Assessment of viability and acrosomal status of Asian elephant (*Elephas maximus*) sperm after treatment with calcium ionophore and heparin

M Sa-ardrit[a,b], N Thongtip[b,c], K Kornkaewrat[c], T Faisaikarm[d], Y Kitiyanant[d,e], S Mahasawangkul[f], A Pinyopummin[c] and K Saikhun[d*]

ABSTRACT

Knowledge about the acrosomal status of Asian elephant (*Elephas maximus*) sperm is extremely limited. The objective of this study was to evaluate the viability and acrosomal status of Asian elephant sperm following induction by calcium ionophore and heparin using propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA). Semen samples were collected from elephant bulls by manual stimulation. Semen was diluted with extender, cooled to 4 °C and transported to a laboratory for the experiment. Sperm cells were incubated in modified Tyrode's medium containing either 1 mM calcium ionophore or 10 mg/mℓ heparin for 5 h at 39 °C. Sperm recovered at the onset (0 h), 1, 2, 3, 4 and 5 h of incubation were simultaneously assessed for the viability and acrosomal status using dual staining of FITC-PNA and PI. Results were confirmed by transmission electron microscopy. A progressive increase in the proportion of live-acrosome reacted sperm was observed within 3 h of incubation in both treatment groups which slightly decreased at 4 to 5 h of incubation. At 1 to 3 h of incubation, the percentage of live-acrosome reacted sperm induced by calcium ionophore was higher ($P < 0.05$) than those induced by heparin and the control. However, there were no statistical differences at 4 to 5 h of incubation. A progressive reduction of the percentage of motile sperm was observed in the control as well as both treatment groups. Sperm motility decreased sharply when they were incubated in calcium ionophore compared with incubation in heparin and control groups. These results indicate that the occurrence of live-acrosome reacted sperm in the Asian elephant was induced by calcium ionophore at a rate higher than that induced by heparin.

Keywords: acrosome reaction, calcium ionophore, elephant, heparin, sperm.

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INTRODUCTION

The Asian elephant (*Eliphas maximus*) is an important animal in Thailand but the elephant population has declined gradually. The Asian elephant is in danger of extinction and has been listed in Appendix I of the Convention on International Trade in Endangered Species (CITES) since 1972. Some of the major threats to elephant survival are reproductive problems. The development of assisted reproductive technologies such as artificial insemination (AI) or *in vitro* embryo production (IVP) is therefore required as an alternative approach for breeding management of this species[26].

Artificial insemination using either fresh or frozen semen is routinely used for breeding management in farm animals. However, this technique requires semen of high quality. Studies on semen collection[9,23,27] and cryopreservation[25,29] of Asian elephant sperm have been reported. Mammalian sperm are unable to fertilise an oocyte immediately[2,4]. The sperm must undergo the capacitation and acrosome reaction (AR) processes for the development of fertilising ability. Capacitation involves a series of biological changes in the sperm that ultimately result in the acrosome reaction[32]. The AR involves the fusion of the apical portion of the sperm plasma membrane with the underlying outer acrosomal membrane at many sites on the sperm head, inducing membrane vesiculation[3,15]. Disruption of the plasma and outer acrosomal membranes releases acrosomal enzymes and exposes the inner acrosomal membrane. The released acrosomal enzymes digest a pathway through the *zona pellucida* allowing the sperm to pass through, bind to the plasma membrane and fertilise the egg[32].

