Original Article

Relationship between Seminal and Serum Calcium Concentration with Semen Quality in the Asian Elephant (*Elephas maximus*)

Somsajee Sivilaikul[1] Amornrat Jitprom[1] Ajaree Kularb[1] Kornchai Kornkaewrut[1] Piyawan Suthanmaphinuth[1] Sittidet Mahasawangkul[2] Kulnasan Saikhun[5] Worawidh Wajjwalku[1] Sitthawee Thongtipsiridech[1, 3, 4*]

Abstract

The purpose of this study is to identify the relationship of calcium concentration in seminal plasma on elephant semen quality including of volume, concentration, pH and percentage of progressive motility, dead sperm and abnormal morphology, respectively. Semen collection and evaluation were done in 9 elephants of Thai Elephant Conservation Centre, National Elephant Institute, Forest Industry Organization at Lampang. Calcium in seminal plasma was measured by using colorimetric method. Data were analyzed by using Linear Regression. The results revealed that amount of calcium in seminal plasma was negative correlated with only percentage of progressive motility ($p<0.05$). Ejaculates were separated into three groups based on their progressive motility percentage including of low, moderate and high-motile semen (0-5%, >5-40% and >50%, respectively) and analyzed by using Repeated measure ANOVA. The results revealed that percentage of dead sperm and abnormal morphology and concentration of calcium in seminal plasma of all groups were significantly difference ($p<0.05$). Percentage of dead sperm and seminal calcium concentration was highest in low-motile group ($p<0.05$). The highest percentage of abnormal morphology was also found in the low-motile group ($p<0.05$). However, serum calciums were not different among each group. Thus, calcium in seminal plasma may be effected to semen quality; i.e. progressive motility of Asian elephant. However, there are other factors that can influence elephant semen quality, thus, more information are needed to improve the better knowledge in male elephant reproductive biology.

Keywords: Asian elephant (*Elephas maximus*), calcium, semen quality

[1]*Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus Nakhonpathom, 73140, Thailand.*
[2]*Thai Elephant Conservation Centre, National Elephant Institute, Forest Industry Organization, Hang Chart, Lampang, 52000, Thailand.*
[3]*Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus Nakhonpathom 73140, Thailand.*
[4]*Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok, Thailand.*
[5]*Institute of Molecular Biosciences, Mahidol University, Nakhonpathom, 73140, Thailand.*
Corresponding author E-mail: *fvetnit@ku.ac.th, nthongtip@yahoo.com*

บทคัดย่อ

ความสัมพันธ์ระหว่างความเข้มข้นของแคลเซียมในน้ำเลี้ยงเซลล์อสุจิและซีรั่มกับคุณภาพของน้ำเชื้อช้างเอเชีย

โสมศจี ศิวิลัยกุล[1] อมรรัตน์ จิตร์พรหม[1] อาจารีย์ กุหลาบ[1] กรไชย กรแก้วรัตน์[1]
ปิยวรรณ สุธรรมาภินันท์[1] สิทธิเดช มหาสาวังกุล[2] กุลณสรรค์ สายขุน[4] วรวิทย์ วัชชวัลคุ[1] สิทธวีร์ ทองทิพย์ศิริเดช[1, 3, 4]*

