIN, combined with increases in VCL and ALH as compared to the untreated control group ( $P < 0.05$ ). Moreover, assessment of the tracks displayed by procaine-treated sperm (Fig. 2B) also suggested that hyperactivation was induced as these sperm exhibited the star-shape pattern typical of hyperactivated stallion sperm (McPartlin et al., 2009).

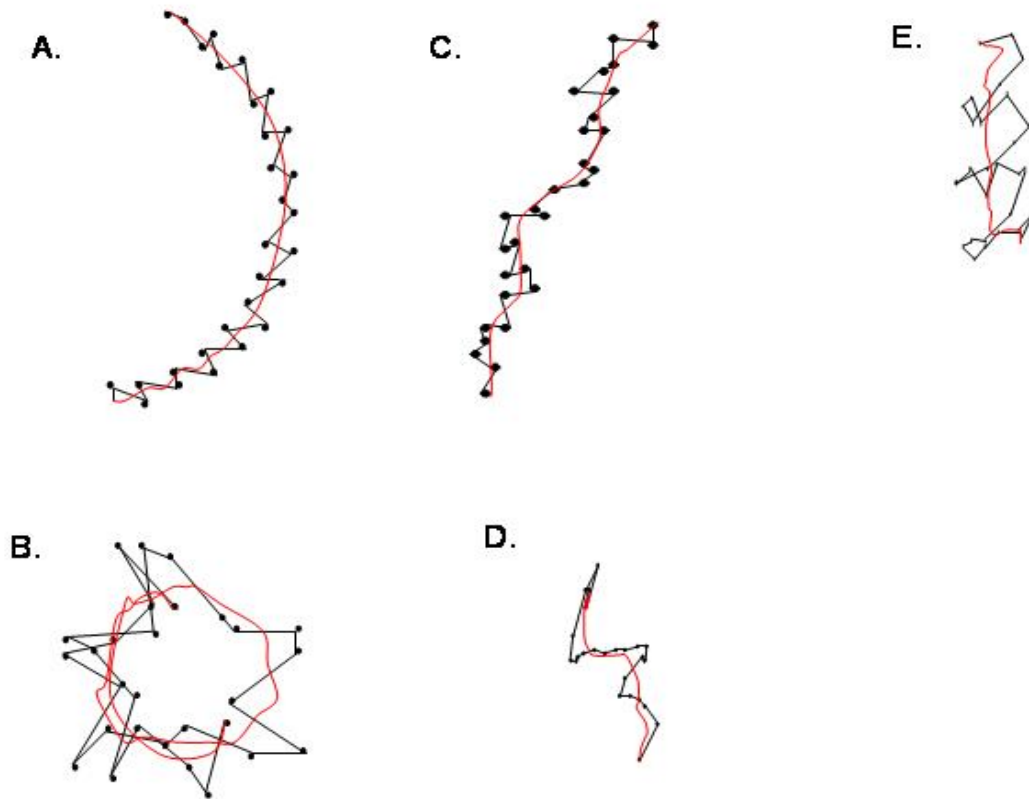
Caffeine has been shown to induce hyperactivation in other mammalian species through activation of  $\text{Ca}^{2+}$ -permeable cation channels found in the plasma membrane (Ho and Suarez, 2001). All the concentrations tested in this study induced a decrease in VSL as compared to untreated (negative control) sperm ( $P < 0.05$ ), as did the procaine-treated samples. While this could suggest hyperactivation, the combined analysis of VSL with other motion parameters did not support this notion (Table 2). That is, since the average VCL did not change ( $P > 0.05$ ) as compared to untreated controls, this would suggest that sperm were not displaying the typical star-shaped pattern. Moreover, ALH did not increase and STR and LIN did not significantly decrease, changes which are typically observed in hyperactivated sperm. Visual observation of the track displayed by caffeine-treated sperm indicated a great deal of similarity to that of the untreated sperm (Fig. 2C). Based on these results, hyperactivation was not induced in caffeine-treated sperm.



**Table 2.** Sperm motion measurements as assessed by computerized sperm motility analysis before and after addition of caffeine to stallion sperm.

