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The effect of platelet activating factor on the motility and acrosome reaction of ram spermatozoa

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Summary

Platelet activating factor (PAF) is a novel signaling phospholipids that in addition to platelet activation has many biological properties. The acrosome reaction, as an essential step in mammalian fertilization, can occur in response to several agents such as PAF. Therefore, the present study aimed to assess the effect of PAF on the motility and acrosome reaction of ram spermatozoa. Semen was collected from 18 fertile rams and incubated with four levels of PAF (10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol) at 37°C for 15, 30, 60 or 120 min. Sperm motility and acrosome reaction were analyzed at varying levels of PAF with different incubation periods. With increasing PAF concentration, acrosome reaction was enhanced, while sperm motility was reduced ($P<0.001$). As the period of incubation increased, there was a gradual decrease in sperm motility and increase in acrosome reaction percentages. There were high correlation between PAF concentrations and incubation times on induction of acrosome reaction ($R^2 = 0.86$) and reduction in sperm motility ($R^2 = 0.82$). In addition, it was found that a PAF level of 10^{-9} and incubation time for 30 min is the best optimum for inducing acrosome reaction in ram spermatozoa without drastically decreases in sperm motility. The present study optimized for the first time the concentration and incubation time of PAF for induction of acrosome reaction in fresh ram spermatozoa.

Key words: Ram, Platelet activating factor, Spermatozoa, Acrosome reaction, Motility

Introduction

Development of successful *in vitro* fertilization (IVF) techniques is essential for the study of the basic aspects of fertilization process. Freshly ejaculated mammalian spermatozoa are not immediately capable of achieving fertilization (Yanagimachi, 1994). During residence in the female tract, the sperm cell undergoes a complex and poorly understood set of modifications which confer fertilization competence, a process collectively called capacitation (Sukardi *et al.*, 1997; Jaiswal *et al.*, 1998; Bedu-Addo *et al.*, 2005; Huang *et al.*, 2007).

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 (Jones, 1997; Sukardi *et al.*, 2001). An unregulated capacitation process causes sperm to undergo a spontaneous acrosome reaction and resulting in loss of sperm fertilization capacity (Huang *et al.*, 2005, 2007).

Fresh ram spermatozoa will spontaneously undergo the acrosome reaction when incubated at 39°C over a period of 4 h in the absence of any inducing agent (Watson *et al.*, 1991). The acrosome reaction occurs in response to natural inducers, i.e., the zona pllucida and oviductal fluids (Yanagimachi, 1994), but it can also be artificially induced by a variety of substances such as Ca^{2+} ionophore

A23187 (Watson *et al*., 1991; Jaiswal *et al*., 1998), heparin (Varner *et al*., 1993), bovine serum albumin (Son *et al*., 2000; Bedu-Addo *et al*., 2005; Huang *et al*., 2005) and mannose-BSA (Blackmore and Eisoldt, 1999). Phospholipids are essential components of all mammalian cells. Platelet activating factor (PAF) is a novel potent signaling phospholipid that has been implicated in a number of biological processes. In reproductive processes, PAF has been shown to influence sperm function by affecting the motility, capacitation, acrosome reaction and fertility of spermatozoa (Roudebush, 2001; Kordan *et al*., 2003; Ali *et al*., 2007; Huang and Li, 2007).

Several studies have researched the effects of PAF on inducing acrosome reaction in spermatozoa of several species. For example, it has been demonstrated that PAF can induce capacitation and acrosome reaction in the spermatozoa of stallion (Odeh *et al*., 2003), boar (Bathgate *et al*., 2007), buffalo (Aravindakshan and Sharma, 1996; Kumar and Sharma, 2005), cattle (Aravindakshan and Sharma, 1995), mouse (Huo and Yang, 2000; Wu *et al*., 2001), human (Angle *et al*., 1993; Krausz *et al*., 1994; Luconi *et al*., 1995; Sengoku *et al*., 1996) and rabbit (Fukuda *et al*., 1994). However, to date the effect of PAF on the motility or acrosome reaction has not been investigated in ram spermatozoa (O'Meara *et al*., 2008). Therefore, the objectives of this experiment were 1) to study the motility and acrosome reaction in ram spermatozoa at different time intervals after treatment with varying levels of PAF and 2) to optimize the level of PAF and incubation time for obtaining maximum acrosome reaction and sperm motility of fresh ram spermatozoa.