การศึกษาหาความสัมพันธ์ระหว่างระดับแคลเซียมในน้ำเลี้ยงเซลล์อสุจิกับค่าพารามิเตอร์ต่างๆที่แสดงถึงคุณภาพของน้ำเชื้อช้าง ได้แก่ ปริมาตร (Volume) ความเป็นกรด-ด่าง (pH) ร้อยละการเคลื่อนที่ไปข้างหน้า (% Progressive motility) ร้อยละตัวตาย (% Dead sperm) ร้อยละความผิดปกติของเซลล์อสุจิ (% Abnormal morphology) และความเข้มข้นของเซลล์อสุจิช้าง (Concentration) ของศูนย์อนุรักษ์ช้างไทย องค์การอุตสาหกรรมป่าไม้ จังหวัดลำปาง จำนวน 9 เชือก วัดระดับแคลเซียมโดย Colorimetric method แล้วนำค่าที่ได้มาทดสอบหาความสัมพันธ์ด้วยวิธี Linear Regression พบว่ามีเพียงร้อยละการเคลื่อนที่ไปข้างหน้าที่มีความสัมพันธ์กับระดับแคลเซียมในน้ำเลี้ยงเซลล์อสุจิอย่างมีนัยสำคัญ ($p<0.05$) ($R^2=0.1721$) โดยเป็นความสัมพันธ์แบบแปรผกผัน จากนั้นเมื่อแบ่งกลุ่มตัวอย่างตามเปอร์เซ็นต์การเคลื่อนที่ไปข้างหน้า 3 กลุ่ม คือ ร้อยละการเคลื่อนที่ไปข้างหน้าต่ำ (0-5%) ร้อยละการเคลื่อนที่ไปข้างหน้าปานกลาง (>5-40%) และ ร้อยละการเคลื่อนที่ไปข้างหน้าสูง (>50%) แล้วนำค่าที่ได้มาทดสอบหาความแตกต่างระหว่างกลุ่มตัวอย่างด้วยวิธี Repeated measure ANOVA พบว่าค่าเฉลี่ยของร้อยละตัวตาย และระดับแคลเซียมในน้ำเลี้ยงเซลล์อสุจิในกลุ่มร้อยละการเคลื่อนที่ไปข้างหน้าทั้ง 3 กลุ่ม มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) โดยพบว่าเซลล์อสุจิในกลุ่มร้อยละการเคลื่อนที่ไปข้างหน้าต่ำสุดจะมีค่าเฉลี่ยของร้อยละตัวตาย และระดับแคลเซียมในน้ำเลี้ยงเซลล์อสุจิสูงสุด นอกจากนี้เมื่อเปรียบเทียบค่าเฉลี่ยร้อยละความผิดปกติของเซลล์อสุจิ พบว่ามีความแตกต่างกันระหว่างกลุ่มที่มีร้อยละการเคลื่อนที่ไปข้างหน้าต่ำกับกลุ่มที่มีร้อยละการเคลื่อนที่ไปข้างหน้าสูงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) แต่อย่างไรก็ตามในการทดลองนี้พบว่าแคลเซียมในซีรั่มของช้างทุกกลุ่มไม่แตกต่างกัน ดังนั้นแคลเซียมในน้ำเลี้ยงเซลล์อสุจิน่าจะมีผลต่อคุณภาพของน้ำเชื้อ แต่อย่างไรก็ตามยังมีปัจจัยที่เกี่ยวข้องกับคุณภาพของน้ำเชื้อช้างอีกหลายปัจจัย ดังนั้นจึงต้องมีการศึกษาเพิ่มเติมต่อไป

คำสำคัญ: ช้างเอเชีย แคลเซียม คุณภาพของน้ำเชื้อ

[1]*คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ. นครปฐม 73140*
[2]*ศูนย์อนุรักษ์ช้างไทย สถาบันคชบาลแห่งชาติ องค์การอุตสาหกรรรมป่าไม้ จ. ลำปาง*
[3]*ศูนย์เทคโนโลยีชีวภาพเกษตร มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ. นครปฐม 73140*
[4]*ศูนย์ความเป็นเลิศด้านเทคโนโลยีชีวภาพเกษตร สำนักพัฒนาบัณฑิตศึกษาและวิจัยด้านวิทยาศาสตร์และเทคโนโลยี (สบว.) สำนักงานคณะกรรมการอุดมศึกษา*
[5]*สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล นครปฐม*
***ผู้รับผิดชอบบทความ E-mail:** fvetnit@ku.ac.th, nthongtip@yahoo.com*

Introduction

The Asian elephant is an important animal of Thailand. Although it risks to extinct has been listed as appendix I of Convention International Trade in Endangered Species (CITES) since 1972, however, their populations were progressively declined. The major causes for the decreasing of wild elephants are habitat loss and poaching. Therefore, it is an important for breeding captive elephants either by natural or artificial insemination (AI) to maintain or increase the populations as well as genetic management. Due to the difficulties in transporting elephant bull for natural breeding, AI which routinely used in other mammalian species is interested.