Condition	VAP( $\mu\text{m}/\text{sec}$ )	VSL( $\mu\text{m}/\text{sec}$ )	VCL( $\mu\text{m}/\text{sec}$ )	ALH( $\mu\text{m}/\text{sec}$ )	BCF(Hz)	STR(%)	LIN(%)
Cap	120.5 $\pm$ 19.8 <sup>a</sup>	95.2 $\pm$ 25.1 <sup>a</sup>	194.5 $\pm$ 22.8 <sup>a</sup>	7.0 $\pm$ 1.2 <sup>a</sup>	35.6 $\pm$ 2.2 <sup>ab</sup>	75.2 $\pm$ 8.2 <sup>a</sup>	50.0 $\pm$ 10.3 <sup>a</sup>
Cap + Procaine	145.1 $\pm$ 37.8 <sup>b</sup>	64.0 $\pm$ 16.5 <sup>b</sup>	285.6 $\pm$ 57.5 <sup>b</sup>	10.9 $\pm$ 1.4 <sup>b</sup>	34.1 $\pm$ 2.6 <sup>a</sup>	43.9 $\pm$ 4.1 <sup>b</sup>	22.7 $\pm$ 1.3 <sup>b</sup>
2mM	106.5 $\pm$ 20.1 <sup>a</sup>	79.0 $\pm$ 21.1 <sup>b</sup>	181.8 $\pm$ 22.9 <sup>a</sup>	6.9 $\pm$ 0.8 <sup>a</sup>	39.5 $\pm$ 1.3 <sup>b</sup>	72.6 $\pm$ 8.2 <sup>a</sup>	46.2 $\pm$ 9.9 <sup>a</sup>
4mM	100.2 $\pm$ 18.5 <sup>a</sup>	76.5 $\pm$ 19.5 <sup>b</sup>	164.0 $\pm$ 16.9 <sup>a</sup>	5.7 $\pm$ 0.5 <sup>a</sup>	38.8 $\pm$ 0.9 <sup>ab</sup>	75.5 $\pm$ 5.8 <sup>a</sup>	49.3 $\pm$ 7.8 <sup>a</sup>
6mM	97.8 $\pm$ 15.4 <sup>a</sup>	72.9 $\pm$ 16.1 <sup>b</sup>	165.1 $\pm$ 13.3 <sup>a</sup>	5.6 $\pm$ 0.5 <sup>a</sup>	38.6 $\pm$ 2.0 <sup>ab</sup>	74.6 $\pm$ 6.0 <sup>a</sup>	47.6 $\pm$ 7.1 <sup>a</sup>
8mM	95.8 $\pm$ 18.9 <sup>a</sup>	74.3 $\pm$ 18.1 <sup>b</sup>	170.4 $\pm$ 15.4 <sup>a</sup>	6.3 $\pm$ 0.4 <sup>a</sup>	38.7 $\pm$ 0.9 <sup>ab</sup>	75.1 $\pm$ 6.3 <sup>a</sup>	46.9 $\pm$ 7.5 <sup>a</sup>
10mM	100.3 $\pm$ 16.4 <sup>a</sup>	74.5 $\pm$ 16.2 <sup>b</sup>	173.1 $\pm$ 15.5 <sup>a</sup>	6.2 $\pm$ 0.5 <sup>a</sup>	38.5 $\pm$ 1.0 <sup>ab</sup>	73.3 $\pm$ 5.1 <sup>a</sup>	45.4 $\pm$ 6.9 <sup>a</sup>
12mM	104.4 $\pm$ 32.0 <sup>a</sup>	70.8 $\pm$ 16.3 <sup>b</sup>	169.9 $\pm$ 15.0 <sup>a</sup>	6.3 $\pm$ 0.5 <sup>a</sup>	38.8 $\pm$ 2.3 <sup>ab</sup>	73.8 $\pm$ 4.5 <sup>a</sup>	44.9 $\pm$ 5.8 <sup>a</sup>

Different superscripts within column denote significant differences (P<0.05)



**Figure 2.** Tracings of the typical tracks displayed by stallion sperm, untreated or treated with agents to induce hyperactivated motility, as assessed by computerized sperm motility analysis (CASA). Black dots represent positions of sperm head, black lines represent true path of sperm, red lines represent average path of sperm. A) Untreated sperm, B) procaine, C) caffeine, D) thimerosal, E) thapsigargin. A and B reprinted with permission from McPartlin et al., 2009.

