Original Article

Effect of Pentoxifylline on the Motility Characteristics and Viability of Spermatozoa in Asian Elephants *(Elephas maximus)* with Low Semen Quality

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Abstract

To investigate the effects of pentoxifylline (PTX) to enhance the motility and fertilization capacity of semen samples with the low-motile sperm in Asian elephants, fourteen semen collection attempts in 9 elephant bulls by manual stimulation were undertaken and eleven ejaculates fitted the criteria of investigation (0-30% motility). They were divided into two groups: poor-motile (0-9% motility) and low-motile (10-30% motility) sperm groups. Fresh semen samples were divided as a control group and 3 experimental groups that were supplemented with PTX at a final concentration of 0.5, 1.0 and 2.0 mg/ml, respectively. The semen samples were incubated at 37°C for 15 and 30 mins and stained with VIADENT media for viability assessment. Sperm motility and viability were tested using computer-assisted semen analysis. PTX added to the semen did not significantly improve the percentage of the total and progressive motility, motility characteristics and viability of sperm in either the poor-or low-motile groups. However, at 30 min, in the low-motile sperm group, PTX treatment could maintain the percentage of total and progressive motility, path velocity and progressive velocity at a higher level than the control group. The present study indicated that PTX added to low motility semen did not increase elephant semen quality. However, it may partially have a tendency to maintain sperm motility and sperm movement characteristics.

Keywords : CASA, elephant, pentoxifylline, semen

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บทคัดย่อ

ผลของ Pentoxifylline ต่อลักษณะการเคลื่อนที่และความมีชีวิตของเซลล์อสุจิช้างเอเชีย *(Elephas maximus)* ที่มีคุณภาพน้ำเชื้อต่ำ

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การศึกษาผลของ Pentoxifylline (PTX) ต่อการเคลื่อนที่ ลักษณะการเคลื่อนที่ และความมีชีวิตของเซลล์อสุจิในน้ำเชื้อช้างที่มีคุณภาพต่ำ จากการรีดเก็บน้ำเชื้อจำนวน 14 ครั้ง ในช้างจำนวน 9 เชือก สามารถคัดเลือกน้ำเชื้อที่มีร้อยละการเคลื่อนที่อยู่ระหว่างร้อยละ 0-30 จำนวน 11 ตัวอย่าง แบ่งน้ำเชื้อออกเป็น 2 กลุ่ม คือ poor-motile (ร้อยละ 0-9) และ low-motile (ร้อยละ 10-30) และน้ำเชื้อจะถูกแบ่งเป็นกลุ่มควบคุม และกลุ่มทดลองอีก 3 กลุ่ม โดยการเสริมด้วย Pentoxifylline (PTX) ขนาด 0.5 1.0 และ 2.0 มก./มล. ตามลำดับ และส่วนที่ 4 ที่ไม่เติม PTX เป็นกลุ่มควบคุม ทำการบ่มที่อุณหภูมิ 37°ซ. แล้วตรวจด้วยเครื่อง CASA ที่เวลา 15 และ 30 นาที ซึ่งย้อมสีด้วย VIADENT media เพื่อประเมินความมีชีวิต ผลที่ได้พบว่า PTX ในทุกๆ ความเข้มข้นไม่สามารถเพิ่มร้อยละและลักษณะการเคลื่อนที่และความมีชีวิตของเซลล์อสุจิอย่างมีนัยสำคัญ แต่อย่างไรก็ตามที่เวลา 30 นาที ใน low-motile group การเติม PTX ในทุกๆความเข้มข้นมีแนวโน้มที่จะสามารถรักษาร้อยละในการเคลื่อนที่เส้นทางการเคลื่อนที่ และความเร็วในการเคลื่อนที่ไปข้างหน้า ให้สูงกว่ากลุ่มควบคุมได้ ดังนั้นการเติม PTX ในน้ำเชื้อช้างที่มีคุณภาพต่ำไม่สามารถเพิ่มร้อยละและลักษณะในการเคลื่อนที่และความมีชีวิตของเซลล์อสุจิ แต่มีแนวโน้มในการรักษาร้อยละและระดับความเร็วในการเคลื่อนที่

