Identification of platelet activating factor (PAF) receptor in equine spermatozoa and its role in motility, capacitation and the acrosome reaction

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By

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ABSTRACT

Platelet activating factor (PAF) is a unique signaling phospholipid that has many biologic properties in addition to platelet activation. PAF roles in reproduction involve ovulation, fertilization, embryo development, implantation and parturition. It may also serve as a biomarker for normal sperm function. The presence of PAF receptor on the spermatozoa of 10 stallions was investigated by immunofluorescence microscopy. Statistical analysis revealed that the fluorescence intensity, FI (Mean+/-SEM), in the post- acrosomal region (FI= 2.60+/-0.15) was significantly higher ($P< 0.01$) than that in any other region of stallion spermatozoa. The effect of synthetic PAF on stallion spermatozoal motility, capacitation, and the acrosome reaction (AR) were evaluated. Treatment of 10 stallion semen samples with 10^{-4} to 10^{-13} M PAF resulted in statistically significant differences in motility and capacitation ($r^2 = 0.81$ and 0.83 respectively). The concentration of PAF, incubation time and their interaction were highly significant ($P< 0.01$) for their effect on motility. Concentrations of PAF ranging from 10^{-9} to10^{-11} M were able to induce capacitation. Following capacitation in vitro with PAF, and induction of the acrosomal reaction by progesterone, transmission electron microscopy (TEM) was conducted on the spermatozoa of 3 stallions, to detect the true

AR. Differences in PAF concentrations were highly significant as indicated by R-square (for intact: 97.2, reacted: 89.8, and for vesiculated: 98.1). Treating spermatozoa from 3 stallions with the PAF antagonist FR-49175 inhibited calcium release and fluorescence intensity with a median inhibitory concentration (IC_{50}) of $10^{-7.5}$ M (r^2=0.82, P<0.01) and 10^{-8} M (r^2=0.92, P<0.01) respectively, suggesting a receptor mediated process for the mechanism of action of PAF. Although the exact mechanisms of PAF action on equine spermatozoa remain unclear, it is widely reported that PAF acts by a receptor-mediated mechanism and that the PAF receptor is a member of the family of G-protein coupled receptors with phospholipase C as the effector. Since the limited success in equine ART (e.g. IVF) is in part due to lack of efficient treatment of stallion spermatozoa for capacitation, PAF may be useful to help capacitate stallion spermatozoa. Without proper capacitation, spermatozoa are unable to initiate the acrosome reaction which is a prerequisite for fertilization.

DEDICATION

To all people who reach towards others in any good means they have

Six years ago I started my journey here in the town of Blacksburg at Virginia Tech. It was quite a challenge to be a mother of two, a wife and a student who lives miles away from family. Each day was a reminder of how much I missed them, but with all the hectic times and the continuous running, they never left my mind. For deep in my heart, I know that I was in their hearts too. It was first God's mercy and care that made this moment possible and that put on my path the right and kind people at the difficult times.

Second, it is the dedication and the willingness of those people that made my crawling a walk toward the continuous knowledge that will not end by a PhD.

As I am finishing and leaving to go back to Jordan, the memory of all will be with me always to guide me and remind me that being a good human is the ultimate achievement, for we will not really be taking a degree or a wealth when we leave this earth. We will only be remembered of what kind human we were.

ACKNOWLEDGMENTS

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I would like to point out that the interpretation of this research is mine, and that no one is responsible for any remaining errors.

This research is completed as partial fulfillment for the Ph.D. requirements at Virginia Polytechnic Institute and State University.

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LIST of ABBREVIATIONS

AI = artificial insemination

ART = assisted reproductive technology

AR = acrosome reaction

AV = artificial vagina

BSA = bovine serum albumin

cAMP = cyclic adenosine monophosphate

C-kinase = protein kinase C

CTC = chlortetracycline

DAG = diacylglycerol

ET = embryo transfer

FI = fluorescence intensity

GAGs = glycosaminoglycan

GIFT = gamete intrafallopian transfer

GTP = guanosine triphosphate

IC_{50} = median inhibitory concentration

ICSI = intracytoplasmic sperm injection

IVF = in-vitro fertilization

$InsP_3$ or IP3= inositol 1, 4, 5-triphosphate

PAF-AH = PAF acetylhydrolyase

PAF = platelet activating factor

TEM = transmission electron microscopy

ZP = zona pellucida

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INTRODUCTION

Fertilization is a fragile process that may vary temporarily or be permanently depressed, thus resulting in infertility. There are many techniques in the management of infertility in mammals including humans; these techniques and procedures were tested and developed in animals, and subsequently transferred for the treatment of human infertility problems. These procedures include artificial insemination (AI), sperm preparation and swim up techniques, ovarian hyper-stimulation, in-vitro fertilization (IVF), gamete intrafallopian transfer (GIFT), oocyte transfer (OT), intra-cytoplasmic sperm injection (ICSI) and round spermatid nucleus injection.

In-vitro production of ruminant embryos has become routine and is increasingly available as a commercial service to dairy, meat, and wool producers. However, the efficiency of producing viable embryos and the development of such embryos after transfer to recipients are perceived to be inferior to that which occurs in vivo.

Thompson (1997) has reviewed the biochemical and morphological similarities and differences between embryos produced in vitro and those produced in vivo. Some measures of metabolism are not markedly different between in vitro and in vivo-derived blastocysts. However, at a cellular level, differences in metabolism, morphology, and ultrastructure have been described, as has susceptibility to manipulation and cryopreservation (Thompson, 1997).

Stallion fertility requires the production of mature and motile spermatozoa that can undergo capacitation and the acrosome reaction to bind and penetrate the zona pellucida of the oocyte, in order to form an embryo. A defect occurring during this process could lead to male factor infertility. Male factor infertility is an area of active research in the

field of assisted reproductive technology (ART). ART, including artificial insemination, embryo transfers (ET), and IVF, is widely applied in reproductive management and for fertility improvement. This kind of improvement can be important for the long-term viability of the cattle, swine, and sheep industry in the United States, and the breeding and viability of the equine industry as well. Technologies like IVF are also important tools in the study of gamete biology, embryo development, cloning, preservation of equine genetics with subsequent fertility, and they also allow using mares that don't have a healthy uterus to carry the embryo to term to be used as oocyte donors. Furthermore, there is a need for in vitro produced equine embryos since those embryos are limited because of a poor success with superovulation. As a result, there is growing interest in these laboratory techniques in the equine industry.

Numerous factors affect the reproductive capacity of stallion and other animals, such as inherent infertility of the individual animal, nutrition, age, health, season, sexual behavior, and, perhaps, breed (Samper, 2000). Sperm output are related to testicular size and function, and daily sperm production are related to frequency of ejaculation. Thus, any seminal evaluation must relate to testicular and epididymal function. An evaluation of testicular function from an examination of one or more ejaculates presupposes that quality and quantity of semen obtained is an accurate reflection of testicular function (Blanchard and Varner, 1993).

For all mammalian species, fertilization and reproduction is the key to their future, because without the successful production of competent spermatozoa and oocytes, and subsequent zygote formation, species are infertile and thus are incapable of passing on their genes. Mammalian fertilization, whether it takes place within the female

reproductive tract or within a laboratory dish, is comprised of many processes that must follow a specific sequence. To initiate embryonic development, the structures of the fertilizing spermatozoa have to be disassembled and transformed into zygotic components by interactions with the cytoplasm of the oocyte. These interactions were studied extensively in domestic animals and the rhesus monkey, and many infertility questions and problems have been solved, but many more problems await resolution in equine reproduction.

In-vitro embryo production is not highly successful in equines. This problem is mainly associated with a lack of methods for successful in-vitro fertilization and embryo production in this species. Research in this area is negatively affected by limitations of methods for oocyte collection, as superovulation is not highly successful yet; additionally, in vivo recovery rates are poor due to the attachment of the oocyte to the follicular wall. Capacitation, AR, and cryopreservation have also proved difficult to achieve in stallion spermatozoa with any reliability (Arthur et al., 1996). In addition, AI of stallions with frozen semen is more technically demanding than that it is for other domestic animals such as the bull, as stallion semen has proved more difficult to cryopreserve than has that of bulls. It seems vital that research and advances be made in the processing, preparation, and induction of the AR and capacitation of stallion spermatozoa.

Mammalian spermatozoa examined immediately following natural mating fail to fertilize oocytes. While these spermatozoa are motile and appear structurally mature, fertility can only be demonstrated following additional maturation either within the

female reproductive tract or under selected conditions in vitro (Florman and Babcock, 1991).

Freshly ejaculated spermatozoa from humans (Lambert et al., 1985), mice (Fraser, 1984), hamsters (Yanagimachi, 1981), boars (Hunter and Hall, 1974), and bulls (Ellington et al., 1993) have all been shown to require a period of incubation and residence within either the female reproductive tract or a specific incubation medium prior to their interaction and fusion with homologous oocytes. During this mandatory incubation period, which varies from species to species, physiological changes and modification of the spermatozoal plasma membrane, occurs, such as lowered membrane-cholesterol concentration and changes in lipid composition, surface properties, fluidity, and permeability to calcium (Davis, 1981). Other changes include increase in cAMP concentrations, protein tyrosine phosphorylation, and changes in spermatozoa swimming patterns (Breitbart and Naor, 1999). This post-ejaculatory modification of the spermatozoa is termed capacitation. Most of these alterations are related to changes in the spermatozoal plasma membrane and have led to the belief that capacitation is a process of membrane maturation that eventually completes the membrane remodeling events initiated during epididymal transit (Jones, 1997).

The biological phenomenon of sperm capacitation has been known for approximately half a century, but the molecular basis of this process is still poorly understood. In general binding of ligand (e.g. EGF) which is present in the female reproductive tract will activate its receptor leading to increase in phospholipase C (PLC). Protein in the female tract will cause cholesterol efflux from the sperm plasma membrane, leading to increase in HCO_3^-, H_2O_2 and $[Ca^{2+}]_i$. Adenylyl cyclase (AC) will be activated by this increase

resulting in production of cAMP that will activate protein kinase A (PKA) to phosphorylate proteins on serine leading to protein tyrosine phosphorylation. Also enhanced K^+ permeability will cause membrane hyper-polarization leading to capacitation. A possible interaction among the activities invoked during capacitation is presented in figure 1 (Visconti and Kopf, 1998). Capacitation can occur in vitro in a defined medium without the addition of biological fluids which suggest that this process is modulated by the sperm itself, as if these cells are preprogrammed to undergo capacitation when they are incubated in the appropriate medium (Visconti and Kopf, 1998). Different media support capacitation of spermatozoa from different species indicating that there is no universal capacitation medium. Nevertheless these media contain energy substrates such as pyruvate, lactate, and glucose (depending on the species); a protein source that usually is serum albumin; $NaHCO^-_3$, and calcium. It is unclear how these compounds are coupled to membrane, transmembrane, and intracellular signaling events regulating capacitation, but a general scheme presented in figure 1 was adapted from work by several researchers (Visconti and Kopf, 1998).