### ***Effect of Thimerosal on Hyperactivation***

Thimerosal is hypothesized to induce sperm hyperactivation via its stimulation of IP3-gated intracellular  $\text{Ca}^{2+}$  stores, specifically the redundant nuclear envelope (RNE; Ho and Suarez, 2001). Table 3 summarizes the data for this experiment; worth noting is that procaine-treated samples (positive controls) displayed all the typical measures and star-shaped pattern indicative of sperm hyperactivation, as expected. At first glance, thimerosal-treated sperm samples appeared to display motion measures typical for hyperactivation when compared to untreated controls, that is: A decrease in VSL and LIN (at  $\geq 40 \mu\text{M}$  concentrations;  $P < 0.05$ ). However, VAP decreased (100 and 120  $\mu\text{M}$ ;  $P < 0.05$ ) and there was no significant difference in ALH or VCL measures when compared to untreated controls; in hyperactivated sperm all of these parameters should increase. Furthermore, while LIN significantly decreased (concentrations  $\geq 40 \mu\text{M}$ ) as compared to the control condition, this response was not as dramatic as the change observed in the positive control (procaine); moreover, thimerosal-treated sperm moved significantly straighter than procaine-treated sperm. Visually, the track displayed by sperm treated with thimerosal appeared similar to untreated sperm at first, although much slower. After approximately 1-2 minute, at visual inspection most of the sperm appeared to vibrate in place rather than exhibit either forward or hyperactivated motility (Fig. 2D). This phenomenon seemed to be dose-dependent, as higher concentrations of thimerosal lead to this effect sooner.

**Table 3.** Sperm motion measurements as assessed by computerized sperm motility analysis before and after addition of thimerosal to stallion sperm.

