Effect of platelet-activating factor (PAF) on stallion sperm motility, capacitation and the acrosome reaction

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Phospholipids are an essential component of all mammalian cells; platelet activating factor (PAF = 1-*O*-alkyl-acetyl-*sn*-glycero-3-phosphocholine) is a signalling phospholipid that has many biological properties in addition to platelet activation. PAF receptors have been detected on stallion spermatozoa; therefore, the aim of this study was to evaluate the effect of synthetic PAF on the motility, capacitation and the acrosome reaction of stallion spermatozoa. Treatment of ten stallion semen samples with 10^{-4} – 10^{-13} mol PAF l⁻¹ resulted in significant differences in motility and capacitation ($r^2 = 0.81$ and 0.83 , respectively). Statistical analysis indicated that PAF also has

an effect on acrosome reaction ($r^2 = 0.20$). PAF concentrations, incubation time and their interaction had a highly significant ($P < 0.01$) effect on motility. After capacitation *in vitro* with PAF, and induction of the acrosome reaction by progesterone, transmission electron microscopy was conducted on the spermatozoa of three stallions to detect the true acrosome reaction. Differences in PAF concentrations were highly significant (r^2 for intact: 97.2; reacted: 89.8; and vesiculated: 98.1). The results indicate that a lower concentration of PAF enhances motility and induces capacitation of stallion spermatozoa, whereas a higher concentration of PAF induces the acrosome reaction.

Introduction

Capacitation is the modification of spermatozoa after ejaculation, and varies from species to species; it involves a series of complex and poorly characterized cellular events that enable spermatozoa to fertilize oocytes (Yanagimachi, 1988; Kopf and Gorton, 1991). Capacitation is believed primarily to involve membrane modifications, including changes in lipid composition, surface properties, fluidity, permeability to calcium and lowered concentration of cholesterol in membranes (Davis, 1981). Most of these alterations are related to changes in the plasma membrane of spermatozoa and have led to the contention that capacitation is a process of membrane maturation that eventually completes the membrane remodelling events initiated during epididymal transit (Jones, 1997).

The acrosome reaction of mammalian spermatozoa is a calcium-dependent exocytotic event in the sperm head and is essential to fertilization (Yanagimachi, 1981). The acrosome reaction can occur only after completion of capacitation and before penetration of the zona pellucida and fertilization. It results from the fusion of the sperm outer-acrosomal membrane and the plasma membrane with the subsequent release of hydrolytic acrosomal

enzymes. The release of these enzymes is via exocytotic mechanisms through fenestrations formed at the points of fusion of both membranes (Barros *et al.*, 1967). These hydrolytic enzymes aid in the penetration of the cumulus oophorus and zona pellucida that surround the oocyte.

Platelet-activating factor (PAF) is a potent signalling phospholipid that has been implicated in several reproductive processes (Yasuda and Johnson, 1992). Induction of capacitation *in vitro* has been demonstrated in fresh and frozen spermatozoa of cattle by using PAF (Aravindakshan and Sharma, 1995). In that work it was noted that about 0.1×10^{-9} mol PAF l⁻¹ is optimal, because at this concentration the acrosomal reaction improved without much loss of motility.

A metabolic pathway is described for the catabolism of PAF in rat spermatozoa, involving PAF-acetylhydrolase (PAF-AH), lysophospholipase D and a phosphohydrolase. The partial inactivation of PAF-AH by the vaginal pH and its detachment from spermatozoa during migration to the site of fertilization may allow increased motility and migration to the site of fertilization. The activity of PAF-AH in bovine and stallion seminal plasma is more than 50-fold greater than that reported from any other source (Hough and Parks, 1994). It has been suggested that a decapacitation factor described previously may be related to PAF-AH (Hough and Parks, 1994; Muguruma and Johnston, 1997).

Investigators have co-cultured spermatozoa with oviductal epithelia (Ellington *et al.*, 1993), exposed and subjected spermatozoa to potential capacitating agents, such as heparin, artificially induced the acrosome reaction by calcium ionophore (Varner *et al.*, 1993) and performed zona pellucida penetration assay using both hamster and equine oocytes (Blue *et al.*, 1989; Samper *et al.*, 1989). From these studies, valuable information concerning the biochemical changes that occur in the sperm head, and also the time required for capacitation, have been obtained. Stallion spermatozoa are thought to require approximately 4–6 h to undergo capacitation. This time period is proposed based on *in vitro* experiments and on conception rates after insemination of mares with semen at various times before and after ovulation (Hinrichs *et al.*, 2002).