Capacitation involves a series of complex and poorly characterized cellular events that enable spermatozoa to fertilize oocytes (Yanagimachi, 1988; Kopf and Gerton, 1991). Capacitated spermatozoa have the ability to initiate hyperactivated motility, to bind and penetrate the oocyte investments (cumulus oophorus, zona pellucida), to undergo the AR, and to fuse with the oolemma. Most of these events are not well characterized in any species, and their impact on stallion fertility is not known (Meyers et al., 1995). One of the difficulties encountered in trying to investigate requirements for capacitation is that no obvious morphological changes accompany changes in the

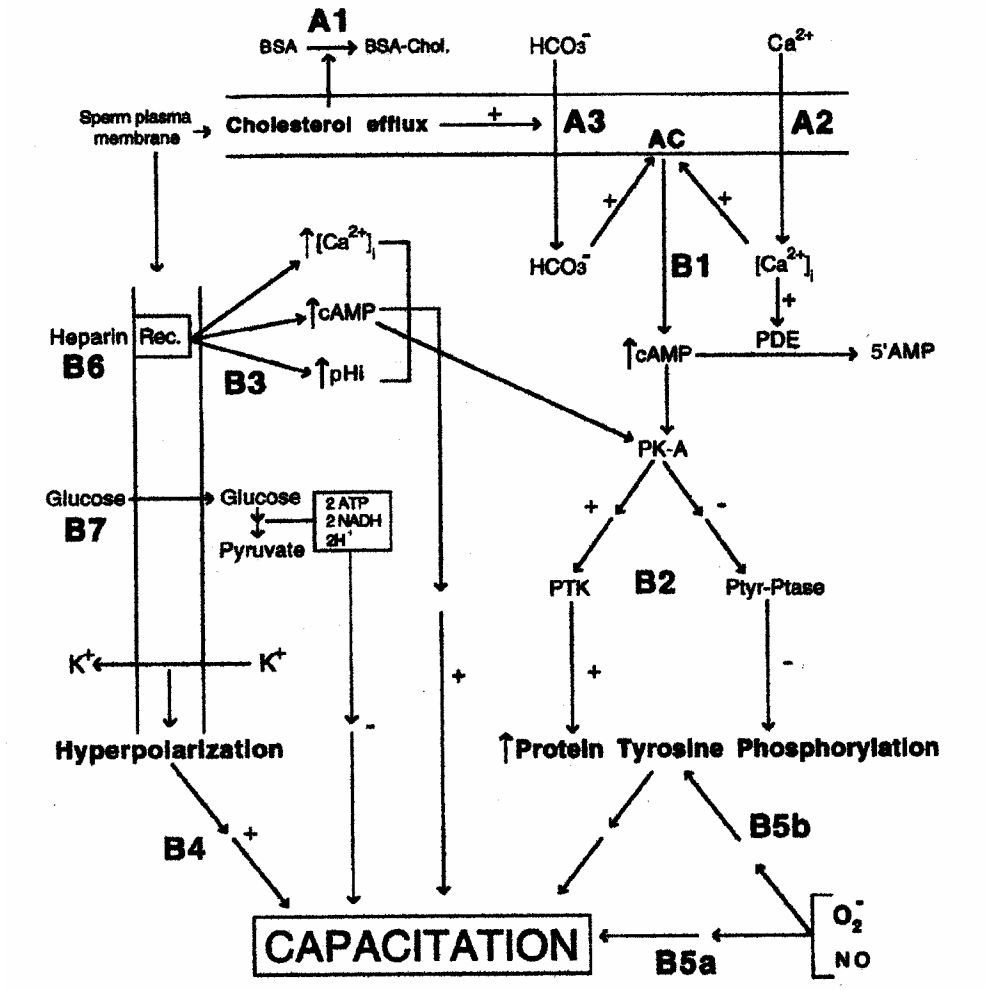


Figure 1: Signaling pathways hypothesized to play a role in regulating spermatozoa capacitation in mammals (Visconti and Kopf, 1998). A1= Serum albumin functions as a sink for removal of cholesterol from the sperm plasma membrane leading to fluidity changes that occur during capacitation. A2= Calcium involved in sperm signal transduction (e.g. adenylyl cyclase; AC). A3= Bicarbonate involved in regulation of sperm cAMP and the increase in the intracellular pH. B1= Cyclic AMP changes in PKA reflect elevation of cAMP. B2 = Protein tyrosine phosphorylation mediates a variety of cellular functions such as growth regulation, cell cycle control, and receptor regulation. B3= Intracellular pH increases during capacitation. B4= Membrane potential involves hyperpolarization of sperm plasma membrane. B5= Free radicals reactive oxygen species up-regulate protein tyrosine phosphorylation of several proteins.

functional potential of spermatozoa. The chlortetracycline (CTC) fluorescence technique was first used by Ward and Storey (1984) to assess the functional status of mouse spermatozoa. It has been also used by DasGupta et al (1993) to assess Ca^{2+}-related changes in the capacitation state of human spermatozoa, by Fraser et al (1995) on bull spermatozoa and by Varner et al (1993) on equine spermatozoa. The major advantage of the CTC is that, besides its discrimination between acrosome-intact cells and acrosome-reacted ones, it also divides acrosome-intact cells into two functionally different categories, uncapacitated and capacitated. DasGupta et al (1993) and Varner et al (1993) reported a high correlation of CTC with transmission electron microscopy (TEM) in human and stallion's spermatozoa respectively.

The other phenomenon in priming the spermatozoa for ova penetration and fertilization is the acrosome reaction. The mammalian spermatozoa AR is a calcium-requiring exocytotic event in the spermatozoa head that is essential to fertilization (Yanagimachi, 1988). The AR can occur only following completion of the capacitation process and prior to zona penetration and fertilization. It results from the fusion of spermatozoa plasma and acrosomal membranes, with the subsequent release of hydrolytic acrosomal enzymes.

Currently, little is known about the exact molecular nature of physiologically relevant AR-inducing components, and even less about the spermatozoa associated sites for the binding of these components. However, recent data have emerged from a few laboratories that might reveal information as to the type of interaction that may occur to bring about this important exocytotic event. These data have emerged from studies of spermatozoa-zona pellucida binding and the zona pellucida induced AR in the mouse, and has evolved

from observations that the zona pellucida glycoprotein (ZP3) is responsible for both of these events. The ZP3 binds to the sperm plasma membrane, leading to signal transduction that induces the enzymatic and ionic changes leading to the AR (Kopf and Gerton, 1991). ZP3 binds to at least two different receptors in the plasma membrane one is G_i-coupled receptor that activates phospholipase C (PLC) β and the other is a tyrosine kinase receptor (TK) coupled to PLCγ (figure 2). Binding to the G_i-receptor will regulate adenylate cyclase (AC) leading to elevation of cAMP and protein kinase A (PKA). Then PKA activates a voltage dependent Ca^{2+} channel in the outer acrosomal membrane which releases $[Ca^{2+}]_i$ from the interior of the acrosome to the cytosol, this small release of calcium will activate PLCγ. The products of hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) by PLC are diacylglycerol (DAG) and inositol-trisphosphate (IP_3) will lead to translocation of protein kinase C (PKC) to the plasma membrane then its activation. PKC opens voltage-dependent Ca^{2+} channels (L) in the plasma membrane leading to a second higher increase in $[Ca^{2+}]_i$. The PKA and IP3 dependent Ca^{2+} channels (I and III) of the outer acrosomal membrane will cause acrosomal depletion, leading to the activation of a capacitative Ca^{2+} entry (CCE) mechanism in the plasma membrane, Na^+- H^+ ion exchanger on the plasma membrane can be activated by TK or G_i leading to alkalization of the cytosol. The increase in $[Ca^{2+}]_i$ and pH_i will lead to fusion of the plasma membrane and the outer acrosomal membrane and acrosomal exocytosis (Breitbart and Naor, 1999).

The release of the acrosomal 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

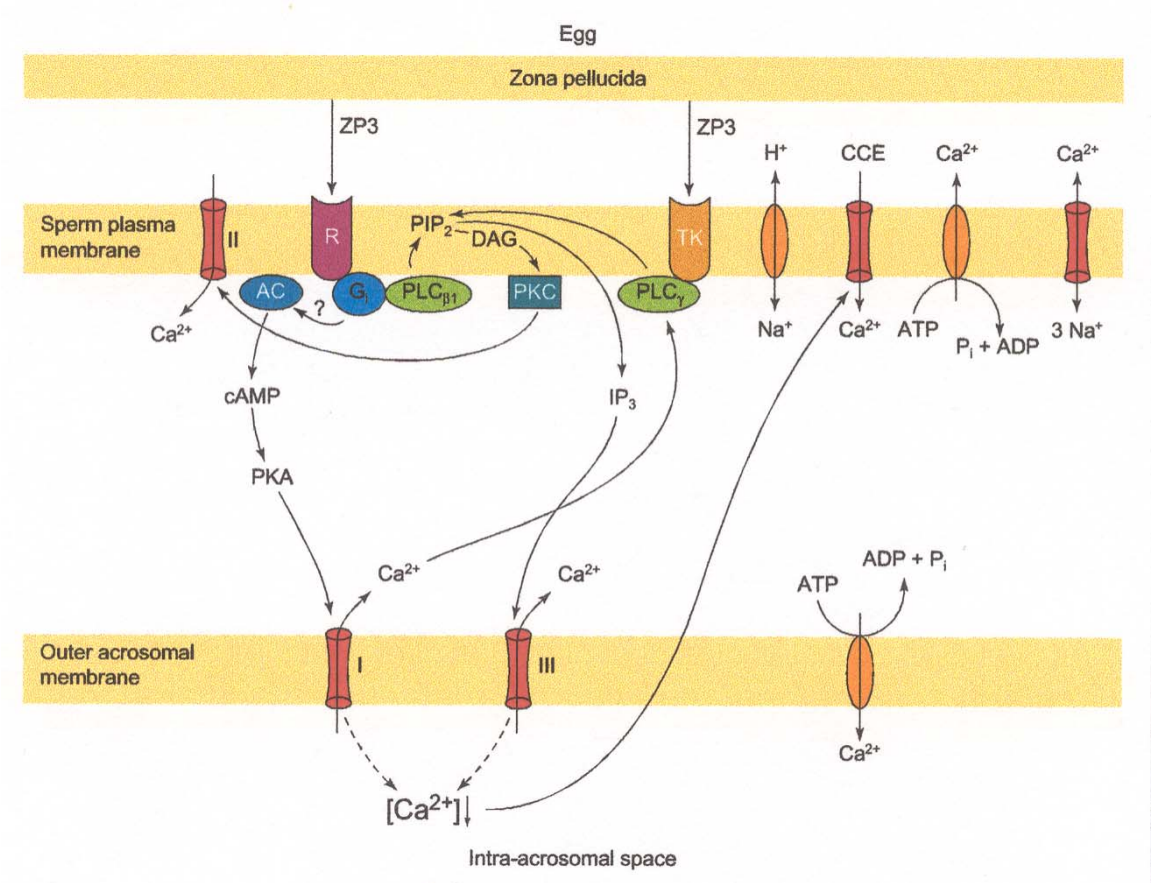


Figure 2: Schematic representation of the events that occur during the acrosome reaction (Breitbart and Naor, 1999). G_i-coupled receptor that activate PLCβ and tyrosine kinase (TK) receptor that coupled to PLCγ, bind egg ZP3 glycoprotein. The resulting signal transduction lead to formation of DAG and IP3; activation of voltage dependent Ca^{+2} channel on the plasma membrane by PKC; IP3 dependent (II and III) Ca^{+2} channel on the outer acrosomal membrane causing acrosomal Ca^{+2} depletion, leading to activation of capacitative Ca^{+2} entry (CCE) mechanism in the plasma membrane. Increase in pH_i as a result to activation of Na^+-H^+ exchanger along with the increase in Ca_i^{+2} will lead to fusion of the membranes causing the AR.

pellucida that surround the oocyte. Measurement of the activity of acrosin (one of the enzymes released during AR) can be used as a method of evaluating of the AR. Acrosin is a spermatozoa acrosomal protease that has an essential role in the fertilization process. Acrosin has been shown to be involved in the binding of spermatozoa to the oocyte's zona pellucida and/or the penetration of spermatozoa through the zona pellucida (Rogers et al., 1982). Low levels of acrosin appear to be associated with subfertility and infertility (Deutsch et al., 1990).

In mice, many substances such as platelet activating factor (PAF) and calcium have been found to stimulate the AR in vitro (Sengoku et al., 1992). The physiological stimulus for the AR in vivo is thought to be an interaction with the oocyte and its surrounding milieu. Prior to the actual sperm-oocyte contact and fusion, the timing of the AR is critical to fertilization. Sperm that undergo this reaction prior to penetration of the cumulus have a reduced ability to further penetrate the zona and adhere to cumulus cells (Talbot, 1985). Spermatozoa that are acrosome-intact participate in zona binding. This process has been documented in mouse (Saling et al., 1979), boar (Peterson et al., 1980), sheep (Crozet and Dumont, 1984), bull (Florman and First, 1988), and rabbit (O'Rand and Fisher, 1987), which further supports the fact that the process of fertilization is a finely tuned and programmed event that we are still trying to understand and duplicate in vitro.

In summary, the AR is a special event that results in the release of acrosomal enzymes that are needed to digest the vestments of the oocyte and expose oocyte receptors found on the inner acrosomal membrane and the plasma membrane of the spermatozoal head.