Condition	VAP( $\mu\text{m}/\text{sec}$ )	VSL( $\mu\text{m}/\text{sec}$ )	VCL( $\mu\text{m}/\text{sec}$ )	ALH( $\mu\text{m}/\text{sec}$ )	BCF(Hz)	STR(%)	LIN(%)
Cap	120.5 $\pm$ 19.8 <sup>a</sup>	95.2 $\pm$ 25.1 <sup>a</sup>	194.5 $\pm$ 22.8 <sup>a</sup>	7.0 $\pm$ 1.2 <sup>a</sup>	35.6 $\pm$ 2.2 <sup>ab</sup>	75.2 $\pm$ 8.2 <sup>a</sup>	50.0 $\pm$ 10.3 <sup>a</sup>
Cap + Procaine	149.7 $\pm$ 22.0 <sup>b</sup>	66.2 $\pm$ 10.0 <sup>b</sup>	297.3 $\pm$ 33.5 <sup>b</sup>	11.0 $\pm$ 1.0 <sup>b</sup>	34.5 $\pm$ 1.4 <sup>ab</sup>	42.8 $\pm$ 2.6 <sup>b</sup>	23.5 $\pm$ 1.7 <sup>b</sup>
20 $\mu\text{m}$	109.9 $\pm$ 20.5 <sup>ac</sup>	81.9 $\pm$ 21.8 <sup>ab</sup>	198.2 $\pm$ 17.9 <sup>a</sup>	6.9 $\pm$ 0.9 <sup>a</sup>	39.3 $\pm$ 2.4 <sup>a</sup>	73.7 $\pm$ 8.3 <sup>a</sup>	43.5 $\pm$ 9.6 <sup>ac</sup>
40 $\mu\text{m}$	101.8 $\pm$ 18.7 <sup>ac</sup>	68.7 $\pm$ 15.5 <sup>b</sup>	210.4 $\pm$ 19.6 <sup>a</sup>	8.1 $\pm$ 0.4 <sup>a</sup>	36.2 $\pm$ 2.5 <sup>ab</sup>	67.0 $\pm$ 4.8 <sup>a</sup>	33.8 $\pm$ 5.1 <sup>bc</sup>
60 $\mu\text{M}$	104.3 $\pm$ 16.0 <sup>ac</sup>	59.7 $\pm$ 20.2 <sup>b</sup>	213.3 $\pm$ 20.7 <sup>a</sup>	8.5 $\pm$ 0.7 <sup>a</sup>	34.6 $\pm$ 3.6 <sup>ab</sup>	65.9 $\pm$ 5.7 <sup>a</sup>	33.9 $\pm$ 5.0 <sup>bc</sup>
80 $\mu\text{M}$	103.9 $\pm$ 18.2 <sup>ac</sup>	67.9 $\pm$ 14.7 <sup>b</sup>	212.7 $\pm$ 29.4 <sup>a</sup>	8.6 $\pm$ 0.6 <sup>a</sup>	32.4 $\pm$ 3.2 <sup>ab</sup>	63.7 $\pm$ 6.5 <sup>a</sup>	33.3 $\pm$ 4.5 <sup>bc</sup>
100 $\mu\text{M}$	89.6 $\pm$ 15.2 <sup>c</sup>	59.0 $\pm$ 10.7 <sup>b</sup>	192.9 $\pm$ 25.8 <sup>a</sup>	8.8 $\pm$ 0.3 <sup>a</sup>	30.5 $\pm$ 2.6 <sup>b</sup>	64.9 $\pm$ 5.0 <sup>a</sup>	31.6 $\pm$ 3.2 <sup>bc</sup>
120 $\mu\text{M}$	95.4 $\pm$ 22.6 <sup>c</sup>	60.4 $\pm$ 17.8 <sup>b</sup>	202.7 $\pm$ 33.4 <sup>a</sup>	9.0 $\pm$ 0.4 <sup>a</sup>	29.7 $\pm$ 3.7 <sup>b</sup>	63.5 $\pm$ 8.0 <sup>a</sup>	30.8 $\pm$ 5.5 <sup>b</sup>

Different superscripts within column denote significant differences (P<0.05)

### *Effect of Thapsigargin on Hyperactivation*

Thapsigargin may exert its effects on sperm hyperactivation by inhibiting the action of  $\text{Ca}^{2+}$ -ATPase pumps, thus precluding replenishment of intracellular  $\text{Ca}^{2+}$  stores which in turn leads to increased intracytoplasmic  $\text{Ca}^{2+}$  concentrations (Ho and Suarez, 2001). As in previous experiments and shown in Table 4, procaine-treated sperm displayed all the characteristics of hyperactivation. In thapsigargin-treated samples, decreases in VSL (concentrations  $\geq 30 \mu\text{M}$ ) and LIN (at  $\geq 30 \mu\text{M}$ ;  $P < 0.05$ ) as compared to the untreated negative control, could suggest sperm hyperactivation when taken alone; however, there was a decrease in VAP (concentrations  $\geq 20 \mu\text{M}$ ;  $P < 0.05$ ), suggesting that sperm were considerably slower than control sperm (Table 4). Moreover, VCL and ALH did not increase ( $P > 0.05$ ) for thapsigargin-treated samples as would be expected with changes in motility supportive of hyperactivation. Upon visual observation of sperm treated with thapsigargin, motility would cease within approximately one minute and sperm would appear to be vibrating in place rather than moving in a straight-line pattern. Consequently, the typical track traced for these sperm samples supports a decrease in overall motion as well as a deviation from the average path, consistent with sperm vibrating in place (Fig. 2E). Therefore, thapsigargin was unable to support hyperactivation in stallion sperm.

**Table 4.** Sperm motion measurements as assessed by computerized sperm motility analysis before and after addition of thapsigargin to stallion sperm.