As PAF is an ether, it might function through destabilization of the sperm plasma membrane and, thus, induce these physiological changes more rapidly. One of the difficulties encountered in attempting to investigate requirements for capacitation is that no obvious morphological changes accompany the changes in functional potential of sperm cells. The chlortetracycline (CTC) fluorescence technique was used to assess the functional status of mouse spermatozoa (Ward and Story, 1984). It has also been used to assess calcium-related changes in the capacitation state of human (DasGupta *et al.*, 1993), bull (Fraser *et al.*, 1995) and equine (Varner *et al.*, 1993) spermatozoa. The major advantage of CTC is that, besides its ability to discriminate between acrosome-intact cells and acrosome-reacted cells, it also divides acrosome-intact cells into two functionally different categories, uncapacitated and capacitated. Therefore, the aim of the present study was to investigate the effect of PAF on the motility, capacitation and acrosome reaction of stallion spermatozoa. These overall objectives will provide a better understanding of the possible physiological role of PAF in sperm function as well as its possible potential in some assisted reproductive technology applications.

Materials and Methods

Sampling and sperm preparation

Spermatozoa were obtained from ten (unless otherwise stated) sexually active light breed stallions with an age range of 5–23 years. Semen was collected on a regular basis from the stallions, depending on individual stallion use, during the active breeding season to decrease the effect of extragonadal sperm storage stasis. Semen was collected using a Missouri model artificial vagina, and filtered using break-proof socks filter (Nasco, Fort Atkinson, WI) to remove debris and gel. In the laboratory, semen was centrifuged for 5 min at 50 g to remove any remaining gel fraction, seminal debris and clumped spermatozoa.

All experimental procedures began within 1 h of collection of semen with the exception of motility, which was assessed as soon as possible after collection. Deficient Ham's F12 HAT variation medium (HAT, Irvine Scientific, Santa Ana, CA), supplemented with 0.03% heat inactivated BSA was used for washing and incubation. This medium was chosen after a longevity test was performed to compare it with commercial equine semen extenders and for its basic components.

PAF (Sigma Chemical Co, St Louis, MO) was dissolved in a stock solution of 10^{-3} mol l $^{-1}$ chloroform and methanol (1:4) and stored at -20°C . PAF at the indicated concentrations was dried under a gentle stream of nitrogen in siliconized tubes and re-dissolved in Ham's F12 medium to the final experimental concentrations. The solutions were mixed for 20 s and tubes were kept at 37°C in water-saturated air before and after the addition of spermatozoa. Siliconized tubes were used to prevent adhering of PAF to glass tubes, which may affect the final concentration of PAF in solution.

After the initial centrifugation, the supernatant was placed in a conical test tube with Ham's F12 + 0.03% BSA medium and centrifuged twice at 600 g for 10 min. The supernatant was discarded and the sperm pellet was re-suspended with the same medium to give a final concentration of $1\text{--}2 \times 10^7$ ml $^{-1}$ for each experiment, unless otherwise stated. Each sample consisting of 1 ml of sperm suspension layered under 2 ml (Ham's F12 + 0.03% BSA) medium was placed into conical tubes and incubated at a 45° angle in a slant rack at 37°C in 5% CO $_2$ in water-saturated air. After 1 h swim-up, the supernatant was collected and spermatozoa were used for evaluations after incubation.

Evaluation of spermatozoa after incubation

Motility evaluation. Swim-up spermatozoa were incubated with 10^{-4} – 10^{-13} mol PAF l $^{-1}$ in 5% CO $_2$ at 37°C in water-saturated air together with the control group (no PAF). Sperm motility was examined at five different time intervals: 30, 60, 90, 120 and 150 min for each PAF treatment together with the control (no PAF) using a Hamilton Thorn IVOS motility analyser. Motility was analysed with low average path velocity (VAP) cutoff of 20.0 $\mu\text{m s}^{-1}$ and a straightness (STR) threshold of 75%. This procedure is the same as that used by other researchers for equine spermatozoa. Microcell counting slides (Conception Technologies, San Diego, CA) of 20 μm in thickness were used. About 10 μl of each sample was loaded on to the slide at the time of analysis. Five fields were analysed and the data were averaged.