PLATELET ACTIVATING FACTOR (PAF)

Phospholipids are an essential component of all mammalian cells; over 100 different phospholipid molecular species are known to be present in mammalian cells and tissues. Fatty acid remodeling systems for phospholipids including acyl-CoA:lysophospholipid acyltransferases, CoA-dependent and CoA-independent transacylation systems and lysophospholipase/transacylase are involved in the biosynthesis of these molecular species (Yamashita et al., 1997). These phospholipids are present in all mammalian cells.

Ether lipids are the 1-O-alkyl derivatives of phospholipids. In contrast to nongerminal tissues where the plasma membrane contents of ether lipids is low, over 40% of the phospholipids present in spermatozoa plasma membranes are ether lipids. In 1997, Jones conducted a study to determine whether ejaculated human sperm could synthesize ether lipids either through reacylation of 1-alkyl-sn-lysophosphotidylcholine or through direct incorporation of 1-hexadecanol into phosphotidylcholine or diacyl phosphotidylethanolamine; he concluded that human spermatozoa can directly synthesize phosphoethanolamine ether lipids that may subsequently undergo exhaustive methylation to form phosphocholine ether lipids (Jones, 1997). One in particular ether lipid, platelet activating factor (PAF = 1-O-alkyl-acetyl-sn-glycero-3-phosphocholine), is promising in the field of reproduction. The structure of PAF is presented in figure 3.

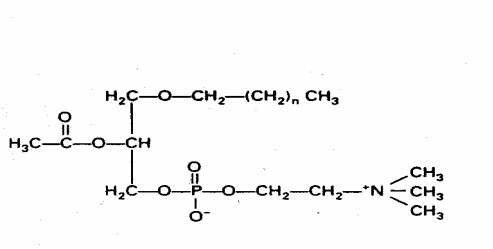


Figure 3: Chemical structure of PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); n=14-16

The structural requirement for PAF biological actions is highly specific. Alterations of the chemical groups, e.g., the alkyl ether group at C_1, acetyl group at C_2, or phosphorylcholine at C_3, or the stereochemical configuration of PAF lead to significant reductions of its potency (Hwang et al., 1988). There are two metabolic pathways involved in the biosynthesis of PAF, the remodeling and the de novo pathways. The synthetic and degradative pathways for PAF are presented in figure 4. Activation of phospholipase A_2 is the first step in the remodeling pathway. This activation appears to be regulated by protein kinase C (Whatley et al., 1989). Phospholipase A (PLA_2) catalyzes the hydrolysis of the sn-2 fatty acyl residue from alkyl choline phosphoglycerides to yield an intermediate, 1-O-alkyl-sn-glycero-3-phosphocholine (lyso-PAF) and a free fatty acid. The PLA_2 prefers a phospholipid that has arachidonic acid at the 2-position. Many of the cells that produce PAF in response to appropriate stimulation also produce metabolites of arachidonic acid under the same circumstances.

In the next step a specific acetyl-coenzyme A, Lyso-PAF acetyltransferase which is activated by phosphorylation, will catalyze the conversion of lyso-PAF to PAF by the addition of acetate. Free fatty acids stimulate the de novo pathway that involves the synthesis of 1-O-alkyl-2-acetyl-sn-glycerol, which then is converted to PAF by CDP-choline: 1-alkyl-2-acetyl-sn-glycerol cholinephosphotransferase (Chao and Olson, 1993).

The percentages of PAF that will be secreted depend on cell types and vary with different conditions, and this finding led to the suggestion that PAF may act at times as an intracellular messenger (Lynch and Henson, 1986). PAF degradation to inactive products is catalyzed by a PAF acetylhydrolase that is highly specific for phospholipids with short acyl chains at the sn-2 position (Toyoshima et al., 1995).

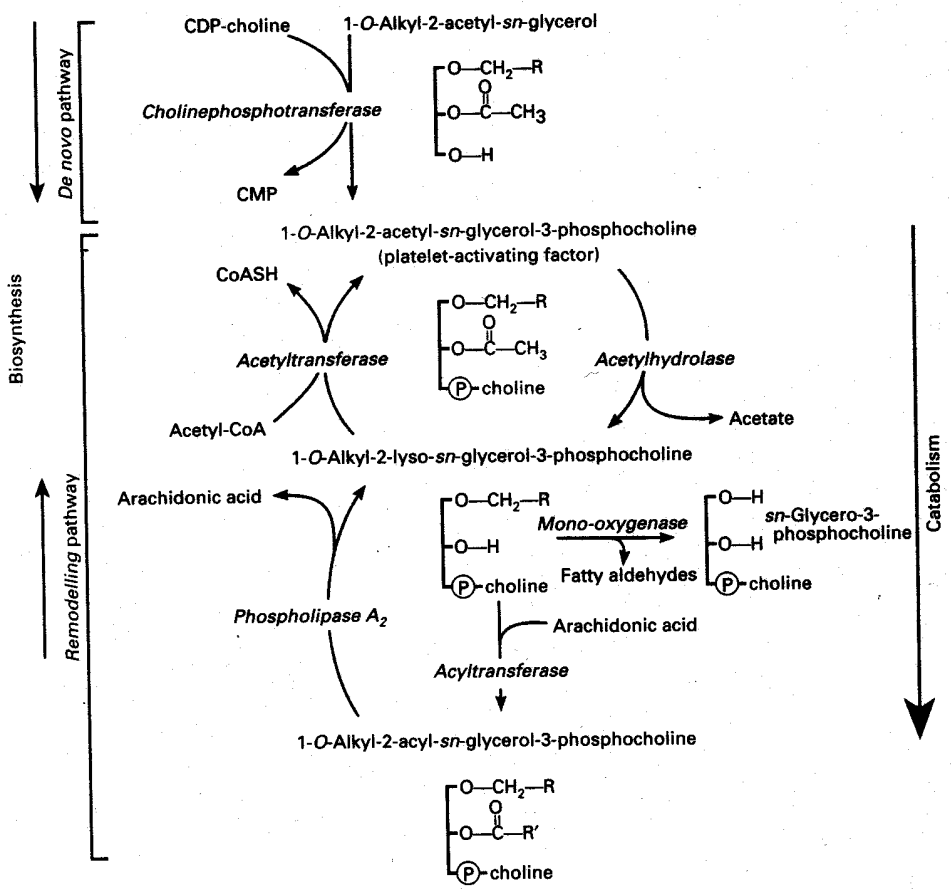


Figure 4: Pathways for synthesis of PAF. The remodeling pathway begins with the hydrolysis of a fatty acid from the sn-2 position of a cholinephosphoglyceride that has an ether-linked fatty alcohol at the sn-1 carbon. The de novo pathway starts with an intermediate in the synthesis of ether-linked membrane phospholipids. It is acetylated by a specific acetyltransferase, a different enzyme than in the remodeling pathway. In the following steps the phosphate is removed from the sn-3 position and phosphocholine is added (Chao and Olson, 1993).

PAF which is an ether phospholipid, is produced by, and influences, a large number of cells and organs (Roudebush et al., 1993; Hanahan, 1986; Toyoshima et al., 1995). PAF is released from basophils, polymorphonuclear neutrophils, monocytes, macrophages, and endothelial cells in culture (Kudolo and Harper, 1990). PAF is known to be a chemoattractant for equine neutrophils in vivo and in vitro (Foster et al., 1992). Specific receptors for PAF have been reported in cell membranes of human and rabbit platelets and human neutrophils (Hwang et al., 1988). The PAF receptor cDNA analysis in guinea pig lung indicates that the PAF receptor has 342 amino acids and a molecular mass of 38982 Da (Chao and Olson, 1993). The cloned PAF receptor from human leukocytes (Kunz et al., 1992) has 83% identity in the amino acid sequences to that of guinea pig lung. From these findings it was suggested that PAF receptor has seven transmembrane segments and that the cytoplasmic tail of the PAF receptor contained four serine and five threonine residues as possible phosphorylation sites. A schematic representation of the PAF receptor is presented in figure 5.

PAF is a phospholipid mediator with various physiological functions, including cellular growth and transformation. PAF exerts its biological activities through G-protein-coupled receptors (Snyder, 1990). It has been shown that PAF induced growth stimulation, inhibition, and suppression of oncogenic transformation in rat fibroblasts (NRK cells) over-expressing PAF receptor (Kume and Shimizu, 1997).

In a study done by Rezaul et al (1997), PAF was shown to stimulate calcium-dependent activation of protein-tyrosine kinase in ASK.0 B lymphoblastoid cells. PAF induced a rapid increase in protein-tyrosine kinase activity, which was insensitive to

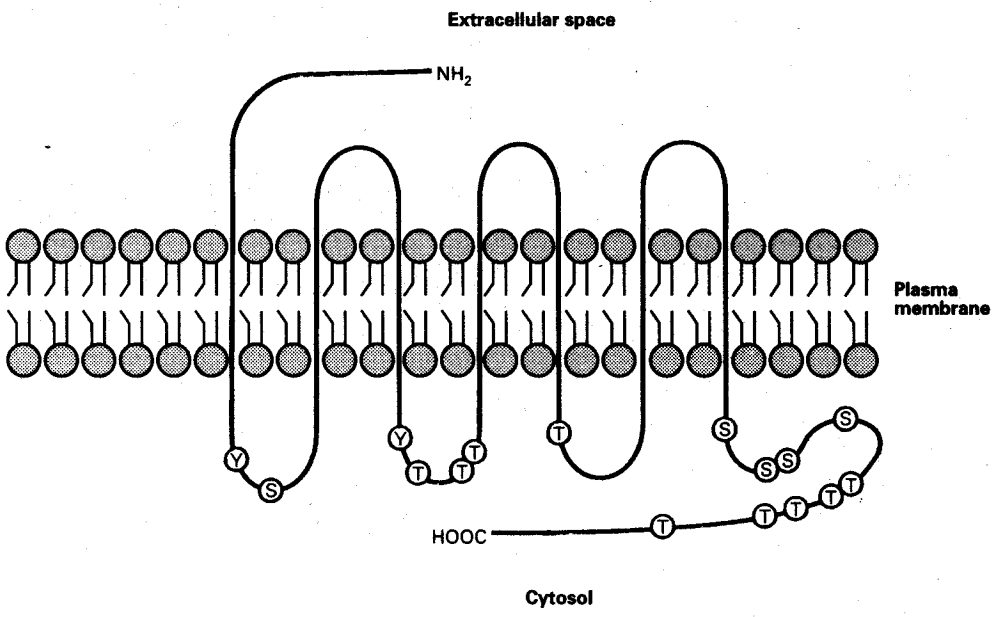


Figure 5: Schematic representation of PAF receptor. There are seven transmembrane segments and the possible intracellular phosphorylation sites of the PAF receptor are shown as S, serine residues; T, threonine residues; Y, tyrosine residues (Chao and Olson, 1993).

pertussis toxin (PTX), but was abolished by the phospholipase C inhibitor, U-73122. PAF-induced Ca^{2+} mobilization was also insensitive to PTX and was almost completely inhibited by U-73122, suggesting that the mechanism of action of PAF involves a Ca^{2+} dependent pathway, probably through the previously mentioned G-protein coupled receptors.

PAF is a potent signaling phospholipid that has been implicated in several reproductive processes. The presence of PAF in rabbit spermatozoa, as demonstrated by Yasuda et al (1992), has led to a series of investigations on the enhancement of sperm motility and penetration into the oocyte by PAF. The synthesis and release of PAF has also been demonstrated in human spermatozoa (Ward and Kopf, 1993). In vitro induction of capacitation has been induced in fresh and frozen spermatozoa of cattle by using PAF (Aravindakshan and Sharma, 1995). In their work it was noted that a PAF level of about 100 micromolar is optimum, as at this level, the acrosomal reaction improved without much loss of motility. A metabolic pathway was 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/or its detachment from spermatozoa during migration to the site of fertilization, may allow increased spermatozoal motility and migration to the site of fertilization. The activity of PAF-AH in bovine and stallion seminal plasma is over 50-fold greater than that reported from any other source (Hough and Parks, 1994). It is suggested that a decapacitation factor previously described may be related to PAF-AH (Muguruma and Johnston, 1997; Hough and Parks, 1994).