Materials and Methods

Animals

Eighteen adult sexually active Bakhtiary rams (the native sheep breed in Iran) were used in this experiment. All animals had been reared under similar conditions at the farm in suburb of Khorramabad. During the experimental period, each ram was fed daily with hay and concentrates and water was freely available. The rams were trained for semen collection into an artificial vagina (42-45°C) using two sexually receptive and restrained ewes treated with estrogen one day before sperm collection (1 mg estradiol benzoate, Aburaihan Pharmaceutical Co., Iran).

Experimental procedure

The spermatozoa were treated with four levels of PAF: no PAF (control), 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol of PAF. The motility and acrosome reaction were examined after 0, 15, 30, 60 and 120 min of incubation. Lyophilized PAF was purchased from Tocris Cookson Ltd. (Bristol, UK).

Sperm preparation

Immediately after semen collection, the samples were kept in a water bath (37°C) and rapidly transported to the laboratory. The fresh semen samples (about 0.5 ml) were initially washed 2 times in 3 ml synthetic oviductal fluid (SOF) medium by centrifugation at 400 g for 10 min. The supernatant was discarded using a sampler and the sperm pellet was re-suspended with the same medium. Concentration of spermatozoa was estimated by haemocytometer and aliquots of the sperm suspension (20 µl, adjusted to about 1×10^8 cells/ml) were transferred into 2 ml of fresh SOF medium containing HEPES buffer (Tervit *et al*., 1972) with various concentrations of PAF. All the preparations were incubated at 37°C up to 120 min.

Evaluation of sperm motility and acrosome reaction

For evaluation of sperm motility, 10 µl of aliquots were placed on clean glass slides at specific time intervals and covered with a coverslip and examined visually under a phase-contrast microscope (Leica, USA) at the magnification of 400 (Sonmez *et al*., 2005). Acrosome reaction was assessed according to the method of Aravindakshan and Sharma (1996). Briefly, smears were made on clean glass slides by transferring 8 µl aliquots with 10^{-7} to 10^{-10} mol of PAF levels separately at the end of specific time intervals (15, 30, 60 and 120 min) and followed by fixing spermatozoa in 15% formaldehyde solution for 10 min. The slides were rinsed with distilled water, air dried in an incubator at 37°C and immersed in 4.5% filtered Giemsa solution for 17 to 18 h.

Screening of slides

To quantify the percentage of acrosome reactions, 100 spermatozoa were randomly counted from each stained preparation for each treatment and classified as completely reacted, partially reacted and nonacrosome reacted spermatozoa. The spermatozoa were considered as completely reacted when the anterior sperm head remained completely unstained and the acrosomal cap was either completely detached from the sperm head or remained attached to it at some points. Spermatozoa were classified as partially reacted when stained less intensely and the acrosome appeared ruffled or loosened, and as nonacrosome reacted when the acrosomal region was evenly stained blue to pink with a clear boundary between the sperm cell and the background. Partially reacted spermatozoa were considered as reacted acrosome in the present study.

Statistical analysis

Data were analyzes using the SPSS 10.0 statistical package (SPSS Inc., Chicago, IL, USA). Data obtained on motility and acrosome reaction were evaluated by linear regression analysis to model motility at different concentrations of PAF and time periods. Pairwise comparisons were also conducted using Bonferroni's test to detect if there were any differences between incubation time and PAF concentrations (Petrie and Watson, 1999). Values were presented as mean ± SEM.

Results

Motility analysis

The results of the effect of PAF on the motility of fresh ram spermatozoa at different concentrations and incubation times are summarized in Table 1. There was a significant correlation (R^2 = 0.82, P<0.001) between the effect of PAF concentrations and incubation times on reduction of sperm motility. A gradual decrease was observed in sperm motility at all levels of PAF, however, motility percentage was severely depressed at 10^{-7} mol (b = -10.62, P<0.0001), the highest concentration of PAF, so that, it reached to 50.8 ± 1.0 and 44.2 ± 0.9 percent at min 15 (b = 14.28, P<0.0001) and 30 (b = 9.30, P<0.0001), respectively.