Statistical analysis. Data obtained on sperm motility were presented as percentages. Data were initially analysed by repeated measures analysis and then log transformed and analysed by multiple linear regression analysis to model motility at different concentrations of

PAF and time periods. The pairwise comparisons were performed by Duncan's method.

Chlortetracycline assessment for capacitation and acrosome reaction

The method used was that described by DasGupta *et al.* (1993) for human spermatozoa, with some modification. CTC (Sigma) was prepared fresh each day at 500 $\mu\text{mol l}^{-1}$ in a buffer of 20 mmol Tris l^{-1} , 130 mmol NaCl l^{-1} and 5 mmol cysteine l^{-1} , (all from Sigma) and the pH was adjusted to 7.8. The solution was kept wrapped in foil to prevent the entry of light and stored at 4°C in a refrigerator. Swim-up spermatozoa were incubated with 10^{-4} – 10^{-12} mol PAF l^{-1} in 5% CO_2 at 37°C in water-saturated air together with the control group (no PAF). Fifty microlitres of sperm suspension was added to 50 μl of CTC solution in a foil-wrapped microcentrifuge tube and mixed for 20 s. After 10 s of incubation, cells were fixed by the addition of 10 μl of 12.5% (v/v) glutaraldehyde in 1 mol Tris buffer l^{-1} (pH 7.8) and mixed thoroughly for 20 s. Slides were prepared by placing 10 μl of fixed spermatozoa suspension on a clean slide. Subsequently one drop of 0.22 mol 1,4-diazabicyclo [2,2,2]-octane l^{-1} (DABCO; Sigma) in a ratio of glycerol: PBS (9:1) was added to retard fading of fluorescence. After the addition of a coverslip, excess fluid was removed gently and the slides were sealed with colourless nail varnish and stored in the dark at 4°C at all times. One hundred spermatozoa from each slide were assessed on the same day. Evaluation of spermatozoa was performed using an Olympus BX51 microscope equipped with phase and epifluorescence optics. For viability staining, after incubation with PAF and before the CTC assay, 10 μl of the nuclear exclusion dye Hoechst bis-benzimide 33258 (Sigma) was added to the sperm suspension at a final concentration of 1 $\mu\text{g ml}^{-1}$ and incubated for 5 min at 39°C and 5% CO_2 . Samples were washed by layering sperm suspension over 2 ml of 45% (v/v) Percoll (Holden *et al.*, 1990) and centrifuged at 300 g for 5 min to remove extracellular stain. The supernatant was removed and the pelleted cells were used for CTC stain. Each cell was first observed under UV illumination (excitation at 330–385 nm and emission at 400 nm) for determination of live and dead status. Cells showing bright blue staining of the nucleus were considered dead and not counted. Live cells were observed under blue-violet illumination (excitation at 400–440 nm and emission at 455 nm) for CTC patterns (Varner *et al.*, 1993).

Statistical analysis. Data for the acrosome reaction and capacitation as detected by CTC were presented as percentages: they were first log transformed, and then analysed by quadratic multiple linear regression where log capacitation and log acrosome reaction were dependent variables.

Ultrastructural visualization of the acrosome reaction after PAF treatment

Swim-up spermatozoa from three stallions were incubated with 10^{-9} , 10^{-10} and 10^{-11} mol PAF l^{-1} for 120 min. Subsequently 1 ml of treated spermatozoa (about $30 \times 10^6 \text{ ml}^{-1}$) was diluted with Ham's F12 medium supplemented with 0.03% BSA and centrifuged at 500 g for 10 min at room temperature (25°C). The pellet was then re-suspended in (HAT+0.03% BSA) medium and processed for electron microscopy. Progesterone, for induction of acrosome reaction, was prepared each day by dissolving 1 mg progesterone (Sigma) in 5 ml dimethyl sulphoxide (DMSO; Sigma) to make solution A. Solution B was prepared by adding 100 μl of solution A to 900 μl distilled water immediately before induction of the acrosome reactions (Meyers *et al.*, 1995). For induction of the acrosome reaction, solution B was added to sperm suspensions at 1 μl per 100 μl sperm suspension to obtain a 3.18 $\mu\text{mol l}^{-1}$ final concentration as described for boar spermatozoa by Melendrez *et al.* (1994). Samples were viewed and 100 spermatozoa were counted. Reacted status was identified as loss of the outer acrosomal and overlying plasma membranes. Vesiculation was identified upon fusion of the plasma and the outer acrosomal membranes. A non-reacted (intact) spermatozoon maintains the acrosomal membrane and its content. The scoring was divided among the intact, reacted and vesiculated spermatozoa and the numbers of spermatozoa were determined for intact, reacted and vesiculated spermatozoa for each concentration of PAF.