The involvement of PAF in ovulation has been described (Prescott and Zimmerman, 1990). PAF has been detected in the uterine tissue of rats (Yasuda et al., 1992), and the presence of PAF in spermatozoa has been reported in various animal species including rabbits (Kumar et al., 1988), mice (Kuzan et al., 1990), cattle (Parks et al., 1990), and humans (Aleozay et al., 1989; Minhas et al., 1991; Angle et al., 1991; Sanwick et al., 1992; Baldi et al., 1993). Radioimmunoassay (RIA) was conducted on spermatozoa from four stallions (personal communication with Dr. Roudebush, see appendix 2) and the result indicate that PAF is present in stallion spermatozoa but additional studies are required to determine what the levels have with the spermatozoal function (e.g. cell motility). The relationship of PAF to implantation has received special attention. O'Neill and colleagues first reported that PAF was secreted by the embryo and that the implantation rate was increased in proportion to the PAF secreted into the culture medium (O'Neill, 1989). Furthermore, Matsubara et al (1997) evaluated the roles of PAF and PAF-AH activity in late pregnancy. Based on their findings, it is suggested that the PAF concentration in the uterus and placenta may be regulated by intracellular PAF-AH and/or plasma PAF-AH activities. Increased PAF activity in the pregnant rat uterus may be related to the initiation of labor due to its known effect on myometrial contraction. Decreased PAF concentration in the placental plasma may contribute to fetoplacental circulation due to its known hypotensive activity and associated increase in vascular permeability (Matsubara et al., 1997).

Since it was suggested that binding of PAF to its receptor sites is a first step in its biological function, considerable impact was given in search for PAF inhibitors or antagonists which are likely to have therapeutic effects in diseases that is mediated by

PAF such as inflammation, anaphylaxis, and endotoxin shock. The PAF antagonists can be classified into four categories. The first group is PAF- related phospholipids having a heteroaromatic quaternary ammonium function such as CV 3988 and SRI 63-441. The second group are synthetic compounds such as 52770 RP, L-652, 731, and WEB 2086. The third groups are natural products, such as BN 52021, that are isolated from the Chinese tree, Gingko biloba. The pathophysiological function of PAF interested Hemmi et al (1991) to search for a PAF antagonist from microbial origin. The activity was found in the culture filtrate of Penicillium terlikowskii No. 5348 and Penicillium citrinum No. 2973, so the fourth group were identified as bisdethiodi(methylthio)gliotoxin, designated FR-49175, and 3,6-bismethylthio-3-hydroxymethy-6-phenylmethylpiperazine-2,5-dione, designated FR-106969, respectively (Hemmi et al., 1991).

In summary, PAF is a phospholipid with diverse potent physiological effects. Its actions are achieved at concentrations as low as 10^{-12} M in some systems and almost always by 10^{-9} M as an intercellular messenger (Prescott et al., 1990).

Platelet activating factor (PAF) receptors are thought to signal via a G-protein, since the binding of PAF to membranes stimulates GTPase activity (Houslay et al., 1986; Hwang et al., 1986). The binding of PAF activates phosphatidylinositol-specific phospholipase C, which results in a rise in intracellular calcium and diacylglycerol. These changes activate protein kinase C, which catalyzes the phosphorylation of target proteins (Prescott et al., 1990).

SIGNAL TRANSDUCTION

The mechanism by which specific regulators of spermatozoa function affect informational flow across the spermatozoal plasma membrane to modulate intracellular second-messenger systems leading to appropriate cellular responses is only beginning to be understood. Presently, the mouse remains the model system about which most is known regarding the signal transduction mechanisms that modulate spermatozoa function in response to specific ligands such as PAF.

The spermatozoa-oocyte interaction is affected by many factors that include the glycoprotein components of the zona pellucida (ZP3), the unique structure of the ZP3, the ZP3 biological potency, and the probable existence of a complementary ZP3 receptor on the spermatozoa surface. These factors satisfy a number of criteria required to control specific cell-cell recognition events in a receptor-mediated fashion, leading to subsequent acrosomal exocytosis through the activation of intracellular protein kinases (Ward and Kopf, 1993).

The mechanism of action of PAF on human and other mammalian platelets is well documented (see below), but similar work is lacking on mammalian spermatozoa, particularly stallion spermatozoa. Therefore, we are conducting this work to investigate the presence of PAF receptors on stallion spermatozoa and PAF effects on spermatozoal motility, capacitation and the AR and whether the actions of PAF involve calcium release.

Specific receptors for PAF have been identified in numerous tissues and cells. The first binding experiment utilizing [^{3}H] PAF was conducted in human platelets in 1982 (Valone et al., 1982). Using [^{3}H] PAF in the absence or presence of excess unlabelled

PAF at 20 ^{o}C, two distinct types of binding sites were revealed. One binding site for PAF on platelets exhibited a high affinity with a K_d value of 37+/-13 nM and had a low capacity of 1399+/-498 sites/platelet. The other binding site possessed nearly infinite binding capacity with a low affinity for PAF. The high affinity sites were responsible for PAF-elicited platelet aggregation.

Research studies have indicated that PAF receptor-induced transmembrane signaling mechanisms involve guanine nucleotide regulatory proteins (G-proteins). The PAF receptor is coupled to various cellular effectors systems such as phospholipase A_2 and phospholipase C through these G-proteins, the end result of which involves subsequent intra-cellular events that includes: (1) phosphoinositide turnover, (2) calcium flux, and (3) protein tyrosine phosphorylation (Chao and Olson, 1993).

PHOSPHOINOSITIDE TURNOVER

PAF stimulates the hydrolysis of phosphatidylinositol in rabbit, human, and equine platelets (Chao and Olson, 1993). Further, it has become clear that one key event in PAF-mediated signaling mechanisms is the hydrolysis of phosphatidylinositol 4,5-bisphosphate, by a specific phospholipase C, yielding two second messengers (figure 6), diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). While IP_3 mobilizes intracellular calcium, DAG has two potential signaling roles. First, it can be further cleaved to release arachidonic acid, which can be used in the synthesis of prostaglandins and eicosanoids that play an important part in inflammation. Second, it activates protein kinase C (C-Kinase) that is Ca^{2+} dependent. It seems likely that C-Kinase is activated by the cooperative effect of diacylglycerol and an increase in cytosolic Ca^{2+} brought about

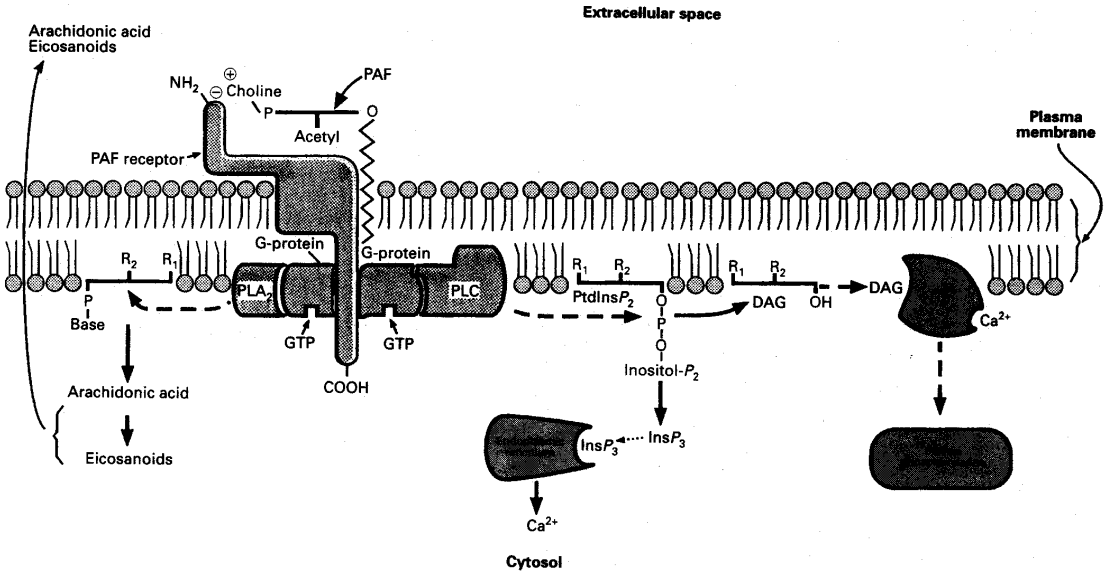


Figure 6: Proposed mechanism of action of PAF (Chao and Olson, 1993)

Interaction of PAF with its specific receptor activates multiple biochemical pathways, each of which mediates in part cellular activation.

by IP_3. Once activated, C-Kinase transfers the terminal phosphate group from ATP to specific serine or threonine residues on target proteins that vary depending on the cell. In many animal cells, C-Kinase phosphorylate then activate the plasma membrane Na^{+}-H^{+} ion exchanger that controls intracellular pH. The resulting increase in intracellular pH may help signal some cells to proliferate. In other cells, activation of C-Kinase leads to the transcription of specific genes. The two branches of the inositol phospholipid signaling pathway can be mimicked using a calcium ionophore, such as A23187 or ionomycin, which allows Ca^{+2} to move into the cytosol from the extracellular fluid. The effects of diacylglycerol can be mimicked by monoacyl derivatives of diacylglycerol or by phorbol esters, plant products that bind to C-Kinase and activate it directly. It has been shown that the two branches of the pathway often collaborate in producing a full cellular response (Albert et al., 1989).

CALCIUM FLUX

Of all of the ions that have been studied in relationship to capacitation and the AR, calcium (Ca^{2+}) appears to be an absolute prerequisite for the successful completion of this exocytotic event in every species studied. Incubation of mammalian spermatozoa in a Ca^{2+} deficient medium prevents the AR from occurring under a variety of conditions. Likewise, incubation of spermatozoa with cation ionophores such as calcium inonophore A23187 that catalyze Ca^{2+}, or incubation of spermatozoa under other condition that promotes Ca^{2+} entries such as treatment with progesterone, zona pellucida or ovarian follicular fluid, will induce the AR (Kopf and Gerton, 1991).

It has been established that intracellular calcium is required for the completion of the AR (Yanagimachi and Usui, 1980). Several studies have observed an increased uptake of calcium by spermatozoa cells undergoing the AR. However, it is not clear what role calcium plays in the capacitation process (Yanagimachi, 1981).

PAF causes an elevation of cytosolic free calcium in various cells such as platelets and endothelial cells. There are at least two mechanisms involved in PAF-induced increases in cytosolic free calcium: (a) calcium influx occurring through a membrane-associated calcium channel regulated by PAF receptors and/or PAF stimulating intracellular calcium mobilization leading to activation of phospholipase A_2 and the liberation of arachidonic acid. (b) Mobilization of calcium instigated from intracellular stores in response to the intracellular second messenger IP_3 produced during PAF receptor stimulation (Chao and Olson, 1993).

PROTEIN TYROSINE PHOSPHORYLATION

Protein kinase C and tyrosine kinase appear to play a major role, together with calcium signaling, in the processes of capacitation, regulation of flagellar motility and the acrosome reaction of mammalian spermatozoa. Tyrosine phosphorylation of proteins is increased during capacitation and acrosome reaction of mice (leyton and Saling, 1989) and human (Naz et al., 1991) spermatozoa. It was also shown that progesterone increases tyrosine phosphorylation in human spermatozoa (Tesarik et al., 1993). PAF is actively synthesized by human spermatozoa in response to progesterone and calcium ionophore (Baldi et al., 1993). The ability of PAF to stimulate tyrosine phosphorylation of proteins has been shown in platelets (Dahr et al., 1990) and human spermatozoa (Luconi et al.,

1995). PAF-induced tyrosine phosphorylation is extracellular Ca^{2+} dependent and may be mediated by G-proteins and protein kinase C activation (Chao and Olson, 1993).