คำสำคัญ : CASA ช้าง pentoxifylline น้ำเชื้อ

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Introduction

Male Asian elephant *(Elephas maximus)* subfertility attributed to poor motility and immotility of spermatozoa, has been in a high proportion of overall ejaculates obtained via the manual collection technique. From twenty samples of domesticated elephant bull semen collected weekly for two months, the medians of progressive motility was 0% (Thongtip et al., 2001; 2004). By contrast, there have been reports of good semen quality obtained via electroejaculation in wild African elephants. Results have shown that eight of nine samples of elephant semen contained high concentrations of progressively motile spermatozoa and the mean ejaculated sperm motility was 70% (Howard et al., 1984). However, due to the anesthetic risk involved, general anesthesia for electroejaculation is not often performed on domesticated elephants. Thus, the manual collection technique has been developed for use with domesticated elephants (Schmitt and Hildebrandt, 1998). An understanding of the underlying causes of poor semen quality in Asian elephant spermatozoa obtained via manual collection technique remains unclear. However, searching for artificial media or chemicals *in vitro* that can initiate elephant sperm motility may solve these problems. Pentoxifylline (PTX) has been used to enhance testicular sperm motility in the *in vitro* culture of testicular tissue in humans (Angelopoulos et al., 1999). Spontaneously immotile epididymal and testicular spermatozoa have also initiated

motility by PTX stimulation (Terriou et al., 2000). In addition, it has been successfully used to enhance sperm movement in electroejaculated baboon sperm (Cseh et al., 2000), to hyperactivate human sperm (Kay et al., 1993), to inhibit the production of reactive oxygen species (Gavella et al., 1991; Yovich, 1993) and to improve acrosome reaction (Tesarik et al., 1992). Probably PTX enhances sperm motility by inhibiting the phosphodiesterase, leading to an increase in intracellular cAMP concentration and activation of cAMP-dependent kinases (Tash et al., 1986). One mg PTX/mL (3.6 mM) has been used by Yovich et al (1990) on human spermatozoa that were treated before *in vitro* fertilization (IVF) in couples with severe male factor infertility. Furthermore, Nassar et al.(1998) demonstrated that the maximum effect of pentoxifylline on sperm motility characteristics and on the penetration of the cervical mucus *in vitro* occurred when spermatozoa were incubated with a dose of 1 mg PTX/mL for 30 minutes. However, there has been no data on PTX doses for elephant spermatozoa. In the present study, we investigated the effects of PTX on sperm motility and motion parameters in elephant semen with low-motile sperm using computer-assisted semen analysis (CASA).

Materials and Method

Animal and semen collection: Ejaculated semen was obtained from 9 Asian elephant bulls held captive at the Thai Elephant Conservation Center, Forest Industry Organization. The elephant bulls were between 10- and 45-year old. Semen samples were obtained by manual collection technique. In brief, before starting, the faeces was removed and the rectum was cleaned with tap water. Then, protrusion and erection of the penis was accomplished by rectal massage of the pelvic portion of the urethra, near the seminal colliculus. Following protrusion, the penis was cleaned and dried to reduce environmental contamination of the semen sample. A collection sleeve was then placed over the end of the penis and massage of the pelvic urethra began. As an ejaculatory response was detected, massage included the region of the ampulla of the ductus deferens and expulsion of the sperm rich fraction was obtained (Schmitt and Hildebrandt, 1998). A total of fourteen ejaculates without urine contamination were collected from 9 elephant bulls. The maximum total motility was 90% while the minimum total motility was 0%. Eleven out of 14 ejaculates met the criteria of 0-30% total motility used in this study. The ejaculated semen was evaluated immediately for concentration and motility by using CASA. Ejaculates showing a total sperm motility of 10-30% were classified as the low-motile group (n=3) whereas ejaculates showing a total sperm motility of 0-9% were classified as the poor-motile group (n=8).