Many substances, such as calcium ionophore, heparin, caffeine, bovine serum albumin, lysophosphatidylcholine and progesterone have been successfully used to induce the AR in sperm of several mammalian species[14,18,21,28]. However, there is only 1 published study on the *in vitro* induction of the acrosome reaction in the Asian elephant using cAMP, caffeine, heparin and PHE (penicillamine, hypotaurine and epinephrine)[12]. There are several staining methods used to determine the acrosomal status in mammalian sperm such as, trypan blue and Giemsa[7], triple-stain[8] and Coomassie Blue[13]. Although these methods were not costly, the staining procedures were complicated. A fluorescent probe, fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA), has been utilised to determine the acrosomal status in various mammalian species such as stallions[5], dogs[10], buffalo[11] and pigs[16]. Previous studies have used FITC-PNA for evaluating the acrosomal damage to elephant sperm after freezing and thawing[25,29]. This method is very easy to perform and effective for assessing the acrosomal status of sperm. Propidium iodide, a fluorescent probe for viability assessment, has been successfully used with FITC-PNA for simultaneous evaluation of viability and acrosomal status of stallion sperm[24]. Nevertheless, there is no report of using FITC-PNA to assess the acrosome reaction in elephant sperm. Therefore, the present study was to determine the acrosomal status and viability of Asian elephant sperm induced by calcium ionophore and heparin using FITC-PNA and propidium iodide.

[a]National Laboratory Animal Center, Mahidol University, Nakorn Pathom 73170, Thailand.

[b]Center for Agricultural Biotechnology, Kasetsart University, Nakorn Pathom 73140, Thailand.

[c]Faculty of Veterinary Medicine, Kasetsart University, Nakorn Pathom 73140, Thailand.

[d]Institute of Science and Technology for Research and Development, Mahidol University, Nakorn Pathom 73170, Thailand.

[e]Department of Anatomy, Faculty of Science, Mahidol University, Nakorn Pathom 73170, Thailand.

[f]The Thai Elephant Conservation Center, The Forest Industry Organization, Lampang 52000, Thailand.

*Author for correspondence.
E-mail: jsaikhun@yahoo.com

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MATERIALS AND METHODS

Chemicals

Fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and propidium iodide (PI) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Heparin was obtained from Leo Pharmaceutical Products (Ballerup, Denmark). All other chemicals in this study were purchased from Sigma Chemical Company (Sigma, St Louis, MO, USA).

Media

The basic culture medium used in this study was modified Tyrode's medium (SP-TALP). It consisted of 100 mM NaCl, 0.4 mM $mgCl_2$, 2 mM $CaCl_2$, 0.3 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, 1 mM pyruvic acid, 21.6 mM lactic acid, 6 mg/mℓ BSA (Fraction V), and 10 μg/mℓ phenol red. The Tyrode's medium was buffered with 1 N NaOH or HCl to pH 7.4 and had a final osmolarity of 285–295 mOsmol/kg. The media stock was sterilised through Millipore filtration (0.22 μm pore size) and used within 2 weeks. FITC-PNA stock was prepared in PBS at a final concentration of 100 μg/mℓ, whereas PI was prepared in double distilled water at a final concentration of 1 mg/mℓ.

Animals, semen collection and analysis

The Asian elephant bulls housed at the Thai Elephant Conservation Center, the Forest Industry Organization, Lampang, Thailand were used in this study. The animals were fed with grass, banana and sugar cane during the day and allowed to roam in the jungle at night. Ejaculates were collected from 10 elephant bulls (age range from 23–50 years old) by manual stimulation as previously described[27,30]. Briefly, the protrusion and erection of the penis was accomplished by rectal massage of the pelvic portion of the urethra, near the seminal colliculus. Following protrusion, a collection sleeve was placed over the end of the penis. As an ejaculatory response was detected, continuing massage of the region of the ampulla and ductus deferens resulted in expulsion of the sperm-rich fraction. After collection, semen samples were immediately evaluated for concentration, progressive motility and viability under a phase contrast microscope. Sperm concentration was assessed using a haemocytometer. Progressive motility was assessed visually at room temperature (28–32 °C) using phase-contrast microscopy (×200) by 2 experienced investigators. Sperm viability was assessed using an eosin-nigrosin staining method (200 sperm cells were counted). A total of 22 ejaculates was collected from 11 bulls (2 ejaculates from each bull), 4 ejaculates obtained from 2 bulls showing high quality (>50 × 10^6 cells/mℓ, >75 % progressive motility and >70 % live spermatozoa) were used in this study. The other 18 ejaculates, either low in semen quality or with urine contamination were discarded. After analysis, semen samples were diluted at a ratio of 1:1 in a TEST extender that consisted of 5.54 % Tes (N-Tris (hydroxmethyl)-methyl-2-aminoethane-sulfonic acid], 1.15 % Tris-(hydroxymethyl)-aminomethane, 0.4 % glucose and 20 % egg yolk. The diluted semen was cooled from room temperature (28–32 °C) to 5 °C at a rate of 1 °C/min using the Biocool II cooling machine (FTS® Systems, Stone Ridge, NY, USA). The cooled semen was then maintained on ice and transported to a laboratory within 6 h.