PAF acts on platelets and other cells through specific receptors and a variety of signal transduction systems to elicit diverse biochemical responses. Most reproduction studies have been conducted on mice, but in attempts to further study the capacitation process in the stallion, work has been done in which investigators have co-cultured spermatozoa with oviductal epithelia (Ellington et al., 1993), exposed and subjected spermatozoa to potential capacitating agents, such as heparin and calcium ionophore, (Varner et al., 1993), and performed zona penetration assay using both hamster and equine oocytes (Samper et al., 1989; Blue et al., 1989). From these studies, valuable information concerning the biochemical changes which occur in the spermatozoa head, as well as the time required for capacitation, have been obtained. Stallion spermatozoa are thought to require approximately 4 to 6 hours to undergo capacitation. This time period was proposed based on in-vitro experiments and on conception rates following insemination of mares with semen at various times before and after ovulation.

Despite the lack of direct experimental evidence for stallion spermatozoa, changes and alterations similar to that occurring in the glycoprotein coating of the sperm plasma membrane of the bull are thought to occur in the stallion (Meyers et al., 1995). In addition to these alterations, the plasma membrane itself also becomes destabilized through loss of cholesterol (Graham, 1996). In summary, PAF being an ether lipid might be functioning through destabilization of the spermatozoa plasma membrane, and thus inducing these physiologic changes such as capacitation and the AR in a more rapid manner.

RATIONALE and JUSTIFICATION

Each year equine owners in Virginia and elsewhere in the USA are frustrated with the enormous expense incurred while breeding infertile or subfertile animals. These problems can involve the mare, the stallion, or both. Lack of embryo development from older mares may involve ovulation failure, poor oocyte quality, failure of fertilization, abnormal oviductal transport, or the presence of an abnormal uterine environment. Problems with stallions may include inadequate semen quality/quantity, decreased spermatozoal longevity, and decreased fertilization potential.

Currently, embryo and oocyte transfer (OT), gamete intrafallopian transfer (GIFT) and in-vitro fertilization (IVF) are the assisted reproductive techniques used to combat these problems. OT is an established clinical program and has helped to obtain pregnancies from subfertile mares. Embryo development using OT differs among type of semen used for insemination (fresh, 82%; cooled, 25%; frozen, 8%) (Coutinho et al., 2002). GIFT requires the surgical transfer of oocytes and spermatozoa to a recipient mare's oviduct; this type of transfer has a reported success rate of only 23% (Carnevale, 1999). IVF has the advantage of avoiding a surgical procedure, allowing the trans-cervical deposition of an embryo. However, IVF rates are low in horses, 27 % (Palmer, 1991), as compared to cattle, 75 % (Leibfried, 1989). In addition, the procedures used with other species may not directly transfer to work with the stallion. This is primarily due to lack of knowledge of the mechanisms specifically involved in the maturation of equine oocytes, the capacitation of equine spermatozoa, and the proper techniques and media to grow equine embryos in vitro.

The equine is one of the few species in which the individual animal is important enough to warrant application of assisted reproductive technologies; this can be due to the following reasons. First, among large domestic animals, the individual equine is generally more expensive due to the racing and show business. Second, subfertility is common in older stallions. Only 55% of mares bred annually in the United States will produce foals (Hinrichs et al., 1998), so understanding the biology of the equine reproductive process should enhance treatment of the problem. Third, ART would help with preservation of gametes where by frozen/thawed oocytes may be fertilized. Lastly, development of a reliable method of ART would improve the management of subfertile, competition and out-of-season mares and stallions, since breeding in the stallion is limited to a specific period of the year in some breeds. The reasons for the slow development of in vitro maturation and in vitro fertilization of equine oocytes are the scarcity of slaughterhouse ovaries, difficulty in capacitating equine spermatozoa in vitro, and reluctance of breed registries to accept these assisted reproductive techniques (Squires, 1996). In vitro studies would allow the requirements for fertilization to be determined and it will help to develop and establish in vitro conditions necessary to capacitate and induce the acrosome reaction in stallion spermatozoa and furthermore would allow the development of early embryo.

ART such as IVF are less developed in the stallion. The poor success rate of IVF may be associated with problems of spermatozoa capacitation and the acrosome reaction, and inadequacy of culture conditions for oocytes, zygotes, and embryos.

Different modifications of IVF have been tried in order to improve success rates of IVF in equine. These modifications included the use of Percoll separated semen,

treatment of spermatozoa with calcium ionophore, partial removal of the oocyte cumulus layer, and partial dissolution of the ZP either mechanically or chemically (Hinrichs, 1998). However, other than oocyte transfer, none of these methods has been refined for use as a clinical tool with a predictable outcome. A further modification of IVF is ICSI, which involves direct injection of the sperm into the oocyte. Use of several agents such as albumin, glycosaminoglycans (GAGs), zona pellucida proteins, calcium ionophores (Graham, 1996), ionomycine, follicular fluid, progesterone, and PAF (Meyers et al., 1995) are also investigated extensively in enhancing capacitation, the AR and binding and penetration of the zona pellucida. Beside its role in inflammation and allergy, PAF also has numerous physiological functions, especially those involved in reproduction (Muguruma et al., 1996).

It has been reported that PAF increases the motility of human spermatozoa (Ricker et al., 1989; Jarvi et al., 1993; Krausz et al., 1994), and facilitates capacitation and the AR in the mouse (Sengoku et al., 1992), human (Sengoku et al., 1993; Angle et al., 1993), and rabbit (Fukuda et al., 1994). Pretreatment of spermatozoa with PAF has been shown to enhance IVF in the mouse (Minhas et al., 1989; Shi et al., 1992), and rabbit (Roudenbush et al., 1990), as well as zona-free hamster oocyte penetration by human spermatozoa (Minhas, 1993).

Therefore, the hypotheses of this study are that (1) PAF enhances stallion spermatozoa motility (2) PAF has the potential to induce the capacitated state in equine spermatozoa (3) PAF has the potential to induce the AR in capacitated stallion spermatozoa (4) the mode of action of PAF on these events is a specific binding to a

receptor on stallion spermatozoa plasma membrane, and (5) the pathway of these events involves a Ca^{2+} dependent mechanism.

The ability to induce capacitation and the acrosome reaction in stallion spermatozoa would provide mechanisms for laboratory assays of spermatozoa function as well as methodologies for ART such as IVF. By testing these hypotheses, information on how to make the equine reproductive process more successful might be obtained through the use of PAF in ART. Research regarding the effects of the PAF on the equine spermatozoal function is needed, as results in other species show an increase in both function and fertilization capacity of spermatozoa after treatment with PAF. The initial step, for us, is to investigate the role that PAF can provide in increasing motility and the events associated with fertilization. This information can then be transferred back to our clinical work to increase in-vitro fertilization success rate in the equine.

MATERIALS and METHODS

Sampling, Semen Collection and Sperm Preparation

Spermatozoa were obtained from ten (unless otherwise stated) sexually active light breed stallions with an age range 5-23 years. Stallions were on a regular collection schedule, 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 (AV), and filtered using break-proof socks filter (Nasco # C 07687N) to remove debris and gel. Once in the laboratory, semen was centrifuged for 5 minutes at 50 x g to remove any remaining gel fraction, seminal debris and clumped spermatozoa.

All experimental procedures began within 1 hour of collection with the exception of motility, which was assessed as soon as possible after collection. Deficient Ham's F-12 HAT variation medium (Irvine scientific cat. # 9447), supplemented with 0.03% heat inactivated bovine serum albumin (BSA) was used for washing and incubation.

PAF (Sigma cat. # P-9525) was dissolved in a stock solution of 10^{-3} M chloroform and methanol (1:4) and kept at -20^0 C. PAF, at the indicated concentration was dried under a gentle stream of nitrogen in siliconized tubes and re-dissolved in HAT medium to the final experimental concentrations. 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, and then was washed by centrifugation with HAT medium twice for 10 minutes at 600 x g. The supernatant was discarded and the spermatozoa pellet was re-suspended with HAT medium to give a final concentration of 1-2 x 10^7/ml for each experiment, unless otherwise stated. Each sample consists of 1 ml of the spermatozoal suspension layered under 2 ml (HAT + 0.03% BSA) medium placed into conical tubes and incubated at a 45^o angle in a slant rack in 5% CO_2 at 37^0 C in water-saturated air. After one hour swim-up, the supernatant was collected and spermatozoa incubated in duplicate with PAF in 5% CO_2 at 37^0 C in water-saturated air along with the control group (no PAF) as indicated in each experiments.

POST-INCUBATION EVALUATION of SPERMATOZOA

Experiment 1: Motility Evaluation: Hamilton Thorn IVOS motility analyzer was used for assessment of motility. Motility was assessed with low average path velocity (AVP) cutoff of 20μm/s and straightness (STR) threshold of 75.0% (table 1). Setup shown in table 1 is the same setup that is used by other researchers for equine spermatozoa. Swim-up spermatozoa were incubated with 10^{-4} to 10^{-13} M PAF, then motility was examined at five different time intervals: 30, 60, 90,120 and 150 minutes for each PAF treatment along with the control (no PAF).

Microcell counting slides (Conception Technologies # 1043011) with 20 micron thickness were used. About 10μl of each sample was loaded into the slide at the time of analysis. Five fields were analyzed then data was averaged.

Statistical Analysis: Data obtained on motility were presented as percentage (%). Data was first analyzed by repeated measures analysis. Then, data were log transformed (Ott, 1993) and analyzed by multiple linear regression analysis to model motility at different concentrations of PAF and time periods. The pairwise comparisons were done by Duncan's method.

Table 1: Analysis setup used for equine spermatozoa in Hamilton Thorn IVOS motility analyzer.

Parameter	Value	Parameter	Value
Frames acquired	40	Brightness for LED	2274
Frame rate	60 Hz	Brightness for ident	3000
Minimum contrast	70	Temperature set	37.0 ºC
Minimum cell size	3 Pixels	Cell type	Slide 1
Minimum statistic contrast	15	Cell depth setup	20.0µm
Straightness (STR) threshold	75.0%	Field selection mode	Auto
Low VAP cutoff	20.0µm/s	Ident active	none
Medium VAP cutoff	50.0µm/s	Ident mode	A
Low VSL cutoff	0.0µm/s	Integrating time	1 frame
Head size, nonmotile	2 Pixels	Static head size	0.13 to 7.40
Head intensity, nonmotile	80	Static head intensity	0.57 to 1.67
Static elongation	1 to 78	Slow cell motile	none
Magnification	1.89	Video source	camera
Video frequency	60	Bright field	none

Experiment 2: Chlortetracycline (CTC) Assessment for Capacitation and the Acrosome Reaction

The method used was the one described by DasGupta et al (1993) for human spermatozoa with some modification. CTC (Sigma cat. # C4881) was prepared fresh each day at a concentration of 500 µM in a buffer of 20 mM Tris, 130 mM NaCl and 5 mM cysteine, (all from Sigma Chemical CO. St. Louis MO) and the pH was adjusted to 7.8. The solution was kept wrapped in foil to prevent the entry of light and stored at 4ºC in a refrigerator. Swim-up spermatozoa were incubated with 10^{-4} to 10^{-12} M PAF at 120 minutes. Fifty µl of sperm suspension was added to 50 µl of CTC solution in a foil-wrapped microcentrifuge tube and mixed for 20 seconds. After 10 seconds incubation, cells were fixed by adding 10 µL of 12.5% glutaraldehyde in 1M Tris buffer (pH 7.8) and mixed thoroughly for 20 seconds. Slides were prepared by placing 10 µL of fixed spermatozoa suspension on a clean slide. Then one drop of 0.22mol 1, 4-diazabycylo [2,2,2]- octane 1^{-1} (DABCO; Sigma cat # D2522) in a 9:1, glycerol: phosphate-buffered saline was added to retard fading of fluorescence. After adding a cover slip, excess fluid was removed by gently, but firmly, compressing the slide and cover slip between tissues. Slides were sealed with colorless nail varnish and stored in the dark at 4ºC at all times. One hundred spermatozoa from each slide were assessed the same day, although no fading of CTC fluorescence was noticed on the following day. Evaluation of spermatozoa was done 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 Cat. # H258) was added to the spermatozoa suspension at a final concentration of 1 µg/ml and incubated for

5 minutes at 39ºC and 5% CO_2. Samples were washed by layering sperm suspension over 2 ml of 45% Percoll (Holden et al., 1990) and centrifuged at 300 x g for 5 minutes to remove extracellular stain. The supernatant was removed and the pelleted cells were used for CTC stain. Each cell was first observed under ultraviolet illumination (excitation at 330-385 nm and emission at 400 nm) for determination of live /dead status. Cells showing bright blue staining of the nucleus were considered dead and not counted. Live cells were then observed under blue-violet illumination (excitation at 400-440 nm and emission at 455 nm) for CTC patterns. The same nomenclature that was observed in the mouse, bull and human spermatozoa was used (Varner et al., 1993). These patterns are: "***F***", for uniform fluorescence over the head which indicates no capacitation;"***B***", for fluorescence free band in the post-acrosomal region which indicates the capacitated state: and "***AR***" pattern with dull head fluorescence or a band of the fluorescence in the equatorial segment, which indicates acrosome-reacted spermatozoa. The midpiece was brightly fluorescent on all cells (table 2 summarizes the different patterns of CTC).