It has been reported that elephant calves were successfully produced following AI with either fresh or chilled semen (Schmitt et al., 2001). We have reported the success of semen cryopreservation in the Asian elephant (Thongtip et al., 2004; Sa-ardrit et al., 2006), however, no elephant calf was born after AI with frozen-thawed semen. Recently, we have reported that pregnancy was established following AI with frozen-thawed semen (Thongtip et al., 2009), unfortunately, the embryo did not develop to term. It

is indicated the need of the improvement of cryopreservation technique. However, the core problem for establishment of semen bank for AI is low semen quality in fresh ejaculate. The underlying causes of poor sperm motility and non-motile sperm are still unclear (Thongtip et al, 2008). Seminal plasma is composed of secretions releasing from testicular, epididymis and accessory sex glands. It provides a nutrient medium in which essential for stimulating capacity and motility of spermatozoa (Mann, 1964; Elzanaty et al., 2002). It has been reported that semen of several mammalian species contained a high level of calcium (Ca), magnesium (Mg), zinc (Zn) and copper (Cu) (Mann, 1964; Abou-Shakra et al., 1989). Aberrant levels of Ca, Mg and other elements may influence semen quality thus affecting motility and fertilizing capacity (Hong et al., 1984; Sørensen et al., 1999; Wong et al., 2001). High concentration of Ca has been found in the prostatic and seminal vesicles secretions (Mann, 1964). It is an activator for the acrosome reaction in mammalian spermatozoa and cooperates in sperm motility (Hong et al., 1984). However, a relationship between Asian elephant semen quality with seminal calcium concentration have not been examined. The objective of this study was to investigate the relationship between calcium level in seminal plasma and semen quality of Asian elephant.

Materials and Methods

Chemicals: All chemicals in the present study were purchased from Sigma Chemical Company (Sigma, St. Louis, MO, USA) unless stated otherwise.

Animals: Nine elephant bulls (age range, 15-to 45-years-old) housed at the Thai Elephant Conservation Center (TECC), Forest Industry Organization (FIO), Lampang, Thailand were used in this study. The elephants were fed with grass, banana, sugar cane and free access to water. The experimental procedures were approved by Ethic Committee of the Faculty of Veterinary Medicine, Kasetsart University, Thailand.

Semen collection and evaluation: Semen samples were collected twice monthly during May to July 2008 manual rectal stimulation as previously described (Schmitt and Hildebrandt, 1998). Ejaculates without urine contamination were analyzed for volume, sperm concentration, progressive motility, sperm viability and pH (Kidd et al., 2001). Semen samples contaminated with urine were excluded from this study. Sperm concentration was assessed using a hemocytometer. Progressive motility was visually assessed under a phase-contrast microscopy by two experienced investigators. Sperm morphology was assessed using a phase contrast microscope. Sperm viability (live/dead) was assessed following an eosin-nigrosin staining and at least 200 spermatozoa were counted per slide (BjoÃ rndahl et al., 2003).

Seminal plasma and serum collection: Seminal plasma was collected from all elephant bulls. Semen was centrifuged at 1,000 g for 10 min and the supernatant fluid was collected and stored at -20°C until analysis. For serum collection, blood sample was collected from an ear vein using a vacuum tube (Venoject®, Terumo, Tokyo, Japan). After blood clot for 1 to 2 hr, it was centrifuged at 1,000 g for 10 min and supernatant serum was collected, and stored at -20°C until analysis.