Fixation and preparation for transmission electron microscopy

For both treatment and control samples, 2 ml of sperm suspension was added to 2 ml of fixative solution (pH 7.6) containing 5% (v/v) glutaraldehyde, 3% (w/v) sucrose and 0.1 mol sodium cacodylate l^{-1} . After 1 h of incubation, spermatozoa were centrifuged at 500 g for 10 min, after which the sperm pellet was sandwiched in 2% (w/v) agar on a glass slide. The agar was cooled on ice and the sample cut into small pieces and transferred to fresh fixative overnight at 4°C. Samples were washed twice for 20 min in 0.1 mol sodium cacodylate buffer l^{-1} , stained with 0.1% (w/v) tannic acid in 0.1 mol sodium cacodylate buffer l^{-1} (pH 7.4) for 1 h at room temperature and washed twice for 1 h with 0.1 mol sodium cacodylate buffer l^{-1} . Post-fixation was performed with 1% (w/v) osmium tetroxide in 0.1 mol sodium cacodylate buffer l^{-1} for 1 h at room temperature. After washing twice for 20 min with 0.1 mol sodium cacodylate buffer l^{-1} , samples were dehydrated in a series of methanol (MeOH) solutions, each of which was saturated with uranyl acetate. Samples were incubated at room temperature in 50% (v/v) MeOH for 20 min,

Table 1. Percentage motility of stallion spermatozoa at different times of incubation and various concentrations of platelet activating factor (PAF)

PAF (mol l ⁻¹)	Percentage motility at:				
	30 min	60 min	90 min	120 min	150 min
10 ⁻⁴	20.3 ± 2.1	17.7 ± 2.9	13.9 ± 3.4	9.7 ± 3.4	5.6 ± 1.8
10 ⁻⁵	20.8 ± 2.3	18.4 ± 3.0	14.0 ± 3.0	10.0 ± 3.3	5.2 ± 1.5
10 ⁻⁶	61.8 ± 2.9	60.2 ± 3.8	54.5 ± 2.6	36.6 ± 2.5	15.3 ± 3.6
10 ⁻⁷	63.4 ± 3.7	56.4 ± 3.9	53.5 ± 2.5	37.9 ± 1.8	17.0 ± 2.2
10 ⁻⁸	67.2 ± 2.8	66.6 ± 2.0	65.9 ± 2.5	64.7 ± 2.3	42.1 ± 4.5
10 ⁻⁹	65.9 ± 2.4	65.0 ± 2.9	65.4 ± 1.9	60.3 ± 2.3	47.7 ± 3.0
10 ⁻¹⁰	76.9 ± 2.5	77.8 ± 2.6	77.8 ± 1.6	78.3 ± 1.9	58.0 ± 1.5
10 ⁻¹¹	74.0 ± 2.4	75.1 ± 2.8	75.5 ± 2.1	80.6 ± 2.5	57.6 ± 1.2
10 ⁻¹²	74.7 ± 2.7	78.3 ± 2.9	78.5 ± 3.4	80.8 ± 4.6	54.6 ± 3.0
10 ⁻¹³	73.6 ± 2.3	78.4 ± 2.6	76.5 ± 3.0	79.6 ± 4.3	53.1 ± 2.9
0	76.1 ± 2.5	75.1 ± 2.6	72.6 ± 2.4	68.8 ± 2.8	62.8 ± 3.2

Values are mean ± SEM, *n* = 10.

in 75% (v/v) MeOH for 20 min, twice each for 45 min in 95% (v/v) MeOH and 100% (v/v) MeOH, and then placed once in propylene oxide for 15 min. Infiltration was carried out with 1 propylene oxide/1 Polybed 812 overnight. Samples were then infiltrated with 100% Polybed 812 overnight, embedded in fresh Polybed 812 and placed into ovens at 60°C for 24–48 h. Ultrathin sections (600–800 Å) were cut on a Leica Ultracut UCT and collected on 200 mesh copper grids. Sections were stained for 6 min with 2% (v/v) aqueous uranyl acetate; 5 min with Reynolds' (Reynolds, 1963) lead citrate; and samples were viewed under a JEOL JEM100 CX II Electron Microscope at 80 KV.