Sperm preparation and incubation in the presence of calcium ionophore and heparin

When semen samples arrived at the laboratory, they were immediately assessed for progressive motility, viability and acrosomal status. Progressive motility was assessed visually by phase-contrast microscopy, whereas viability and acrosomal status were simultaneously assessed by PI and FITC-PNA as described below. The sperm cells with high motility were selected by a swim-up technique. Briefly, 75 μl of semen mixture was placed in a polystyrene tube containing 1 mℓ of SP-TALP medium and incubated for 1 h at 39 °C, 5 % CO_2, 95% air. The supernatant (0.8 mℓ) medium containing motile sperm from each tube was gently removed into a 15 mℓ conical centrifuge tube. It was centrifuged at 500 g for 10 min. After removal of the supernatant, the sperm pellet was resuspended with SP-TALP medium to a concentration of 50 × 10^6 cells/mℓ. After swim-up, sperm cells were divided into 3 groups: 1) control, sperm cells were incubated in SP-TALP; 2) calcium ionophore, sperm cells were incubated in SP-TALP containing 1 μM of calcium ionophore A23187 and 3) heparin, sperm cells were incubated in SP-TALP containing 10 μg/mℓ heparin. Samples from the control and treatment groups were then incubated at 39 °C, 5 % CO_2, 95 % air for 5 h. Sperm cells were assessed for progressive motility, viability and acrosomal status at the onset (0 h), 1, 2, 3, 4 and 5 h of the incubation period.

Assessment of viability and acrosomal status

To evaluate simultaneously for viability and acrosomal status, sperm cells were stained with a combination of FITC-PNA (marker of acrosome leakage) and PI (marker of membrane damage)[24]. Sperm suspension was stained with FITC-PNA and PI at a final concentration of 10 and 25 μg/mℓ, respectively. After incubation at 37 °C for 30 min, a 10 μl aliquot of the double-stained sperm suspension was removed and placed onto a microscopic slide, covered with a cover slip and observed under a fluorescence microscope (×1000) at room temperature. Two hundred sperm cells were recorded per slide.

Transmission electron microscopy (TEM)

Sperm suspensions from control, calcium ionophore and heparin groups were collected at 3 h of incubation to study the ultrastructural changes of the acrosome using TEM. The sperm samples were prefixed at 4 °C overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer. After washing in 0.1 M cacodylate buffer, the sperm pellet was postfixed in 1 % osmium tetraoxide at 4 °C for 1 h. They were then dehydrated in an ascending alcohol series (50 to 100 %), infiltrated with propylene oxide and embedded in Araldite resin mixture. Ultrathin sections were obtained using a diamond knife. They were stained with uranyl acetate and lead citrate[12] and acrosomal status of sperm was examined under a H-7500 Hitachi TEM.

Statistical analysis

The experiments were repeated 4 times. Data relating to acrosomal status and viability were analysed after arc sine transformation using ANOVA with Duncan's New Multiple Range Test. Differences of $P < 0.05$ were considered significant. The SAS statistical software package (SAS Institute, Cary, NC, USA) was used for analysing the data.