Condition	VAP( $\mu\text{m}/\text{sec}$ )	VSL( $\mu\text{m}/\text{sec}$ )	VCL( $\mu\text{m}/\text{sec}$ )	ALH( $\mu\text{m}/\text{sec}$ )	BCF(Hz)	STR(%)	LIN(%)
Cap	120.5 $\pm$ 19.8 <sup>ab</sup>	95.2 $\pm$ 25.1 <sup>a</sup>	194.5 $\pm$ 22.8 <sup>a</sup>	7.0 $\pm$ 1.2 <sup>a</sup>	35.6 $\pm$ 2.2 <sup>a</sup>	75.2 $\pm$ 8.2 <sup>a</sup>	50.0 $\pm$ 10.3 <sup>a</sup>
Cap + Procaine	133.5 $\pm$ 40.2 <sup>a</sup>	59.2 $\pm$ 15.0 <sup>ab</sup>	272.6 $\pm$ 62.2 <sup>b</sup>	10.4 $\pm$ 1.3 <sup>b</sup>	35.5 $\pm$ 2.6 <sup>a</sup>	45.1 $\pm$ 4.2 <sup>b</sup>	23.4 $\pm$ 1.4 <sup>b</sup>
10 $\mu\text{m}$	82.0 $\pm$ 23.9 <sup>bc</sup>	62.8 $\pm$ 23.6 <sup>ab</sup>	146.6 $\pm$ 30.8 <sup>a</sup>	5.9 $\pm$ 0.5 <sup>a</sup>	33.3 $\pm$ 5.4 <sup>a</sup>	73.4 $\pm$ 7.1 <sup>a</sup>	43.1 $\pm$ 8.1 <sup>ac</sup>
20 $\mu\text{m}$	70.0 $\pm$ 19.2 <sup>c</sup>	77.6 $\pm$ 53.2 <sup>ab</sup>	135.1 $\pm$ 30.5 <sup>a</sup>	6.1 $\pm$ 0.6 <sup>a</sup>	32.7 $\pm$ 6.3 <sup>a</sup>	72.6 $\pm$ 7.6 <sup>a</sup>	40.0 $\pm$ 7.2 <sup>ac</sup>
30 $\mu\text{M}$	58.9 $\pm$ 12.4 <sup>c</sup>	43.7 $\pm$ 12.5 <sup>b</sup>	120.1 $\pm$ 20.5 <sup>a</sup>	6.5 $\pm$ 0.6 <sup>a</sup>	32.3 $\pm$ 7.1 <sup>a</sup>	71.3 $\pm$ 6.7 <sup>a</sup>	37.6 $\pm$ 5.7 <sup>c</sup>
40 $\mu\text{M}$	54.3 $\pm$ 10.8 <sup>c</sup>	39.3 $\pm$ 11.9 <sup>b</sup>	117.6 $\pm$ 16.3 <sup>a</sup>	6.5 $\pm$ 0.5 <sup>a</sup>	34.4 $\pm$ 4.7 <sup>a</sup>	70.7 $\pm$ 6.4 <sup>a</sup>	35.0 $\pm$ 6.2 <sup>c</sup>
50 $\mu\text{M}$	49.2 $\pm$ 6.0 <sup>c</sup>	33.7 $\pm$ 7.8 <sup>b</sup>	108.2 $\pm$ 14.5 <sup>a</sup>	6.4 $\pm$ 1.2 <sup>a</sup>	30.4 $\pm$ 6.4 <sup>a</sup>	66.4 $\pm$ 7.0 <sup>a</sup>	32.6 $\pm$ 5.5 <sup>c</sup>

Different superscripts within column denote significant differences (P<0.05)