Statistical Analysis: Data for the acrosome reaction and capacitation as detected by CTC were presented via percentage (%), so they were first log transformed, and then analyzed by quadratic multiple linear regression where log capacitation and log AR were dependent variables.

Table 2: Types of equine spermatozoa patterns visualized with Chlortetracycline Fluorescence (CTC) assay (Varner et al., 1993)

Fluorescence Characteristics	Functional State	Name of Pattern
Moderate uniform fluorescence over entire sperm head	Uncapacitated, acrosome-intact	"F"
Enhanced fluorescence over acrosomal area of sperm head, with a fluorescence-free band in the postacrosomal region	Capacitated, acrosome-intact	"B"
Fairly dull fluorescence over the whole sperm head, except for the thin, bright punctuate band of fluorescence in the equatorial segment. Fluorescent-free over most or all of acrosomal area, with retained fluorescence over post acrosomal portion of sperm head. Nonfluorescent over entire sperm head (acrosomal/postacrosomal areas).	Acrosome-reacted	"AR"

Experiment 3*:* Ultrastrucural Visualization of the Acrosome Reaction (AR) after PAF Treatment.

Swim-up spermatozoa from 3 stallions were incubated with 10^{-9}, 10^{-10}, and10^{-11} M PAF for 120 minutes. Then 1 ml of treated spermatozoa (concentration about 30 million /ml) was diluted with HAT medium and centrifuged at 500 x g for 10 minutes at room temperature. The pellet is then re-suspended in HAT medium and processed for electron microscopy. Progesterone for induction of acrosome reaction was prepared daily by dissolving 1 mg progesterone (Sigma cat. # P 6149) in 5 ml dimethyl sulfoxide (DMSO; Sigma cat. # D 2650) to make solution A. Solution B was prepared by adding 100 µl of solution A to 900 µl distilled water immediately prior to induction of the acrosome reactions (Meyers et al., 1995). For induction of the AR, solution B was added to spermatozoa suspensions at 1 µl per 100 µl sperm suspension to obtain a 3.18 µmol/L 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 or undergoing acrosomal exocytosis with differing amounts of acrosomal matrix still present. A non-reacted (intact) sperm maintains the acrosomal membrane and its content. The scoring was divided between the intact, reacted and vesiculated spermatozoa and the number of spermatozoa was clarified for each level of PAF.

Statistical analysis: Data for the acrosomal status (intact, reacted, and vesiculated) as detected by TEM were presented as percentage (%), so they were first log transformed. Then they were analyzed by linear regression analysis using SAS program.

Fixation and Preparation for Transmission Electron Microscopy (TEM).

Day 1

1. 2 ml sperm suspension was added to 2 ml fixative;

(5%Glutaraldehyde/3%Sucrose/0.1M Sodium Cacodylate (NaCaco), pH 7.6) for 1 hour at room temperature.

2. Samples were centrifuged at 500 x g for 10 minutes;

3. Sperm pellet was sandwiched in 2% agar on a glass slide;

4. Agar was cooled on ice and sample was cut into small pieces;

5. Samples were transferred to fresh fixative (#1) in refrigerator (4° C) overnight;

Day 2

Samples were:

1. Washed twice for 15 to 20 minutes in 0.1M NaCaco. Buffer;

2. Enblock stained with 0.1% Tannic Acid in 0.1M NaCaco. Buffer (pH 7.4) for 1 hour at room temperature;

3. Washed twice for1 hour with 0.1M NaCaco. Buffer;

4. Post fixed in 1% Osmium Tetroxide in 0.1M NaCaco. Buffer for 1 hr. at room temperature;

5. Washed twice at 15 to 20 minutes with 0.1M NaCaco. Buffer;

6. Dehydrated as follows

50% Methanol saturated with Uranyl Acetate ------20 minutes

75% Methanol saturated with Uranyl Acetate ------20 minutes

95% Methanol saturated with Uranyl Acetate ------2 x 45 minutes

Absolute Methanol saturated with Uranyl Acetate ------2 x 45 minutes

7. Treated with Propylene Oxide for15mins;

8. Infiltrated with 1 propylene oxide/1 Polybed 812 overnight.

Day 3

Samples were:

1. Infiltrated with 100% Polybed 812 for overnight (or all day);

2. Embedded in fresh Polybed 812; placed into 60°C oven and cured 24 to 48 hours;

3. Ultra thin sections (600-800 A) were cut on a Leica Ultracut UCT and collected on 200 mesh copper grids;

4. Sections were post stained for 6 minutes with 2% aqueous uranyl acetate; 5 minutes with Reynolds's Lead Citrate (Reynolds, 1963);

5. Samples were viewed with a JEOL JEM100 CX II Electron Microscope at 80kV.

Experiment 4: Intracellular Calcium Measurement by Flow-Cytometry.

Swim-up spermatozoa from 4 stallions were incubated with 10^{-7} to 10^{-12} M PAF for 2 minutes. Then spermatozoa at 1-2 x 10^7/ml were loaded simultaneously with 4 μM Fluo-3 + 10 μM Fura Red esters and incubated for 45 minutes at 37ºC (Novak and Rabinovitch, 1994). Spermatozoa were then washed once with HAT medium and re-suspended in fresh medium at 2.0 x 10^6/ml. Spermatozoa were kept warmed at 37ºC during flow cytometry analysis. The intracellular concentration of calcium was assessed using the ratio of green to red fluorescence. Measurements were performed using a blue 488nm-laser line on the Beckman-Coulter ultra flow cytometer and cell sorter.

The PAF antagonist FR-49175 (Sigma cat. # D- 8541) at a concentration of 10^{-5} to 10^{-10} M was used on swim-up spermatozoa from three stallions. Control (0 PAF and 0 antagonist). After one minute incubation with the antagonist, PAF at 10^{-7} M was added for two minutes, and then spermatozoa were loaded simultaneously with 4 μM Fluo-3 + 10 μM Fura Red esters and incubated for 45 minutes at 37ºC. After incubation, spermatozoa were washed once with HAT medium and the concentration was adjusted to 2.0 x 10^6 / ml. Spermatozoa were kept warm during the flow cytometry analysis.

Flow Cytometry: Fluo-3 and Fura Red were excited at 488nm with Fluo-3 emission detected at 515-535 nm and Fura Red emission detected at 665-685 nm. Fluo-3 fluoresces with increasing intensity in the green region when bound to calcium while Fura Red exhibits inverse behavior, fluorescing most intensely in the red region when not calcium bound. Spermatozoa were maximally stimulated by addition of ionomycin (Sigma cat# I0634) at a concentration of 25μM.

Statistical Analysis of Cytometry: General linear models procedure (GLM) was used to analyze the data and the dose was linearized using $\log_{10}$ transformation.

Experiment 5: Immunofluorescent Evidence of the PAF Receptor on Stallion Spermatozoa.

Spermatozoa from fresh semen samples were washed twice with PBS. The pellet was then suspended with PBS and treated with 10^{-7}M PAF for 30 minutes at 4 C° in the refrigerator. Spermatozoa were then washed with PBS and exposed to (1:100; v/v) sheep PAF antibody at 4 C°. Sheep PAF antibody was kindly provided by Dr. William E. Roudebush at Reproductive Biology Associates (Atlanta, Georgia, USA). After a 60-minute exposure period they were washed with PBS and exposed to fluorescein

isothiocyanate-conjugated rabbit anti-sheep immunoglobulin G antibody (1:20; v/v) for 30 minutes at 4 C°. Immunofluorscence microscopy was performed and the fluorescent intensity (FI) graded on a scale of 0 - 3, where 0 is the background and 3 is the strongest intensity of spermatozoal fluorescence. The intensity of fluorescence was graded at six locations on the spermatozoal (acrosomal region of the head, post-acrosomal region of the head, neck region where the centriole is located, midpiece, principal region of the tail, and end region of the tail). Figure 7 demonstrates normal stallion spermatozoa structure. Sheep non-immune and FITC rabbit non-immune antibodies were used as negative controls for the first and second antibodies, respectively.

Fluorescence intensity was recorded for 100 spermatozoa of each stallion in both the PAF and control groups. Net FI (PAF FI minus control FI) was determined for each sample. Fresh semen was obtained from a local swine farm and a human volunteer as two positive control samples. PAF is thought to act via specific receptors on the plasma membrane and specific receptor antagonists can inhibit PAF effect (Hwang, 1990). Therefore, spermatozoa from three stallions were washed with PBS then treated with 10^{-5} to 10^{-10} M PAF antagonist FR-49175 (Sigma cat. # D-8541). After one minute exposure, the experiment was conducted as described previously.

Statistical Analysis: Data were analyzed using a randomized complete block design where the stallion is the blocking factor. Data were also analyzed with one-way ANOVA with significance declared at $p \leq 0.05$ for multiple comparisons between different regions of stallion spermatozoa.

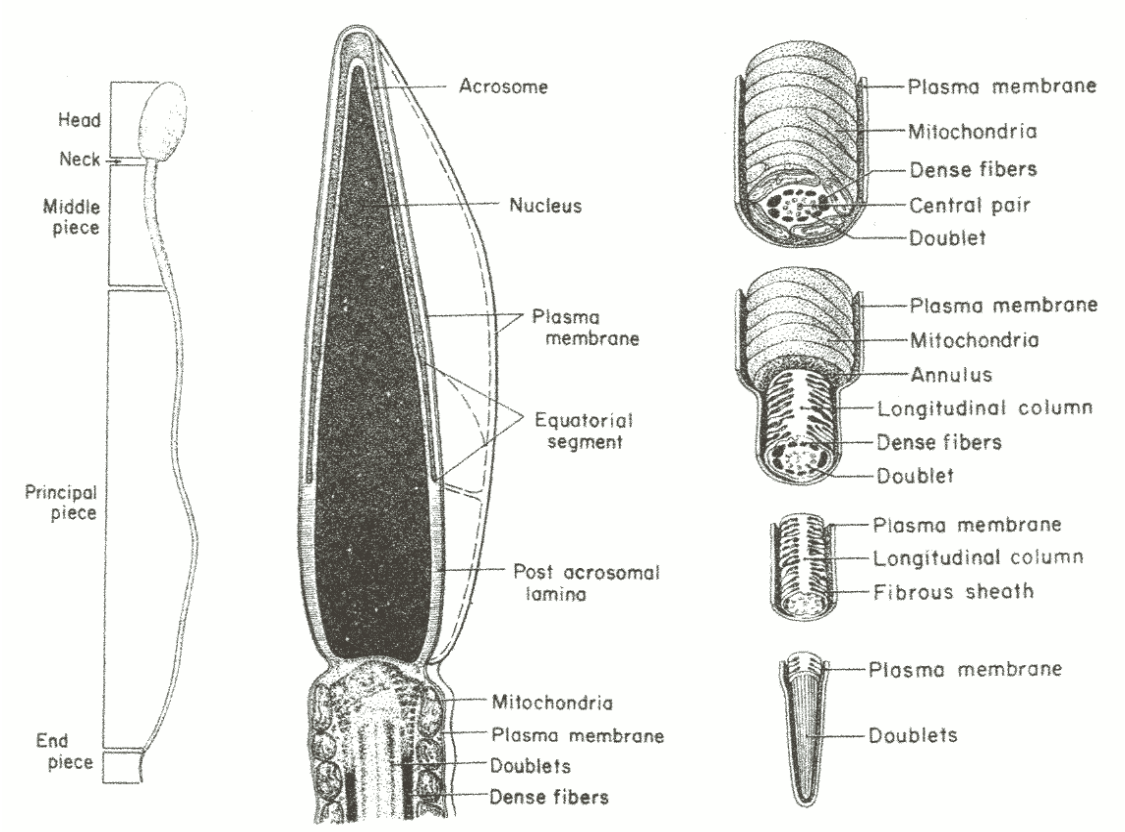


Figure 7: Diagram of stallion spermatozoa. Dimensions of stallion spermatozoa are approximately as follows: head length, 7 μm, middle piece length, 10 μm, middle piece diameter, 0.9 μm; principal piece length, 40 μm; principal piece diameter, 0.6 < 0.5 μm; and end piece length, 4 μm. The entire sperm cell is covered by the plasma membrane (atypical phospholipids bilayer incorporating cholesterol, complex carbohydrates, and proteins). The head includes the nucleus which contain the genetic information in highly condensed DNA, the acrosome which contain enzymes necessary for fertilization, the equatorial segment, and the postacrosomal lamina. The neck is the point of attachment of the tail to the head. The axonem which is composed of the central pair and nine doublets of microtubules. The mitochondrial sheath is membranous structure where the energy for spermatozoal motion is produced (Samper, 2000).