Statistical analysis. Data for the acrosomal status (intact, reacted and vesiculated) as detected by transmission electron microscopy were presented as percentages, so they were first log transformed. Subsequently they were analysed by linear regression analysis using SAS.

Results

Motility analysis

The results of the effect of PAF on motility of fresh stallion spermatozoa at different time intervals are presented (Table 1). There was a quadratic relationship between concentrations of PAF and incubation times on their effect on motility, and this effect was significant ($r^2 = 0.81$). Motility was severely depressed at higher concentrations of PAF and longer time of incubations, whereas lower concentrations ranging from 10⁻¹⁰ to 10⁻¹³ mol PAF l⁻¹ enhanced motility and the best motility was maintained at 120 min of incubation. The PAF concentration, incubation time and their interaction were highly significant ($P < 0.01$) for their effect on

motility. Pairwise comparisons were conducted using Duncan's method to detect if there were any differences between incubation time and PAF concentrations. The test revealed that a PAF concentration ranging from 10⁻¹⁰ to 10⁻¹³ mol l⁻¹ at 120 min of incubation was the best condition in which motility was enhanced. Duncan's method was conducted again to see whether there were differences between PAF concentrations at 120 min of incubation. The analysis showed that the motility of spermatozoa was significantly higher at concentrations from 10⁻¹⁰ to 10⁻¹³ mol PAF l⁻¹ than from 10⁻⁴ to 10⁻⁹ mol PAF l⁻¹ at 120 min, but there were no significant differences among the concentrations from 10⁻¹⁰ to 10⁻¹³ mol PAF l⁻¹ at 120 min of incubation.

CTC staining

As motility, which is an important parameter in sperm function, was best maintained at 120 min, the effect of 10⁻⁴–10⁻¹² mol PAF l⁻¹ on capacitation and the acrosome reaction using CTC was conducted at 120 min. At 10⁻⁴–10⁻⁶ mol PAF l⁻¹, the sperm acrosome reaction increased in contrast to the number of spermatozoa that undergo capacitation at the same concentrations (Fig. 1). Capacitation was achieved at lower concentrations of 10⁻⁷–10⁻¹² mol PAF l⁻¹ and was highest at 10⁻¹⁰ and 10⁻¹¹ mol PAF l⁻¹. There was a significant effect of PAF on the percentage of spermatozoa that were capacitated ($r^2 = 0.83$, $P < 0.01$).

The concentration of PAF that caused the maximum capacitation (B) was from 10⁻⁹ to 10⁻¹² mol PAF l⁻¹ at 120 min of incubation; however, only 20% of evaluated data accounted for the acrosome reaction ($r^2 = 0.20$, $P < 0.01$). The types of sperm CTC pattern that were visualized with the CTC assay are shown (Fig. 2.)

Fig. 1. Chlortetracycline assay patterns of stallion spermatozoa showing (a) capacitated (B), acrosome-reacted (A, C); and (b) uncapacitated (D) patterns. Magnification $\times 1000$.

Fig. 2. The effect of platelet activating factor (PAF) on the (a) capacitation and (b) acrosome reaction status of stallion spermatozoa as detected by chlortetracycline stain. Data are presented as percentages, so they were first log transformed then analysed by regression analysis. $\log_{10}(\text{capacitation}_B) = \beta_0 + \beta_1 \log_{10}(\text{concentration}_B) + \beta_2 [\log_{10}(\text{concentration}_B)]^2$. $\log_{10}(\text{acrosome reaction}_{AR}) = \beta_0 + \beta_1 \log_{10}(\text{concentration}_{AR}) + \beta_2 [\log_{10}(\text{concentration}_{AR})]^2$.

Transmission electron microscopy

The results for transmission electron microscopy analysis of spermatozoa show that the number of intact

spermatozoa decreased as the concentration of PAF increased (Table 2, Fig. 3). The largest number of reacted spermatozoa was achieved at $10^{-9} \text{ mol l}^{-1}$ (47.7 ± 1.45 , Table 2), whereas vesiculation was highest

Table 2. Effect of platelet activating factor (PAF) on the acrosomal status of fresh equine spermatozoa as detected by transmission electron microscopy

PAF (mol l ⁻¹)	Intact	Reacted	Vesiculated
10 ⁻⁹	9.33 ± 1.20	48.33 ± 0.88	42.33 ± 1.85
10 ⁻¹⁰	10.67 ± 0.67	27.00 ± 1.52	62.33 ± 1.45
10 ⁻¹¹	17.00 ± 1.00	22.67 ± 1.33	60.33 ± 1.20
0	72.33 ± 2.40	20.67 ± 2.40	7.00 ± 1.15

Values are mean ± SEM; *n* = 3.