RESULTS

The mean percentage of progressive motility and viability of sperm in fresh semen immediately assessed after collection were 85.3 ± 10.4 and 75.6 ± 2.8, respectively. Four patterns of elephant sperm following staining with PI and FITC-PNA are shown in Fig. 1. Sperm with red fluorescence on the heads and green fluorescence over the acrosomal cap (PI and FITC-PNA positive) were classified as dead-acrosome reacted whereas red fluorescent sperm heads but not green fluorescence over the acrosomal cap (PI-positive but FITC-PNA-negative) were classified as dead-acrosome intact. Sperm without fluorescence on the heads but green fluorescence over the acrosomal cap (PI negative but FITC-PNA positive), were classified as live-acrosome

reacted whereas non-fluorescent sperm heads and acrosomal cap (PI and FITC-PNA negative) were classified as live-acrosome intact. After being diluted with TEST extender and transported to the laboratory, percentages of sperm progressive motility and live-acrosome intact were 70.1 ± 5.9 and 65.3 ± 4.5% respectively. The percentage of progressive motility after swim-up was 75.3 ± 9.5. The investigation of the acrosomal status was also revealed by TEM. The plasma membrane and acrosome of normal sperm appeared intact, whereas the acrosome reacted sperm could be identified either by fusion and vesiculation of plasma and outer acrosomal membranes over the apical sperm head or the leakage of acrosomal content as well as the absence of acrosome membranes (Fig. 2).

At the onset (0 h), the percentage of live-acrosome reacted sperm was not different between treatment and control groups. At 1–3 h of incubation, a progressive increase in the proportion of live-acrosome reacted sperm was observed in both treatment groups (Fig. 3). However, the percentage of live-acrosome reacted sperm was not increased further by prolonging incubation to 4 and 5 h. At 1–3 h of incubation, the percentage of live-acrosome reacted sperm induced by calcium ionophore was higher ($P < 0.05$) than that induced by heparin or in the control (Fig. 3). At 4 –5 h of incubation, no significant differences in the proportion of live-acrosome reacted sperm were found between treatment and control groups. Average percentage of sperm motility at the onset and at 1–5 h of incubation in the control and heparin groups was 75.1 ± 8.3, 71.4 ± 10.5, 66.1 ± 11.4, 63.3 ± 12.3, 55.9 ± 8.7, 48.7 ± 9.0 and 74.2 ± 9.5, 68.6 ± 12.2, 62.6 ± 9.1, 53.6 ± 7.8, 45.9 ± 6.5, 41.7 ± 10.2, respectively. By contrast, the percentage of progressive motility of sperm decreased sharply when incubated in calcium ionophore (73.4.2 ± 10.5, 52.6 ± 9.2, 30.6 ± 7.9, 21.3 ± 6.7, 17.5 ± 7.6, 14.4 ± 6.3, at the onset and at 1 to 5 h of incubation respectively).