## Discussion

This study tested the effect of caffeine, thimerosal and thapsigargin, three agents known to support sperm hyperactivation in other species (Mujica et al., 1994; Ho and Suarez, 2001, 2003; Ishijima et al., 2006; Marquez et al., 2007), in their ability to induce this pattern of motility in stallion sperm. Unexpectedly, all three pharmacological agents failed to induce a pattern of motility consistent with hyperactivation in stallion sperm. For instance, the track traced by sperm treated with caffeine closely resembled that displayed by untreated sperm (Fig. 2A, 2C); similarly, most CASA measurements did not differ between these two conditions (Table 2), suggesting that caffeine had little to no effect on sperm motility. Conversely, thimerosal and thapsigargin exerted a negative effect on sperm motility. This started as a slow non-progressive motion that eventually led to sperm barely vibrating in place or not moving at all, after approximately 1-2 minutes of exposure to the reagent (Fig. 2D, 2E). The inability of these agents to induce stallion sperm hyperactivation was in contrast to the effects observed in sperm treated with procaine which was used as a positive control in this study (McPartlin et al., 2009). All CASA measurements taken from procaine-treated sperm showed the decrease in VSL, STR and LIN and the increase in VCL and ALH that is a characteristic for hyperactivated sperm. Moreover, procaine-treated sperm displayed the star-shaped pattern track that is typical for stallion sperm hyperactivation (Fig. 2B; McPartlin et al., 2009).

While caffeine did not have any noticeable effects on stallion sperm motility in this study, it has been shown to induce hyperactivation in bovine and cynomolgus macaque monkey sperm (Ho and Suarez, 2001; Ishijima et al., 2006). Interestingly, caffeine is believed to induce sperm hyperactivation by promoting the activation of  $\text{Ca}^{2+}$ -permeable cation channels found in

the plasma membrane (Ho and Suarez, 2001). Since procaine also relies on increasing the membrane permeability to extracellular  $\text{Ca}^{2+}$  and is able to support stallion sperm hyperactivation (McPartlin et al., 2009), it is unlikely that the mechanism of action of caffeine is responsible for its lack of effect in stallion sperm.

In contrast to caffeine and procaine, both thimerosal and thapsigargin depend upon intracellular  $\text{Ca}^{2+}$  stores to induce sperm hyperactivation. Thimerosal, which can induce hyperactivation in murine and bovine sperm (Ho and Suarez, 2001; Marquez et al., 2007), is an  $\text{IP}_3$  agonist. In this regard, this agent is postulated to activate  $\text{IP}_3$ -gated intracellular  $\text{Ca}^{2+}$  stores in the sperm, such as the RNE (Ho and Suarez, 2001). Similarly, thapsigargin, an agent reported to hyperactivate bovine sperm, increases the sperm's intracytoplasmic  $\text{Ca}^{2+}$  concentrations through its action as a  $\text{Ca}^{2+}$ -ATPase inhibitor, thus precluding the replenishment of these stores (Ho and Suarez, 2001, 2003).

While we expected at least some of the agents tested in this study to induce stallion sperm hyperactivation similarly to what has been shown for procaine (McPartlin et al., 2009), it is not surprising that stallion sperm may display different physiological responses to pharmacological reagents as compared to other species. For instance, work in our laboratory has shown that agents widely used to support or inhibit protein tyrosine phosphorylation, a marker of sperm capacitation, did not exert the same effects when incubated with stallion sperm. A clear example is the ability of dbcAMP and IBMX to support murine and bovine, but not stallion sperm capacitation (Visconti et al., 1995ab; Galantino-Homer et al., 1997; McPartlin et al., 2008). Similarly, the protein kinase A inhibitor H89 was unable to abrogate time-dependent increases in protein tyrosine phosphorylation in stallions sperm incubated in capacitating conditions



(McPartlin and Bedford-Guaus, *unpublished results*), as reported in other species (Visconti et al., 1995ab; Galantino-Homer et al., 1997). Alternatively, the inability of these three agents to induce stallion sperm hyperactivation could potentially arise from species-specific differences in the mechanisms that regulate hyperactivation. However, the molecular pathways controlling hyperactivation appear to be consistent across all other species studied (Brokaw and Nagayama, 1985; Bannai et al., 2000; Ho and Suarez, 2003; Ignatz and Suarez, 2005; Marquez et al., 2007; Schlingmann et al., 2007) making the possibility of true physiological differences between these and the equine rather unlikely.

In conclusion, caffeine, thimerosal and thapsigargin were found to be unsuccessful in inducing hyperactivation in equine sperm. The regulation of stallion sperm hyperactivation requires additional studies to investigate the possibility of true physiological differences that may exist between the horse and other species known. To date, procaine is the only reagent known to effectively and consistently stimulate sperm hyperactivation in the stallion.

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