The motility percentages were improved as the concentration of PAF decreased. The best motility rate was obtained at the lowest level of PAF. However, its interaction with higher acrosome reaction was to be noted in the present study. Pairwise comparisons revealed that the optimum motility percentage was at min 30 of 10^{-9} mol of PAF level.

Acrosome reaction assessment

Platelet activating factor significantly induced acrosome reaction in ram spermatozoa compared to the control group (P<0.01). The effect of PAF on induction of acrosome reaction at different PAF levels and incubation periods are presented in Table 2. There was a high correlation (R^2 = 0.86, P<0.001) between PAF level and incubation time on induction of acrosome reaction.

The maximum reacted acrosome was seen at 10^{-7} mol of PAF (b = 12.48, P<0.0001). It reached to 78.1 ± 1.3 *vs* 2.2 ± 0.2% at min 15 (b = -11.29, P<0.0001) in the treated spermatozoa compared to the control group, respectively. However, it was highlighted that this concentration of PAF suppressed sperm motility rate dramatically. The optimum interaction between high acrosome reaction percentage without much loss of motility was observed at min 30 with 10^{-9} mol of PAF level (b = -3.22, P<0.01).

At all levels of PAF, the number of reacted acrosome was elevated as incubation time increased. In contrast, acrosome reaction percentages were decreased as PAF levels attenuated. The reduction in reacted acrosome rate ranged from 78.1 to 37.3% at PAF concentrations of 10^{-7} and 10^{-10} mol at min 15, respectively. While the maximum spontaneous values of acrosome-reacted spermatozoa in the control group varied from 2.2 up to 19.6% at min 15 through min 120 (Fig. 1). Overall, the changes in sperm motility and acrosome reaction in samples

Table 1: Sperm motility percentages obtained at various concentrations of PAF and different time intervals in fresh ram spermatozoa

PAF level (mol)	Incubation times (min)				
	0	15	30	60	120
Control (no PAF)	88.3 ± 0.5^a	88.0 ± 0.8^a	82.2 ± 0.8^b	75.5 ± 0.7^c	64.5 ± 1.0^d
10^{-7}	87.7 ± 0.4^a	50.8 ± 1.0^b	44.2 ± 0.9^c	37.8 ± 0.6^d	32.8 ± 1.1^e
10^{-8}	86.6 ± 0.4^a	63.6 ± 1.0^b	58.3 ± 0.9^c	54.3 ± 1.2^c	48.7 ± 0.8^d
10^{-9}	85.7 ± 0.6^a	77.7 ± 1.1^b	73.7 ± 1.2^b	68.0 ± 1.4^c	65.8 ± 1.4^c
10^{-10}	86.2 ± 0.6^a	78.6 ± 1.0^b	74.6 ± 0.9^b	68.0 ± 1.3^c	66.2 ± 1.2^c

Means with different superscripts (a, b, c, d, e) within each row are significantly different (P<0.05). Values are presented as mean ± SEM

Table 2: Percentages of reacted acrosome obtained at various concentrations of PAF and different time intervals in fresh ram spermatozoa

PAF level (mol)	Incubation times (min)				
	0	15	30	60	120
Control (no PAF)	2.1 ± 0.2^a	2.2 ± 0.2^a	8.7 ± 0.7^b	13.2 ± 0.8^c	19.6 ± 0.9^d
10^{-7}	3.0 ± 0.3^a	78.1 ± 1.3^b	84.5 ± 1.5^c	85.9 ± 1.3^c	86.4 ± 1.5^c
10^{-8}	3.3.± 0.3^a	61.1 ± 1.1^b	68.2 ± 1.4^c	70.6 ± 1.3^c	71.7 ± 1.4^c
10^{-9}	2.6 ± 0.2^a	51.2 ± 1.3^b	62.5 ± 1.1^c	64.2 ± 1.2^c	66.6 ± 1.3^c
10^{-10}	2.8 ± 0.3^a	37.3 ± 1.1^b	44.7 ± 0.9^c	47.3 ± 1.0^c	48.2 ± 1.4^c