Intact: spermatozoa that maintain the acrosome and its content; reacted: loss of the outer acrosomal and overlying plasma membranes; vesiculated: the fusion of the plasma and the outer acrosomal membranes.

at 10⁻¹⁰ and 10⁻¹¹ mol PAF l⁻¹ (Table 2). The number of vesiculated sperm cells matches the amount of type B CTC-stained cells. The linear regression analysis indicated that differences in PAF concentrations were highly significant as indicated by the *R*² values (for intact: 0.97.2; reacted: 0.89.8; and for vesiculated: 0.98.1). The structural changes in stallion spermatozoa under the effect of PAF treatment are shown (Fig. 3)

Discussion

Other studies have demonstrated that treatment of mammalian spermatozoa with PAF affects motility (human: Ricker *et al.*, 1989; Hellstrom *et al.*, 1991; bovine: Aravindakshan and Sharma, 1995). The PAF content in human spermatozoa processed for use in IVF correlates positively with motility indices and pregnancy rates (Roudebush and Purnell, 2000). PAF is present in squirrel monkey spermatozoa (Roudebush and Mathur, 1998) and in stallion spermatozoa (W. E. Roudebush, personal communication), and PAF concentrations in rhesus monkey spermatozoa were found to be directly correlated with motility and progression of spermatozoa (Roudebush *et al.*, 2002). The amount of PAF receptor mRNA was higher in abnormal than in normal bull spermatozoa (Roudebush *et al.*, 2001). High fertility boars have significantly more PAF in their spermatozoa than low fertility boars (Roudebush and Diehl, 2001). Spermatozoa from a PAF receptor knock-out mouse strain failed to express the receptor and had a significantly reduced rate of capacitation as assessed by the spontaneous onset of the acrosome reaction *in vitro* (Wu *et al.*, 2001). Treatment of human spermatozoa for 5 min with synthetic PAF at concentrations from 10⁻⁷ to 10⁻¹³ mol l⁻¹ resulted in a significant (*P* < 0.05) increase in motility, whereas treatment with ≥ 10⁻⁵ mol l⁻¹ resulted in immediate cell death (Ricker *et al.*, 1989). Human spermatozoa exposed to exogenous PAF concentrations of (0.5–100.0) × 10⁻⁹ mol l⁻¹ resulted in a significantly increased linear velocity and the greatest increase in

linear motion was observed at 50 × 10⁻⁹ mol PAF l⁻¹ (Jarvi *et al.*, 1993). A threefold improvement in motility of human spermatozoa was reported after exposure to 10 × 10⁻⁹ mol PAF l⁻¹ for 4 h (Krausz *et al.*, 1994). The effect of PAF on mouse epididymal spermatozoa indicated that 10⁻⁴ mol PAF l⁻¹ reduced the motility of spermatozoa and decreased (*P* < 0.05) the fertilization rate (Kuzan *et al.*, 1990).

Motility results conducted in the present study indicate that the effects of PAF on motility of stallion spermatozoa are time- and dose-dependent. At higher concentrations of PAF, and as incubation time increased, motility was severely depressed. Interaction between time of incubation and PAF concentration was significant (*P* < 0.01). Sperm motility was best maintained at 120 min of incubation at 10⁻¹⁰–10⁻¹³ mol PAF l⁻¹; however, there were no significant differences among this range of concentrations at 120 min of incubation. These concentrations were lower than that reported for humans; the time of incubation may be a factor, as human spermatozoa were incubated for 5 min whereas stallion spermatozoa were incubated for 120 min. Species differences and the medium conditions too may affect results.

As sperm motility is an important parameter of a semen analysis, and motility was best maintained at 120 min, this duration was chosen for the capacitation and acrosome reaction experiments. The final stages of capacitation are associated with hyperactivated motility and Ricker *et al.* (1989) suggested that this change in motility is necessary for fertilization both *in vivo* and *in vitro*. Wu *et al.* (2001) confirmed that exogenous PAF acts in a specific manner to induce sperm motility changes associated with capacitation.