RESULTS

Experiment 1: The results of the effect of PAF on motility of fresh stallion spermatozoa at different time intervals are presented in table 3. Data were log transformed and analyzed by multiple linear regressions in which motility was the dependent variable. There was a quadratic relationship between concentrations of PAF and incubation times on their effect on motility, and this effect was significant (R-square=0.81, figure 8). Motility was severely depressed at higher concentrations of PAF and longer time of incubations, while lower concentration, that range from 10^{-10} to 10^{-13} M PAF, enhanced motility and the best motility was maintained at 120 minutes incubation time (figure 9). The PAF concentration, incubation time and their interaction were highly significant ($P< 0.01$) for their effect on motility. To see if there were any differences between incubation time and PAF concentrations, pairwise comparisons were conducted using Duncan's method. The test revealed that a PAF level that ranged from 10^{-10} to 10^{-13} M at 120 minutes was the best time where motility was enhanced. Duncan's method was conducted again to see if there were differences between PAF concentrations at 120 minutes. The analysis showed that spermatozoal motility was significantly higher at concentrations that range from 10^{-10} to 10^{-13} M PAF than the concentration that ranged from 10^{-4} to 10^{-9} M PAF at 120 minutes, but there was no significant differences among the concentrations that range from 10^{-10} to 10^{-13} M PAF at the same time.

Table 3: Percent motility for stallions spermatozoa at different time intervals and various concentrations of PAF (n=10)

Concentration (M) PAF	% Motility at 30 min	% Motility at 60 min	% Motility at 90 min	% Motility at 120 Min	% Motility at 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)

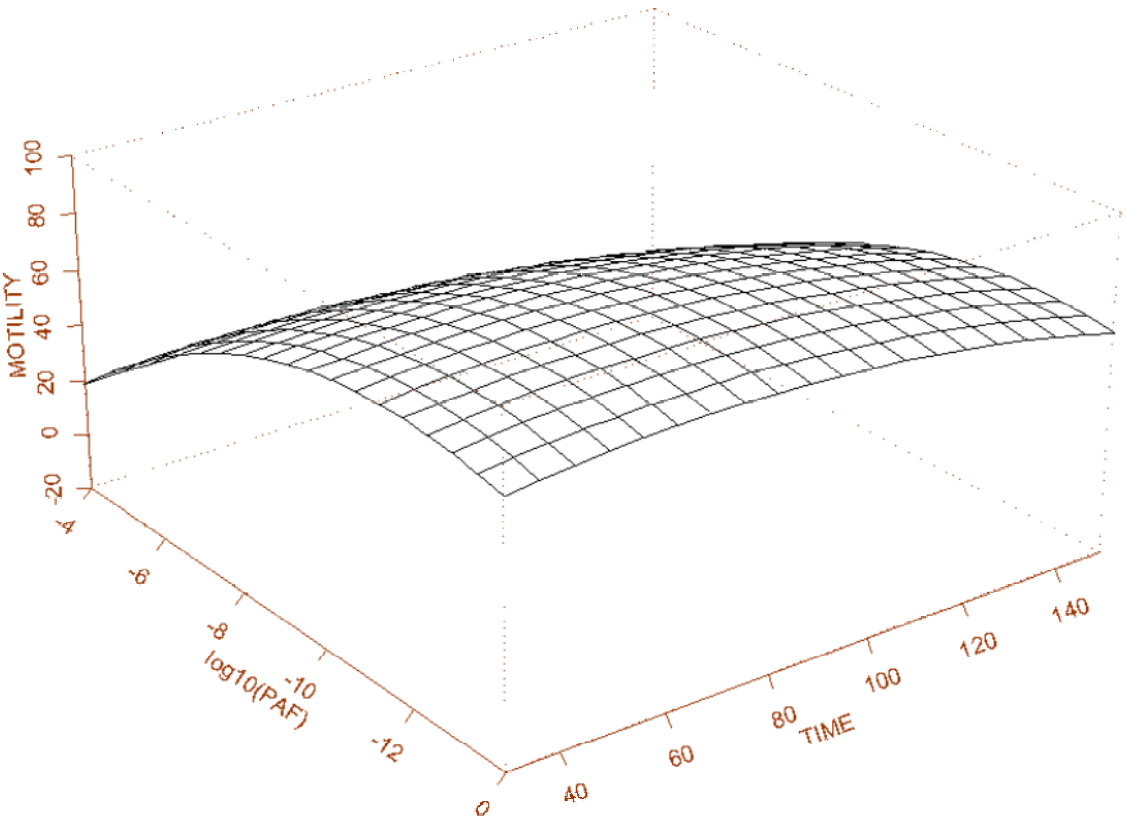


Figure 8: Regression plot for the effect of time and PAF on stallion spermatozoa motility. The multiple regression equation after log transformation is

*MOTILITY=-63.05615 + -23.13681*LOGPAF + -1.01291*LOGPAF^2 + 0.20614*TIME + -0.00278*TIME^2 + -0.01337*LOGPAF TIME

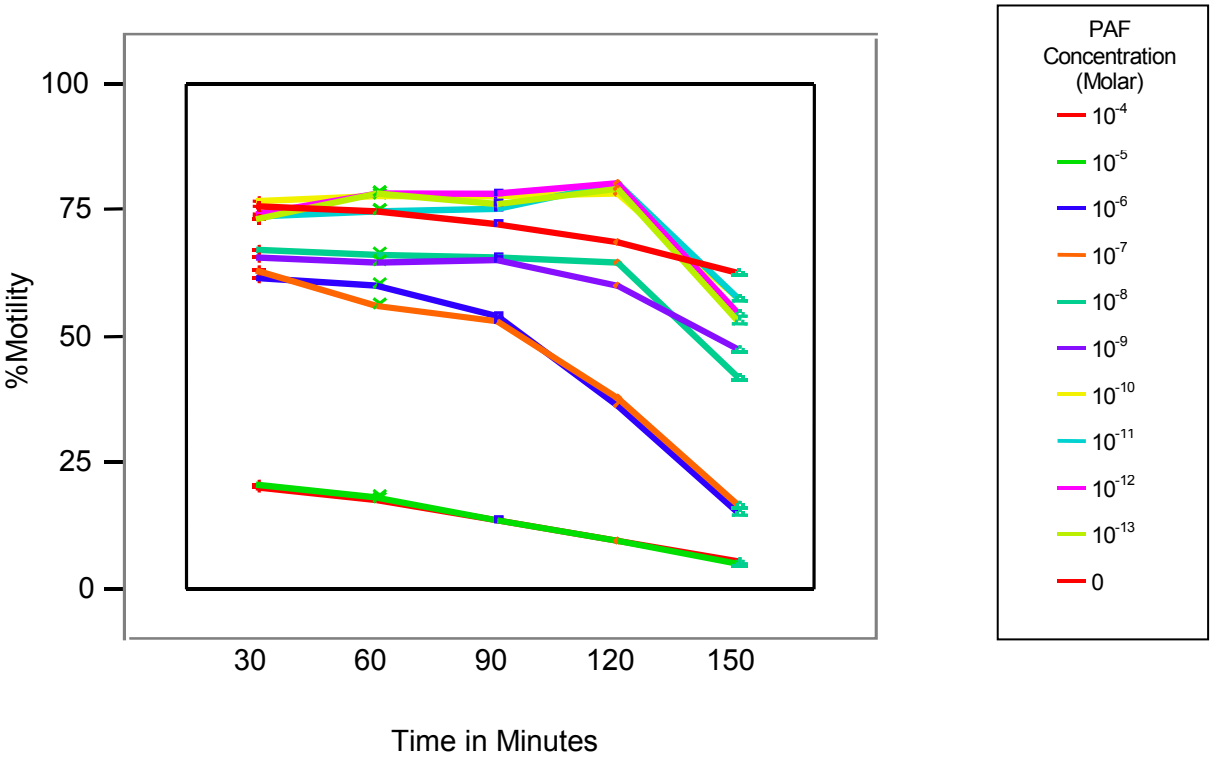


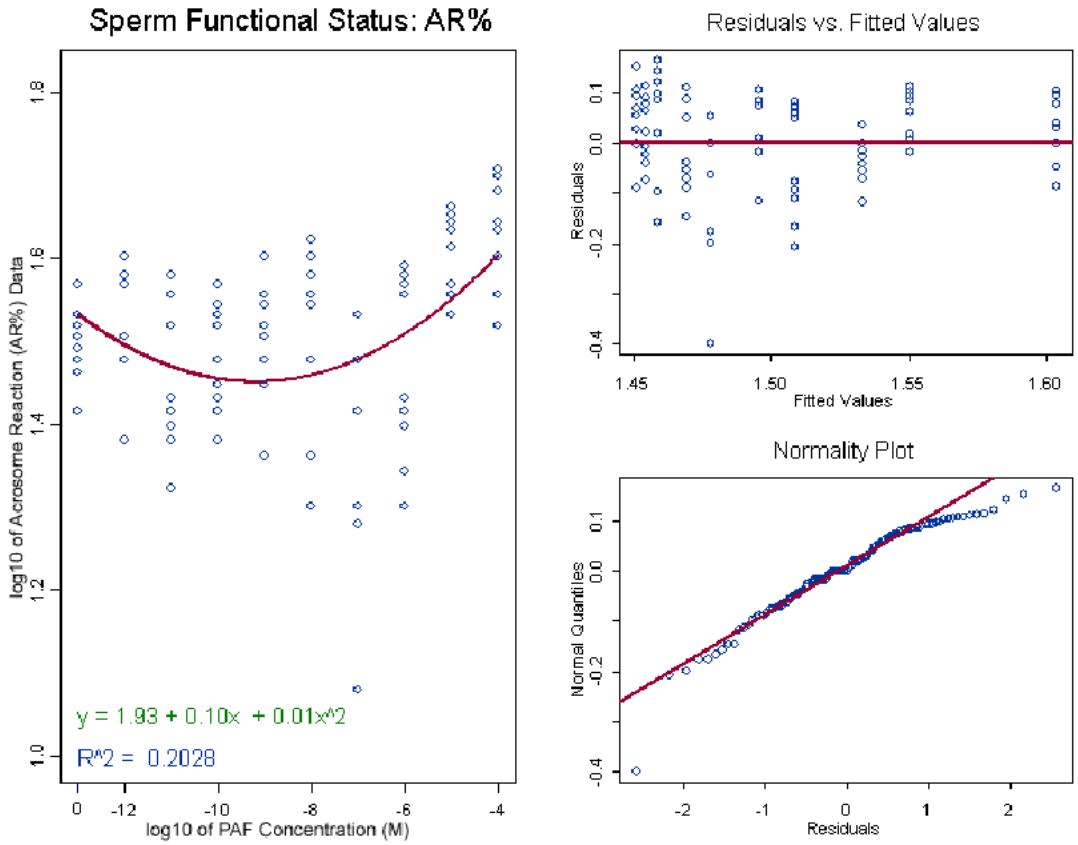
Figure 9: Effect of time in minutes and PAF concentrations in molar on spermatozoal motility of ten stallions, the plot represents the averages of the data shown in table 3.