Means with different superscripts (a, b, c, d) within each row are significantly different (P<0.05). Values are presented as mean ± SEM

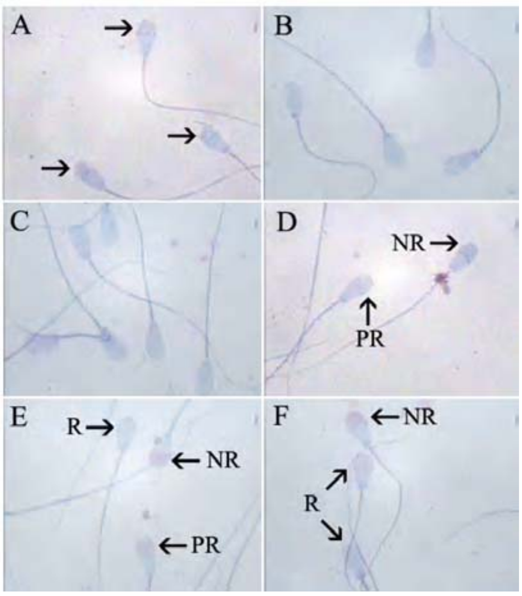

Fig. 1: Evaluation of acrosome reaction in ram spermatozoa (A) Non-reacted acrosome (NR) in the control group (arrows) without PAF treatment at min 15. (B and C) Completely reacted acrosomes in 10^{-7} mol of PAF at min 15 (B) and 10^{-8} mol (C) at min 60. (D) Partially reacted (PR) and non-reacted acrosome in the control group at min 120. (E and F) Reacted (R), non-reacted and partially reacted acrosome in 10^{-9} mol of PAF at min 15 (E) and 30 (F)

with no PAF (control) were negligible up to min 120.

Discussion

To our knowledge, this is the first study to standardize the optimum PAF concentration and incubation time for inducing acrosome reaction in ram spermatozoa. The results of the present experiment indicated that 10^{-9} mol of PAF and 30 min of incubation time is the best condition for inducing acrosome reaction without much loss of sperm motility. This strongly suggests that PAF can be considered as an accelerating factor for ram sperm capacitation.

The response of ram spermatozoa to different concentrations of PAF from 10^{-7} to 10^{-10} mol in our investigation is similar to those reported for boar (Bathgate *et al.*, 2007), stallion (Odeh *et al.*, 2003), buffalo (Aravindakshan and Sharma, 1996) and cattle (Parks and Hough, 1990), who found that higher levels of PAF induced acrosome reactions in the above-mentioned species and caused a reduction in sperm motion. Such a similar finding was observed in our study in the ram with an increase in the percentage of acrosome-reacted spermatozoa and a corresponding decrease in sperm motility.

The acrosome reaction of mammalian spermatozoa is a calcium dependent exocitotic event in the sperm head and is essential for fertilization. The acrosome reaction can occur only after completion of capacitation and before penetration of the zona pellucida. It has been suggested that binding of PAF to target cells is followed by an increase in phosphatidyl inositol 4-5 bisphosphate breakdown and subsequent formation of diacyl glycerol, an important intracellular second messenger. The breakdown of this agent generates inositol 1, 4, 5 triphosphate, which is involved in calcium mobilization (Minhas *et al*., 1989). It is possible that calcium mobilization may promote the capacitation and acrosome reaction in spermatozoa. This interpretation is supported by the fact that PAF has been observed in spermatozoa of ram (O'Meara *et al*., 2008), bull (Diehl *et al*., 2001; Brackett *et al*., 2004), stallion (Roudebush, personal communication), rhesus monkey (Roudebush *et al*., 2002) and human (Roudebush and Purnell, 2000). High fertility boars have significantly more PAF in their spermatozoa than low fertility boars (Roudebush and Diehl, 2001).

The results obtained in the present study indicate that the effects of PAF on motility and acrosome reaction of ram spermatozoa are time- and dose-dependent. At higher levels of PAF, motility was depressed as incubation time increased. Whereas at lower concentrations (10^{-9}), sperm motility was best maintained at 30 min of incubation with relatively high percentages of reacted acrosome, therefore, this level and time were optimal for the acrosome reaction induced by PAF. This incubation time was lower than that reported for stallion and higher than the buffalo, as the best stallion and buffalo incubation periods were 120 and 15 min, respectively. Species differences and the medium conditions too may affect the results.