Pentoxifylline treatment: A working solution of 100 mg/ml PTX was prepared in SP-TALP (2.0mM $CaCl_2$, 3.1mM KCl, 0.4mM $MgCl_2$, 100mM NaCl, 25mM, $NaHCO_3$, 0.3mM NaH_2PO_4, 1.0mM sodium pyruvate, 21.6mM sodium lactate, 10mM Hepes, and 6 mg/ml BSA) (Parrish et al., 1988) and 5, 10 and 20 µl of PTX solution were added to 995, 990 and 980 µl of fresh s emen containing $20x10^6$/ml spermatozoa yielding a final concentration of 0.5, 1, and 2 mg PTX/ml, respectively. An aliquot of 1 ml of fresh semen without PTX was used as the control. The control and treated semen samples were mixed for 1 min and incubated at 37°C without shaking. One hundred µl of treated semen were stained, after 15 and 30 min with VIADENT media (1:1) and incubated at 37°C in the dark for 2 min; and 5 µl was then placed in the CASA evaluation chamber (MicroCell slide; Conception Technologies, La Jolla, CA, USA) and allowed to warm for 1 min before the start of the analysis. A working solution of 10 µg/ml VIADENT media (Hoechst 33258; Hamilton-Thorne Biosciences, Beverly, MA, USA) was freshly prepared before the experiment (Wessel and Althouse, 2006).

Computer assisted semen analysis (CASA): An IVOS motility analyzer (IVOS model 12.0, Hamilton-Thorne Biosciences, Bevery, MA, USA) was used for semen analysis. Total and progressive motility, sperm motion

parameters [path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR) and linearity (LIN)] and viability of each sample were determined using the "VIADENT" option. The VIADENT stain (Hoechst 33258) is a vital stain that marks only cells with non-intact membranes. The VIADENT option using visible (blue light emitting diode) light to determine cell count and motility was performed prior to determination of the number of non-viable cells with fluorescent light.

CASA analysis set up: The settings used for semen analysis are shown in Table 1. At the onset of each experiment, it was verified that the setting permitted accurate differentiation of motile sperm and non-motile sperm or debris utilizing the "playback" option. During "playback", the motions of sperm in the previous field were replayed: a green dot was located over the head of all motile spermatozoa and a red dot was positioned over those of non-motile sperm. When an error was detected, the setting was adjusted until the problem was corrected (Nassar et al., 1999).

Statistical analysis: Data is expressed as mean±SD. The percentage of motility, viability and motion parameters between control and treatment groups was compared using the Kruskal-Wallis Multiple-Comparison Z-Value Test. The level of significance was set at $p<0.05$.

Results

The percentages of total and progressive motility, viability and motion parameters (VAP, VSL, VCL, ALH, BCF, STR and LIN) of spermatozoa in the low- and poor-motile groups immediately observed after semen collection are shown in Table 2. The percentages of total motility, viability and STR in the low-motile group were significantly ($p<0.05$) higher than those in the poor-motile group.

The effects of PTX on the motility and viability of the low- and poor-motile groups are presented in Table 3 and 4. There was no statistically significant difference in the percentages of total and progressive motility, viability and motion parameters between control and PTX treated groups evaluated at 15 and 30 min incubation periods. In the low-motile group, the percentages of total and progressive motility, VAP and VSL of spermatozoa treated with 0.5, 1, 2 mg/ml and incubated for 30 mins had a tendency towards higher values than those of the control group (Table 4).