Calcium measurement: Calcium concentration both in serum and seminal plasma were analyzed using colorimetric method. The method is based on the metallochromogen Arsenazo III (Thermo Fisher Scientific Inc, Middletown, VA, USA). Arsenazo III was combined with calcium ions at pH 6.75 to form a highly colored chromophore and the absorbance was measured at 650 nm (Microlab 300, Vital Scientific NV, The Netherlands). Arsenazo III has high affinity ($K° = 1x10^{-7}$) for calcium ions (Bauer, 1981) without interference with other cations present in serum, plasma or urine. The sensitivity of the assay is 0.26 ΔA per mmol/l (0.065 ΔA per mg/dl).

Statistical analysis: The data were expressed as mean±SE and were analyzed using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA). The correlation between semen characteristics and seminal calcium level was analyzed using Linear Regression. The comparison of mean±SE of seminal and serum calcium and semen characteristics among low-, moderate- and high motile were done using Repeated measure ANOVA. Differences of $p<0.05$ were considered significant.

Results

A total of 54 attempts of semen collection were performed (Figures 1 and 2). Forty one ejaculates (75.92%) without urine contamination were obtained and 13 ejaculates (24.08%) contaminated with urine were discarded. The level of seminal calcium concentration was 9.21±0.85. The results showed that calcium level in seminal plasma was negative correlated with progressive motility (R^2 = 0.1721) ($p<0.05$). Seminal calcium was not significantly correlated with sperm concentration, volume, pH and percentages of abnormal morphology and dead sperm (R^2 = 0.0037, 0.0125, 0.0267, 0.1110 and 0.0442, respectively) (Table 1).

Figure 1. Semen collection by manual rectal stimulation in Asian elephant (*Elephas maximus*).

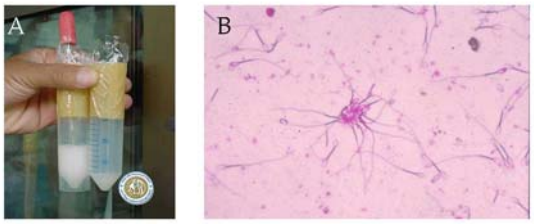

Figure 2. (A) Asian elephant (*Elephas maximus*) fresh semen collected by manual rectal stimulation (B) Asian elephant spermatozoa stained with Eosin-Nigrosin dye.

We have divided ejaculates obtained from each bull into 3 groups according to their progressive motility to examine the relationship between seminal parameters and serum and seminal calcium levels. Semen samples contained spermatozoa with 0-5%, >5-40% and >50% progressive motility were classified as low-, moderate- and high-motile groups, respectively. Percentage of sperm with abnormal morphology in low-motile group was significantly higher (p<0.05) than of the high-motile group. The percentage of dead sperm was highest in low-motile group (p<0.05) and lowest in high-motile group (p<0.05). Similarly, seminal calcium was highest (p<0.05) in low-motile group and lowest (p<0.05) in high- motile group (Table 2). The serum calcium was not different among each group.

Table 1. Linear regression and Correlation reports of seminal calcium on semen qualities.

Parameters	Mean ± SD	*R*-squared	Correlation
Progressive motility (%)	21.59±24.61	0.1721	-0.4149
Volume (ml)	7.48±8.08	0.0125	0.1116
Concentration ($\times10^6$/ml)	1,327.85±643.04	0.0037	-0.0610
Semen pH	7.15±0.79	0.0267	0.1634
Abnormal morphology (%)	33.71±22.13	0.1110	0.3332
Dead sperm (%)	59.15±22.12	0.0442	0.2103

Table 2. Mean+SE of seminal and serum parameters of samples obtained during May through July 2008 in Low, Moderate and High-motile groups.