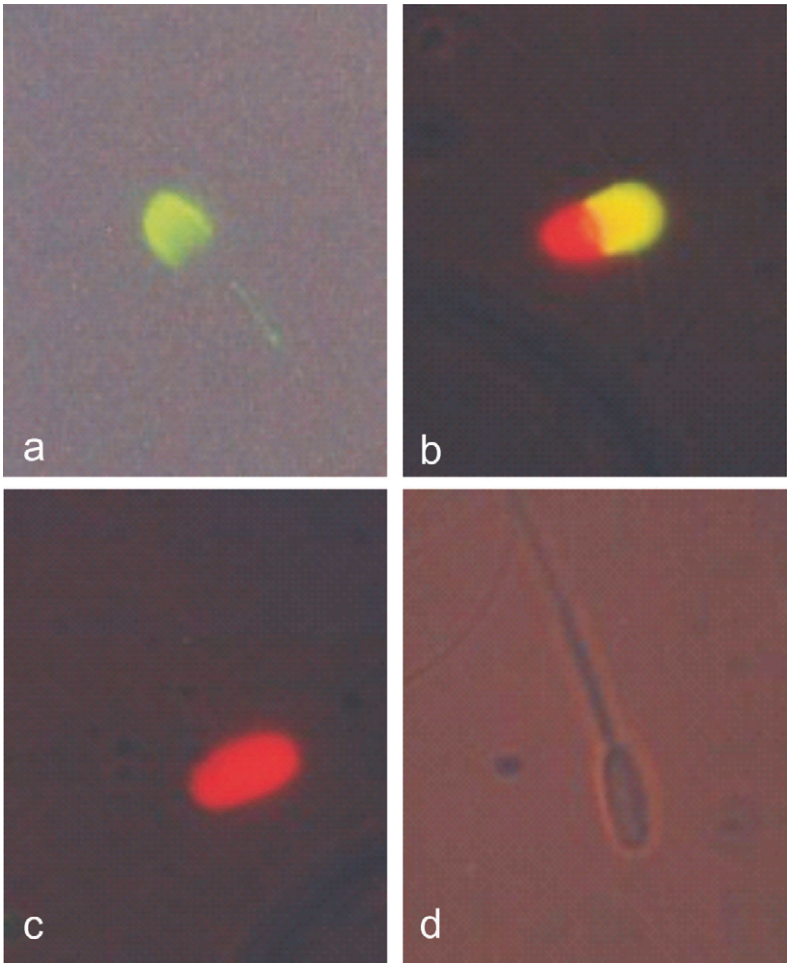

Fig. 1: **The 4 patterns of elephant sperm after being stained with FITC-PNA and PI and observed under a fluorescence microscope** (×1000)**.** a, **Live-acrosome reacted;** b, **dead-acrosome reacted;** c, **dead-acrosome intact;** d, **live-acrosome intact.**

DISCUSSION

In the present study, it has been shown that calcium ionophore was significantly more effective than heparin in the induction of the acrosome reaction in elephant sperm. To our knowledge, only 1 article reported that caffeine and penicillamine-hypotaurine-epinephrine (PHE) were more effective than cAMP and heparin in the induction of the acrosome reaction in elephant sperm[12]. The results reported here are in agreement with previous reports with buffalo sperm[11]. Calcium ionophore has been shown to induce the acrosome reaction of sperm from several mammalian species, including stallions[1], buffalo[11], boar[16] and ram[22]. However, the effect of calcium ionophore in the induction of the acrosome reaction in elephant sperm has not been reported. The acrosome reaction in mammalian sperm is

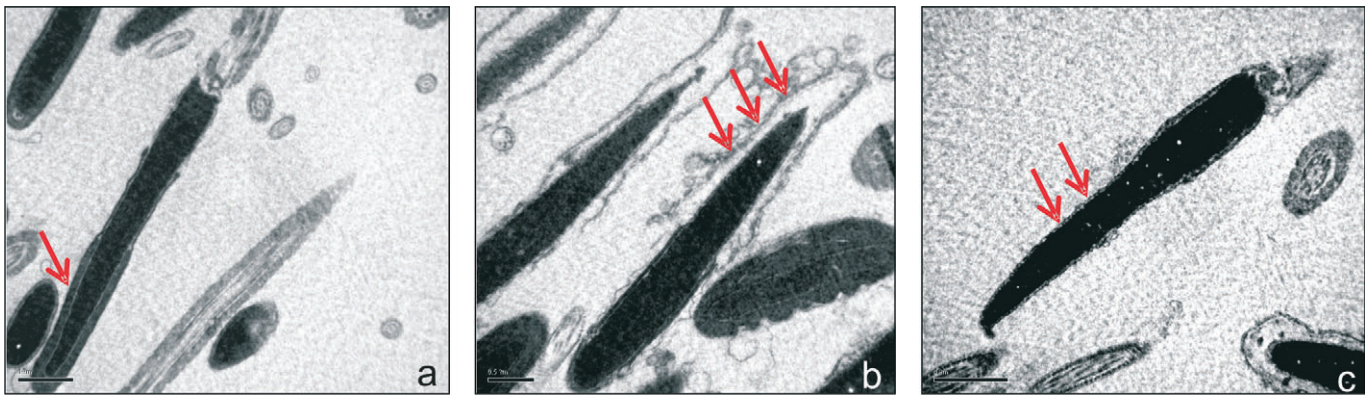

Fig. 2: **TEM micrographs of elephant sperm:** a, **acrosome intact** (arrow) (×15000)**;** b, **vesiculation of plasma and outer acrosomal membranes** (arrows) **indicate acrosome reacting** (×25 000); c, **absence of plasma and outer acrosomal membranes indicates acrosome reacted** (arrow) (×25 000)**.**