Discussion

Poor motility and immotility of spermatozoa in the male Asian elephant *(Elephas maximus)* has frequently been found after manual semen collection and the use of various chemicals to enhance the activity of spermatozoa may help to improve semen quality for storage and artificial insemination. In the present study, we tested the beneficial effects of Pentoxifylline (PTX) in improving the motility of the spermatozoa from the poor- and low-motile semen of Asian elephants. Supplementation of PTX in low quality semen (poor- or low- motile semen) did not significantly effect the percentages of total and progressive motility, viability and sperm motion parameters. The results of our study in Asian elephant semen were different from that previously reported in human sperm after PTX stimulation (McKinney et al., 1994). However, our results were in agreement with a previous investigation in normospermic human sperm treated with 3.6 mM PTX (1 mg/ml) which suggested that mean sperm motility did not increase after PTX incubations *in vitro* (Lewis et al., 1993). Furthermore, investigators also found that the number of motile sperm did not increase after PTX treatment (Yovich et al., 1990; Tesarik et al., 1992). Nassar et al.(1999) had exposed PTX to human spermatozoa for examining its effects on sperm motility characteristics. They found that PTX (1 mg/ml; 3.6mM, incubation period: 30 min) did not significantly change the sperm motility percentage, average path velocity (VAP), straight-line velocity (VSL) and beat cross frequency (BCF) of spermatozoa in normozoospermic or asthenozoospermic samples. However, it significantly

Table 1. CASA set up for elephant semen analysis

Analysis set up	Values
Frames Acquired	30
Frame rate	60 Hz
Minimum cell size	5 pixels
VAP Cutoff	20 μm/s
Prog. Min VAP	80 μm/s
VSL Cutoff	0 μm/s
Cell Size	5 pixels
Cell Intensity	90
Magnification	1.89
Video Frequency	60
LED Illumination Intensity	2194
IDENT Illumination Intensity	3788
Integrating Time	1 Frames

Table 2. Percentages of motility, viability and motion characteristics of elephant spermatozoa in the poor-and low-motile groups, observed immediately after semen collection.

Parameter	Poor-motile sperm (motility 0-9%)	Low-motile sperm (motility 10-30%)
Total motility (%)	3.25 ± 3.01[a]	19.00 ± 7.00[b]
Progressive motility (%)	0.62 ± 0.91	6.33 ± 2.08
Viable (%)	59.28 ± 21.28[a]	66.33 ± 21.36[b]
Path velocity (VAP; μm/s)	63.55 ± 36.34	76.30 ± 10.15
Progressive velocity (VSL; μm/s)	50.82 ± 33.45	60.93 ± 11.39
Track speed (VCL; μm/s)	104.80 ± 47.25	122.33 ± 12.34
Lateral amplitude (ALH; μm)	5.96 ± 3.65	5.86 ± 0.72
Beat frequency (BCF; Hz)	33.68 ± 16.45	35.13 ± 2.27
Straightness (STR; %)	62.12 ± 29.11[a]	70.33 ± 11.59[b]
Linearity (LIN; %)	40.62 ± 24.14	56.00 ± 25.94

Note : values are means±SD, superscripts in the same rows differ significantly ($p<0.05$)

Table 3. Effects of PTX treatment on motility, viability and motion characteristics of spermatozoa in poor-motile semen.