CTC was highly correlated with transmission electron microscopy for estimation of acrosome reacted spermatozoa when the effect of heparin on capacitation and acrosome reaction of stallion spermatozoa was investigated (Varner *et al.*, 1993). The Ca²⁺-related changes in the capacitation state of human spermatozoa were evaluated using CTC. The results of CTC were comparable to fluorescein-conjugated *Pisum sativum* agglutinin (PSA) staining which was verified by transmission electron microscopy (DasGupta *et al.*, 1993). Huo and Yang (2000) investigated the effects of PAF on capacitation and acrosome reaction of mouse spermatozoa by CTC and Coomassie blue staining, respectively. Their results showed that the percentage of capacitated mouse spermatozoa was increased (*P* < 0.05) by incubation with 92.3 × 10⁻⁹ mol PAF l⁻¹ for 20–120 min, whereas the acrosome reaction was increased at 9.2 × 10⁻⁶ mol l⁻¹ at 90 min. The effects of PAF on the acrosome reaction of human spermatozoa as detected by fluorescein iosthiocyanate (FITC)–PSA assay indicate that PAF concentrations from 10⁹ to 10⁻¹¹ mol l⁻¹ affect acrosome reaction in a dose-dependent manner (Sengoku *et al.*, 1996). In bovine spermatozoa,

Fig. 3. Transmission electron micrographs of stallion spermatozoa capacitated *in vitro*. Capacitation was performed *in vitro* by platelet activating factor (PAF) and the acrosomal reaction was induced by progesterone. (a) Vesiculated, (b) acrosome-reacted and (c) intact spermatozoa. Scale bars represent 1 μm .

0.1×10^{-9} mol PAF l^{-1} was observed to be most optimal, as at this concentration, the acrosome reaction detected by Giemsa stain improved significantly without much loss of motility (Aravindakshan and Sharma, 1995).

In the present study, the statistical analysis for capacitation indicated that 83% of spermatozoa were characterized as live-capacitated after treatment with PAF ($r^2 = 0.81$, $P < 0.01$). Statistical analysis for the acrosome reaction indicated that 20% of spermatozoa were characterized as live and acrosome reacted after treatment with PAF ($r^2 = 0.20$, $P < 0.01$). Enhancement of sperm

motility from 10^{-10} to 10^{-13} mol PAF l^{-1} at 120 min of incubation was compatible with the CTC result for capacitation at the same time and concentrations of PAF.

Progesterone induces the acrosome reaction in human (Wistrom and Meizel, 1993) and pig (Melendrez *et al.*, 1994) spermatozoa by increasing intracellular calcium. As the equine preovulatory follicle secretes progesterone before ovulation and luteinization, Meyers *et al.* (1995) evaluated the ability of progesterone to stimulate the acrosome reaction in equine spermatozoa, and they concluded that progesterone-induced acrosome

reactions were physiological. Therefore, as a follow-up to the CTC results that were obtained in the present study on capacitation, and the previous conclusion that indicates the ability of PAF at lower concentrations to induce capacitation, transmission electron microscopy was conducted to find the true acrosome reaction on spermatozoa that were capacitated by PAF and induced for acrosome reaction by progesterone. The sperm acrosome-reacted in response to progesterone, leading to the conclusion that capacitation of stallion spermatozoa had occurred by treatment with PAF. The results also indicated that the number of vesiculated sperm cells correlated with the number of type B CTC stained cells, and this finding provides new insights into CTC staining that need to be addressed by future studies.

The main barrier to IVF in the stallion appears to be penetration of the zona pellucida (Hinrichs *et al.*, 2002). It is possible that the failure of penetration of the equine oocyte by spermatozoa is related to the difficulty in capacitating spermatozoa and then their inability to undergo the acrosome reaction at the surface of the zona pellucida. As the limited success of equine IVF is in part due to lack of efficient treatment of stallion spermatozoa for capacitation, PAF may be used to help capacitate stallion spermatozoa. More insight into the effect of PAF on stallion spermatozoa will also be obtained by treating spermatozoa with PAF before assisted reproductive technology such as IVF. The action of PAF on spermatozoa has been studied in different mammalian species. To our knowledge this is the first study that extensively investigates the effect of PAF on stallion spermatozoa.

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