Parameters	Progressive motility		
	Low-motile (15 ejaculates)	Moderate-motile (15 ejaculates)	High-motile (11 ejaculates)
Volume (ml)	5.73±1.50	10.33±2.91	5.95±1.21
Concentration ($\times10^6$/ml)	1,306.80±183.73	1,469.67±167.84	1,163.18±163.16
Semen pH	7.43±0.24	6.94±0.17	7.06±0.22
Abnormal morphology (%)	44.67±6.81^a	28.23±4.53^{a,b}	26.23±4.92^b
Dead sperm (%)	72.87±5.21^a	54.90±4.44^b	46.23±6.60^c
Seminal calcium (mg%)	11.17±1.62^a	9.67±1.41^b	5.91±0.50^c
Serum calcium (mg%)	10.14±0.25	10.47±0.28	10.51±0.23

Discussion

Due to the problem of poor semen quality attributed from manual collection of Asian elephant remains unclear. The contributions for solving this problem are going on. In the present study, the non-urine contaminated ejaculates were successfully obtained similar to our previous study (75.92%) (Thongtip et al., 2008). An average of percentage of progressive motility (21.59±24.61) and over all semen characteristics revealed the same trend as previously reported (Thongtip et al., 2008). Even though, from Linear Regression analysis showed the weak relationship between seminal plasma calcium and semen parameters. However, in this study we found the interesting results, the low-motile group had a significantly higher level of seminal calcium than moderate-motile and high-motile groups. Furthermore, moderate-motile sperm had a significant higher level of seminal calcium than high-motile sperm. It seems that calcium may be utilized by the elephant sperm during their movement and subsequently leads to the lowest level seminal calcium in the high-motile group. In contrast, the low-motile group had a significant highest of seminal calcium level. It might indicate that seminal calcium was not used for sperm movement in this group. The reason may come from the percentage of dead sperm that was significantly highest in the low-motile group. Viable sperm has an ability to use both extracellular and intracellular calcium for stimulating the producing of the signaling of movement via 2 reactions including of cAMP/protein kinase A signaling pathway and calcium signaling pathway (Meseguer et al., 2004; Turner, 2006). Thus, it might be assumed that the non-viable sperm in low-motile group cannot use calcium in seminal plasma for movement leading to the higher level accumulated in seminal plasma. Furthermore, the leakage of intracellular calcium of dead sperm may be increased the extracellular concentration. Of these, seminal calcium level may be important factor for sperm movement in Asian elephant. In human, calcium has been shown to involve in sperm motility with apparently paradoxical effect as reported by Hong et al. (1984). Those authors suggested that during in the epididymis, calcium ions stimulated developing sperm, whereas, calcium ions inhibited sperm motility in ejaculated semen, contrary, a lower level of seminal calcium was found in the spinal cord injury men who had a lower motility than the neurological intact men (Salsabili et al., 2009). In the present study, in the low-motile, seminal calcium level was higher than serum

calcium. Our finding was in agreement with that reported in human semen (Salsabili et al., 2009). However, the serum calciums were not different among each group. The maintenance of serum calcium has been reported in the orphan elephant (Yartbantoong et al., 2000). However, more studies are needed for investigating the role of both serum and seminal calcium on semen parameters. In conclusion, calcium in seminal plasma may influence the semen quality; i.e. progressive motility of Asian elephant. However, there are other factors that can be affected to elephant semen quality, thus, more information were needed to improve the better knowledge in male elephant reproductive biology.

Acknowledgement

We thank Dr. Wah Wah Thein-Han and Asst. Prof. Dr. Naris Thengchaisri for critical reading and editing the manuscript. This project was funded by Thailand Research Fund (MRG5280087) and Faculty of Veterinary Medicine, Kasetsart University. This research is supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office (PERDO), Commission on Higher Education, Ministry of Education.

References

Abou-Shakra, F.R., Ward, N.I. and Everand, D.M. 1989. The role of trace elements in male fertility. Fertil. Steril. 52: 307-310.

Bauer, P.J. 1981. Affinity and stoichiometry of calcium binding by arsenazo III. Anal Biochem. 110(1): 61-72.

BjoÃ rndahl, L., SoÃ derlund, I. and Kvist, U. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum. Reprod. 18: 813-816.

Elzanaty, S., Richthoff, J., Malm, J. and Giwercman, A. 2002. The impact of epididymal and accessory sex gland function on sperm motility. Hum. Reprod. 17: 2904-2911.