Motion characteristics	15 min				30 min			
	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml
Motility (%)	4.1 ± 7.3	4.0±5.0	4.3 ± 7.2	5.0 ± 8.2	3.4 ± 7.1	2.7 ± 2.7	2.9±3.8	2.5±3.9
Progress (%)	1.0 ± 1.6	1.0±1.7	0.9 ± 1.5	1.9 ± 4.6	1.1 ± 1.9	0.8 ± 0.8	1.0±1.3	0.6±0.8
Viable (%)	64.0 ± 30.2	60.0±31.2	63.0 ± 27.8	61.0 ± 34.0	66.0 ± 26.7	68.0 ± 23.2	68.0±23.1	68.0±24.0
VAP (μm/s)	61.0 ± 39.1	57.0±40.8	45.0 ± 31.5	47.0 ± 40.6	50.0 ± 37.2	46.0 ± 39.8	59.0±51.2	38.0±47.4
VSL (μm/s)	50.0 ± 34.6	48.0±36.5	35.0 ± 31.5	39.0 ± 40.6	43.0 ± 32.3	39.0 ± 36.7	50.0±43.5	32.0±42.8
VCL (μm/s)	92.0 ± 54.0	95.0±54.9	76.0 ± 2.5	72.0 ± 57.0	80.0 ± 56.0	77.0 ± 61.1	83.0±67.8	52.0±59.0
ALH (μm)	4.0 ± 3.6	5.0±3.9	2.5 ± 2.5	3.0 ± 2.8	2.6 ± 2.5	5.0 ± 4.1	3.3±2.6	2.3±2.5
BCF (Hz)	30.0 ± 18.2	29.0±15.9	27.0 ± 15.9	23.0 ± 18.7	30.0 ± 21.9	21.0 ± 17.1	24.0±18.3	18.0±19.1
STR (%)	57.0 ± 34.0	57.0±23.7	56.0 ± 33.9	52.0 ± 40.0	59.0 ± 39.0	50.0 ± 38.6	55.0±38.6	40.0±42.0
LIN (%)	38.0 ± 24.5	36.0±2.3	37.0 ± 33.9	34.0 ± 27.5	38.0 ± 27.2	31.0 ± 26.1	40.0±28.6	29.0±31.9

Note: values are means±SD, motility: total motility, progress: progressive motility, viable: viable sperm

Table 4. Effects of PTX treatment on motility, viability and motion characteristics of spermatozoa in low-motile semen.

Motion characteristics	15 min				30 min			
	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml	Control	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml
Motility (%)	15.0 ± 9.5	13.7±6.4	15.5 ± 12.0	16.3 ± 9.4	8.3 ± 7.6	10.3 ± 4.0	11.3±5.7	13.67±7.4
Progress (%)	9.3 ± 7.8	7.7±8.1	10.0 ± 9.9	9.0 ± 9.6	2.7 ± 1.1	4.7 ± 3.8	5.0±4.4	7.7±9.8
Viable (%)	66.7 ± 18.8	61.3±28.6	82.5 ± 14.8	67.0 ± 24.2	63.3 ± 24.0	62.3 ± 24.1	61.7±24.0	58.7±28.5
VAP (μm/s)	112.8±26.0	95.3±22.8	105.7 ± 21.8	85.4 ± 19.8	77.6 ± 10.5	98.1 ± 31.8	89.3±27.4	84.1±27.5
VSL (μm/s)	99.5 ± 23.0	81.6±24.8	93.5 ± 20.2	71.2 ± 19.9	60.4 ± 12.8	84.1 ± 36.9	74.8±29.2	69.4±30.4
VCL (μm/s)	159.1 ± 31.7	138.1±14.3	143.7 ± 33.0	128.4 ± 24.4	131.6 ± 12.1	135 ± 18.6	126.3±21.5	125±18.8
ALH (μm)	6.0±0.4	6.4±1.0	5.3 ± 0.5	5.6 ± 0.6	5.6 ± 1.3	5.6 ± 1.1	4.9±1.2	5.4±1.1
BCF (Hz)	33.2 ± 5.1	35.1±6.8	31.5 ± 3.7	34.9 ± 4.9	34.5 ± 4.1	32.8 ± 5.9	34.6±4.9	34.3±6.2
STR (%)	80.0 ± 2.6	76.3±7.5	80.0 ± 1.4	74.3 ± 6.5	69.3 ± 4.0	77.0 ± 13.7	74.7±11.1	73.7±13.0
LIN (%)	43.0±7.2	53.3±11.1	60.5 ± 0.7	51.3 ± 18.5	57.6 ± 3.0	57.3 ± 20.1	54.3±16.3	51.0±6.0