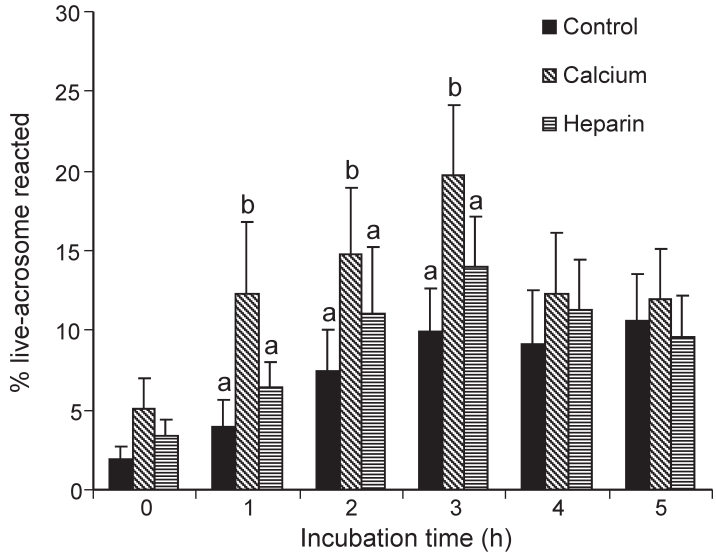

Fig. 3: **Percentage of live-acrosome reacted spermatozoa induced by calcium ionophore or heparin. Different letters above the bars in the same hour represent significant differences** ($P < 0.05$).

Ca^{2+} dependent. The differences between calcium ionophore and heparin in the induction of the acrosome reaction may be due to their mechanisms in increasing intracellular Ca^{+2}. Calcium ionophore causes a rapid influx of Ca^{2+}, producing high intracellular Ca^{+2} levels which trigger the acrosome reaction[6,33]. Heparin, a glycosaminoglycan, has also been used to induce the acrosome reaction in elephant sperm but its effect was less than caffeine, PHE and cAMP[12]. Heparin may initiate calcium influx, which increases the c-AMP as a second messenger to initiate changes in the composition of the plasma membrane and cause the acrosome reaction. It has been widely used in order to induce the acrosome reaction of sperm from buffaloes[11], bulls[20] and goats[21]. It was first shown to capacitate sperm as evidenced by the ability of sperm incubated with heparin to penetrate oocytes within 4 h[19].

The percentage of live-acrosome reacted sperm in treatment groups, was progressively increased at 1 to 3 h of incubation; however, it was not increased further by prolonging incubation. These results confirmed previous reports that the life span of acrosome-reacted sperm is likely to be short[6,17]. The relatively short life span of acrosome-reacted sperm contributed to the decrease in motility during incubation. The reasons for greater decrease in progressive motility induced by calcium ionophore than by heparin are unclear. Calcium ionophore mobilises calcium-dependent regulatory mechanisms by promoting a massive influx of Ca^{2+} which could be harmful to sperm[31]. By contrast, heparin may promote capacitation by binding to and removing seminal plasma proteins that are absorbed into the sperm plasma membrane. Heparin seems to have less effect on membrane permeability and could, therefore, maintain the motility of elephant sperm better than calcium ionophore[1].

In conclusion, calcium ionophore is more effective than heparin to induce the acrosome reaction in elephant sperm. Further studies are required to assess the fertilising ability of acrosome-reacted sperm either by sperm penetration assay or *in vitro* fertilisation.

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