Hong, C.Y., Chiang, B.N. and Turner, P. 1984. Calcium ion is the key regulator of human sperm function. Lancet. 2: 1449-1451.

Kidd, S.A., Eskenazi, B., Wyrobek, A.J. 2001. Effects of male age on semen quality and fertility: a review of the literature. Fertil. Steril. 75: 237-248.

Mann, T. 1964. The Biochemistry of Semen and of the Male Reproductive Tract. Methuen and Co. London, 493.

Meseguer, M., Garrido, N., Pellicer, A., Simon, C., and Remohi, J. 2004. Relationship between standard semen parameters, calcium, cholesterol contents, and mitochondrial activity in ejaculated spermatozoa from fertile and infertile males. J. Assisted Reproduction and Genetics.21: 445-451.

Sa-Ardrit, M., Saikhun, J., Thongtip, N., Damyang, M., Mahasawangkul, S., Angkawanish, T., Jansittiwate, S., Faisaikarm, T., Kitiyanant, Y., Pavasuthipaisit, K. and Pinyopummin, A. 2006. Ultrastructure alteration of frozen-thawed Asian elephant (*Elephus maximus*) spermatozoa. Int. J. Androl. 29: 346-352.

Salsabili, N., Mehrsai, A. R. and Jalaie, S. 2009. Concentration of blood and seminal plasma elements and their relationships with semen parameters in men with spinal cord injury. Andrologia. 41: 24-28.

Schmitt, D.L., and Hildebrandt, T.B. 1998. Manual collection and characterization of semen from Asian elephants (*Elephas maximus*). J. Anim. Reprod. Sci. 53: 309-314.

Schmitt, D.L, Hildebrant, T.B., Hermes, R., Goritz, F. 2001. Assisted reproductive technology in elephants. Proc 1st Int Symp Assisted Reproductive Technology for Conservation Genetic Management of Wildlife, Omaha's Henry Doorly Zoo. 15-17.

Sørensen, M.B., Bergdahl, I.A., Hjøllund, N.H., Bonde, J.P.E., Stolten-berg, M. and Ernst, E. 1999. Zinc, magnesium and calcium in human seminal fluid: relations to other semen parameters and fertility. Mol. Hum. Reprod. 5: 331-337.

Thongtip, N., Saikhun, J., Damyang, M., Mahasawangkul, S., Suthunmapinate, P., Yindee, M., Kongsila, A., Angkawanish, T., Jansittiwate, S., Wongkalasin, W., Wajjwalkul, W.,Kitiyanant, Y., Pavasuthipaisit, K. and Pinyopummin, A. 2004. Evaluation of post-thaw Asian elephant (*Elephus maximus*) spermatozoa using flow cytometer: the effect of extender and cryoprotectant. Theriogenology. 62: 748-760.

Thongtip, N., Saikhun, J., Mahasawangkul, S., Kornkaewrat, K., Pongsopavijitr, P., Songsasen, N. and Pinyopummin, A. 2008. Potential factors affecting semen quality in the Asian elephant (*Elephas maximus*). Reprod. Biol. Endocrinol. 17(6): 9.

Turner, R.M. 2006. Moving to the beat: a review of mammalian sperm motility regulation. Reprod. Fert. Dev.18: 25-38.

Wong, P.Y.D., Lee, W.M. and Tsang, A.Y.F. 1981. The effects of sodium and amiloride on the motility of the caudal epididymal spermatozoa of the rat. Cell. Mol. Life Sci. 37: 69-71.

Wong, W.Y., Flik, G., Groenen, P.M.W., Swinkels, D.W., Thomas, C.M.G., Copius- Peereboom, J.H.J., Merkus, H.M.W. M and Steegers-Theunissen, R.P.M. 2001. The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. Reprod. Toxicol. 15: 131-136.

Yartbantoong, N., Thongtip, N., Sanyathitiseree, P., Kasemsuwan, S. and Viriyarumpa, J. 2000. Diet formulary and feeding in orphan elephants. Kasetsart Veterinarians J. 10(2): 22-27.