Note: values are means±SD, motility: total motility, progress: progressive motility, viable: viable sperm

increased curvilinear velocity (VCL), the amplitude of lateral head displacement (ALH) and hyperactivated motility (HA), and significantly decreased linearity (LIN) of spermatozoa from both samples. Although some motion characteristics were significantly increased, the percentage of sperm motility and some other motion characteristics were not changed. The reasons for unchanged percentage of sperm motility was discussed. PTX-treated sperm that underwent an intrauterine insemination (IUI) preparation program were not evaluated immediately after washing or before separation, where it might have been possible to observe more significant effects. For elephant sperm, the duration effect of PTX on sperm motility may be short and PTX may lead to a premature reduction of motility due to the rapid loss of cellular energy source. However, further studies need to be done to verify this hypothesis. Furthermore, even 30 mins. of incubation time has been proposed for the maximum effect of PTX (Yovich et al., 1990; Kovacevic et al., 2006). Here, in our present study, both 15 and 30 min, incubation times had no impact on sperm motility and motion parameters. Thus, immediate evaluation after PTX is added may be tried in the future.

A concentration of 1†mg/ml or 3.6mM of PTX has been accepted for its effect on enhancing human sperm motility (Lewis et al., 1993; Nassar et al., 1999). In this present study, the concentrations added including of 0.5, 1.0 and 2.0 mg/ml of PTX did not significantly effect elephant sperm motility and motion parameters. It may be assumed that the efficiency of added PTX may be interfered with by some factors in the elephant semen which remain unclear. So, further studies need to be done.

In the present study, the percentage of sperm viability or membrane intact sperm in both the poor- and low-motile groups was higher than percentage of total motility. The occurrence of immotile but membrane intact sperm in fish (Stoss, 1983) and human spermatozoa has been reported (Angelopoulos et al., 1999). PTX has been utilized to stimulate sperm motility *in vitro* without any effect on the sperm membrane integrity (Mladenovic et al., 1994). The underlying cause of immotile sperm with the membrane intact may, in part, be due to the low metabolic state (Makler et al., 1980). In addition, pre-freeze PTX treatment fails to improve post-thaw motility and this may due to the low metabolic state of post-thaw sperm (Stanic et al., 2002). A decreasing of cAMP concentration after the freeze-thaw procedure has been reported in human sperm (Wang et al., 1993). Immotile spermatozoa in ejaculates have been attributed to pathologic phenomena including abnormalities of the sperm tail, senescent degeneration, sperm mixing with seminal plasma or delayed epididymal transport (Wilson et al., 1988). In elephants, spermatozoa are stored in the ampulla gland before ejaculation. The storage time may be prolonged if the elephants have no chance to ejaculate. It may be possible that a low metabolic state, senescent degeneration and being un-diluted or un-mixed with seminal plasma due to an incomplete ejaculate may be among the culprits of this phenomenon in elephant spermatozoa. Thus, the underlying mechanisms of low motility as well as immotile sperm and how to improve the sperm motility of the ejaculated semen in Asian elephants require further study. In our previous data, some bull elephants whose semen had not been collected for a long time would ejaculate semen of poor quality with low sperm motility. In domesticated elephants, the bull elephant can mate naturally everyday for one month while the female is in heat (Ronnachit Rugsri, personal communication). Thus, it is possible to expect that frequent ejaculation during a series of matings releases poor quality semen and when the female ovulates at the end of the heat period (one month), the semen of good quality is ejaculated too. There has been a report on three collections per week with selected 30-day periods, by manual collection from one Asian elephant bull (Schmitt and Hildebrandt, 1998). Unfortunately, in that report, they showed only the volume of obtained semen and the results at each time were varied. Conversely,

in Gulf Coast Native rams in Louisiana, USA, three collections per week by electroejaculation for 3 weeks always revealed good sperm motility (>70%) (Nel-Themaat et al., 2006). Thus, collection time interval and compareson of semen collection techniques may require further studies.

In conclusion, ejaculated Asian elephant semen treated with PTX did not significantly increase the percentage of sperm motility and motion parameters in either the poor- or low-motile groups. However, PTX appears to maintain sperm motility and sperm movement characteristics.

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