Platelet activating factor is an ether and it is believed that it might function through destabilization of the sperm plasma membranes and, thus, induce these physiological changes more rapidly (Odeh *et al*., 2003). It is the possible reason of this phenomenon in our study that why the number of acrosome-reacted spermatozoa elevated or sperm motility suppressed as the PAF concentration increased. This role of PAF provides a better understanding of its probable physiological action in sperm function as well as its possible potential in some assisted reproductive technology applications. Furthermore, this function of PAF justified our findings in the present study, for which the PAF level positively correlated with the number of sperm reacted acrosome and negatively with sperm motility percentages. Such a negative effect of high PAF concentration on sperm motility has been supported by some researchers in the other species. For example, treatment of human spermatozoa for 5 min with synthetic PAF at concentrations from 10^{-7} to 10^{-13} mol resulted in a significant increase in motility, whereas treatment with $\geq 10^{-5}$ mol resulted in immediate cell death (Ricker *et al*., 1989). Moreover, the effect of PAF on mouse epididymal spermatozoa indicated that 10^{-4} mol/PAF reduced the motility of spermatozoa and decreased ($P<0.05$) the fertilization rate (Kuzan *et al*., 1990). Similar results also were found in stallion (Odeh *et al*., 2003) and buffalo (Aravindakshan and Sharma, 1996) spermatozoa, so that, the lowest sperm motility and highest acrosome reaction were observed at greater level of PAF (10^{-4} mol in stallion and 200 µmol in buffalo).

Very recently, O'Meara *et al*. (2008) indicated that the amounts of platelet activating factor recovered from ram spermatozoa display extreme variability among rams. The mean value obtained for total PAF from 40×10^6 spermatozoa was 868.2 pg with a range of 5 to 3749 pg. They also indicated that PAF was positively related to the percentage of live cells and capacitated spermatozoa as well as negatively correlated with the percentage of dead cells. Unfortunately, as yet there is no literature concerning laboratory investigation on the addition of exogenous PAF to sperm medium of ram spermatozoa to compare with our study and only limited paper is involved for detection and measurement of PAF levels in normal ram spermatozoa (O'Meara *et al*., 2008).

In a study by Odeh *et al*. (2003) on the effect of synthetic PAF on the acrosome reaction, it was shown that PAF induced the acrosome reaction in stallion sperm, in which the lower concentration of PAF ranging from 10^{-10} to 10^{-11} enhanced motility and induced capacitation at 120 min of incubation. Induction of capacitation *in vitro* has been demonstrated in fresh and frozen spermatozoa of cattle by using PAF. Parks and Hough (1990) found that at concentration between 80 to 100 µmol PAF, bull spermatozoa underwent acrosome reaction without a rapid loss of motility and penetrated *in vitro*-matured bovine oocytes. Furthermore, Aravindakshan and Sharma (1995) were also noted that about 0.1×10^{-9} mol/PAF is optimal in bulls, because at this concentration the acrosome reaction improved without much drop in sperm motility. They also demonstrated in another study that a PAF level of 100 µmol and an incubation period of 15 min are the best values for inducing acrosome reaction in buffalo spermatozoa without dramatically decreases in sperm motility (Aravindakshan and Sharma, 1996).

This study optimized the exogenous PAF level and incubation time for inducing acrosome reaction in fresh ram spermatozoa. Based on the relative alterations and correlations between motility and acrosome reaction, it was concluded that 1 nmol of PAF for 30 min of incubation is the optimum combination for induction of acrosome reaction in the ram spermatozoa, since significant improvement in acrosome reactions with good motility percentages was observed. Although, the present experiment indicates that PAF may be used to help capacitation of ram spermatozoa before assisted reproductive technology such as IVF, however, before ultimate suitability of using PAF for inducing acrosome reactions in ram spermatozoa can be established, it is first necessary to test the PAF-treated spermatozoa for *in vitro* fertilization and to confirm the penetration efficiency of these treated spermatozoa.

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