Experiment 2: Since motility, which is an important parameter in sperm function, was best maintained at 120 minutes, the effect of 10^{-4} to 10^{-12} M PAF on capacitation and the AR using CTC was conducted at 120 minutes. Table 4 shows that at 10^{-4} to 10^{-6} M PAF, the sperm AR increased in contrast to the number of spermatozoa that undergo capacitation at the same concentrations. Capacitation was achieved at lower concentrations of 10^{-7} to 10^{-12} M PAF with the highest shown at 10^{-10} and 10^{-11} M PAF. Results were presented as (mean+/- SEM). The regression plot for AR is shown in figure 10, however only 20% of evaluated data was accounted for the acrosome reaction (R-Squared=0.20, $P < 0.01$). The effect of PAF on the capacitated state (B), as detected by CTC is shown in figure 11 along with the fitted values and the regression equation. There was a significant effect of PAF on the percentage of spermatozoa that were capacitated (R-Squared = 0.83, $P < 0.01$). The concentration of PAF that caused the maximum level of capacitation (B) ranges from 10^{-9} to 10^{-12} M PAF at 120 minutes incubation time. The types of sperm CTC patterns that were visualized with the CTC assay are shown in figure 12.

Table 4: Dose response of PAF on capacitation and the acrosome reaction of viable equine spermatozoa at120 minutes (n=10).

PAF (M)	Functional Status (F)	Functional Status (B)	Functional Status (AR)
10^{-4}	42.4 ± 1.55	15.5 ± o.97	42.1 ± 1.96
10^{-5}	47.3 ± 1.8	13.2 ± 0.68	39.4 ± 1.55
10^{-6}	43.7 ± 1.90	26.6 ± 1.4	29.7 ± 2.2
10^{-7}	43.7 ± 1.6	31.6 ± 1.00	24.7 ± 2.1
10^{-8}	23.9 ± 2.1	43.7 ± 1.3	32.4 ± 2.3
10^{-9}	19.1 ± 2.3	49.0 ± 2.2	32.2 ± 1.5
10^{-10}	11.1 ± 0.98	58.8 ± 1.5	30.1 ± 1.4
10^{-11}	11.2 ± 0.71	59.1 ± 1.8	28.7 ± 2.0
10^{-12}	23.2 ± 1.4	43.0 ± 0.71	33.8 ± 1.7
0	37.5 ± 1.4	30.3 ± 1.3	32.0. ± 0.99

F= uncapacitated, acrosome intact B= capacitated, acrosome intact, AR= acrosome reacted. Values (Mean +/- SEM)

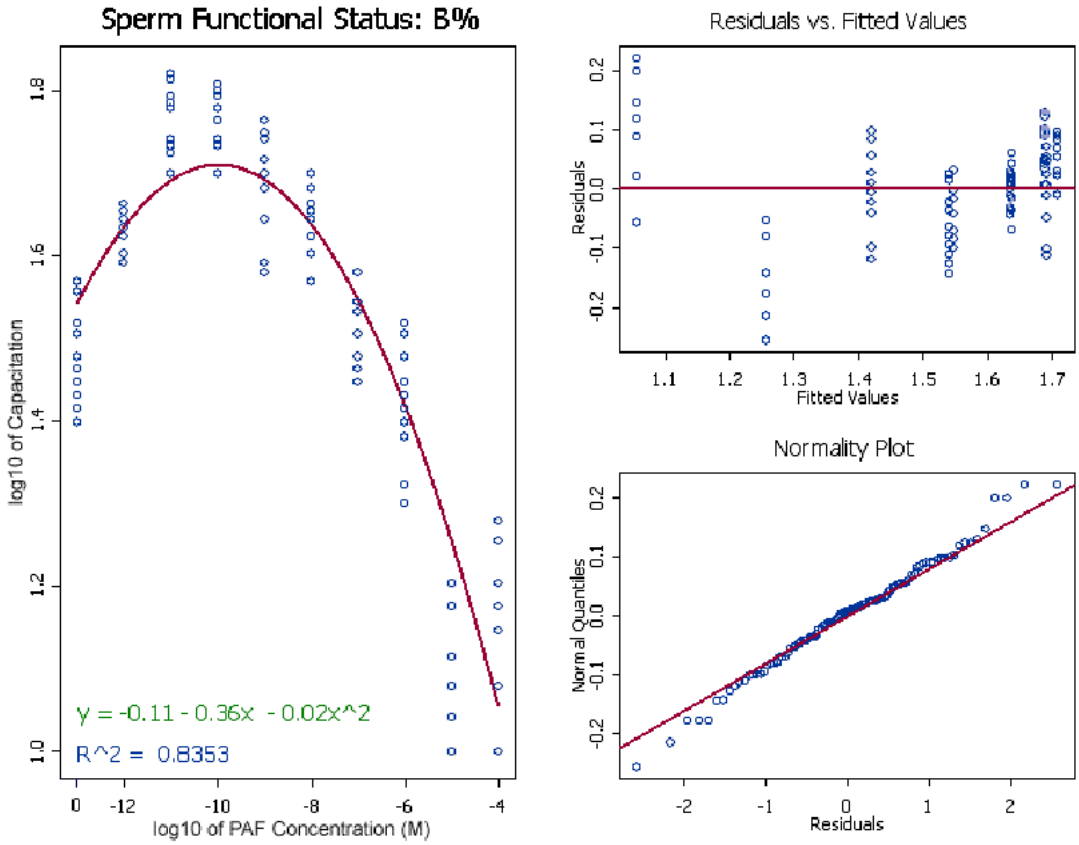


$$\log_{10}(\text{Acrosome Reaction}_{AR}) = \beta_0 + \beta_1 \log_{10}(\text{CONCENTRATION}_{AR}) + \beta_2[\log_{10}(\text{CONCENTRATION}_{AR})]^2$$

```
Coefficients:
               Value Std. Error t value Pr(>|t|)
 (Intercept)  1.9307  0.0886    21.8018  0.0000
     logCONC  0.1045  0.0224     4.6601  0.0000
I(logCONC^2)  0.0057  0.0013     4.3564  0.0000

Residual standard error: 0.09476 on 97 degrees of freedom
Multiple R-Squared: 0.2028
F-statistic: 12.34 on 2 and 97 degrees of freedom, the p-value is 0.00001686
```

Figure 10: The effect of PAF on the acrosome reaction (AR) of stallion spermatozoa as detected by CTC. Data of AR was percentage (%), so they were first log transformed then analyzed by regression analysis.



$$\log_{10}(\text{CAPACITATION}_B) = \beta_0 + \beta_1 \log_{10}(\text{CONCENTRATION}_B) + \beta_2 [\log_{10}(\text{CONCENTRATION}_B)]^2$$

```
Coefficients:
                Value Std. Error  t value Pr(>|t|)
 (Intercept)  -0.1105   0.0860    -1.2853   0.2017
     logCONC  -0.3650   0.0218   -16.7679   0.0000
I(logCONC^2)  -0.0183   0.0013   -14.4483   0.0000

Residual standard error: 0.09202 on 97 degrees of freedom
Multiple R-Squared: 0.8353
F-statistic: 246 on 2 and 97 degrees of freedom, the p value is 0
```

Figure 11: The effect of PAF on the capacitation status (B) of stallion spermatozoa as detected by CTC. Data of B was percentage (%), so they were first log transformed then analyzed by regression analysis.

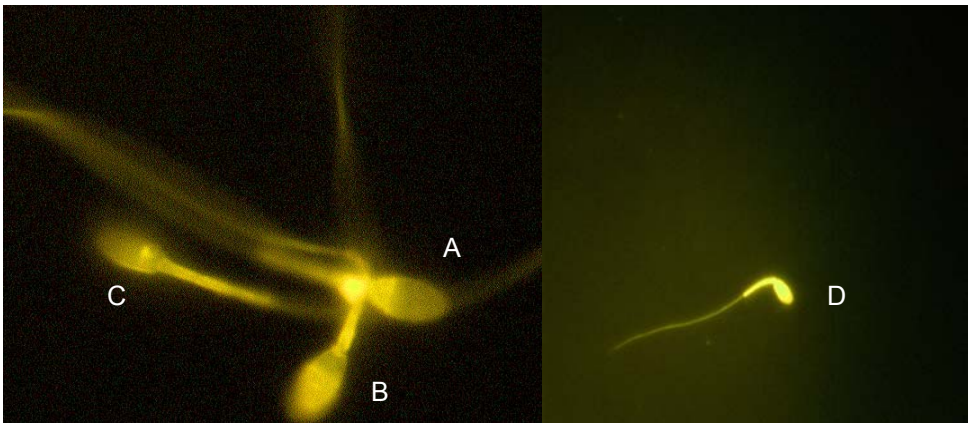


Figure 12: Chlortetracycline (CTC) assay patterns of stallion spermatozoa, shows capacitated (B), acrosome reacted (A&C) and uncapacitated(D)patterns.

Experiment 3: Since motility was best maintained at 120 minutes (experiment 1) and capacitation was best enhanced at lower concentration of PAF (experiment 2), the evaluation of the true acrosome reaction using transmission electron microscopy (TEM) was conducted using 10^{-9}, 10^{-10} and 10^{-11} M PAF. The results for TEM analysis of spermatozoa, shows that the number of intact spermatozoa decreased as concentration of PAF increased (table 5; figure 13). The highest number of reacted spermatozoa was achieved at 10^{-9} M (47.7+/- 1.45, table 5 and figure 14) while vesiculation was highest at 10^{-10} and 10^{-11} M PAF (table 5 and figure 15). The linear regression analysis (figures 13, 14 and 15) showed that differences in PAF concentrations were highly significant as indicated by the R-square values (for intact: 0.97.2, reacted: 0.89.8, and for vesiculated: 0.98.1).

The fusion between the plasma membrane and the outer acrosomal membrane called vesiculation is shown in the TEM graphic in figures 16, 17. In figure 18, the membrane vesicles were shown on the right spermatozoa and acrosome reacted spermatozoa was shown on the left one. There was no vesiculation in the equatorial segment as shown in figure 19. All these changes can be compared to the intact spermatozoa in figure 20.

Table 5: Effect of PAF on the acrosomal status of fresh equine spermatozoa as detected by TEM (n=3).

PAF dose (M)	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 (Mean+/- SEM).

Intact: Sperm maintain the acrosome and its content.

Reacted: The loss of the outer acrosomal and overlying plasma membranes.

Vesiculated: The fusion of the plasma and the outer acrosomal membranes.

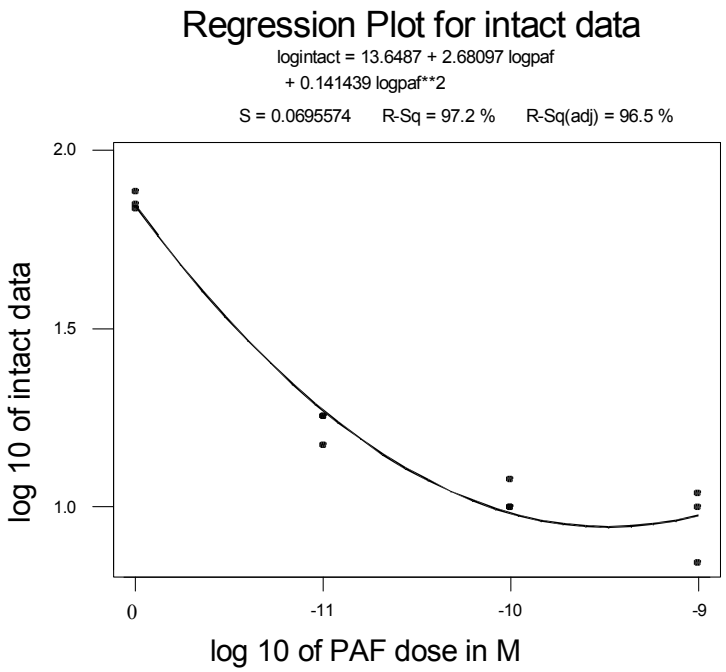


Figure 13: Effect of PAF concentration 10^{-9}, 10^{-10}, and 10^{-11} M on the acrosomal status (intact) of stallion spermatozoa as detected by TEM. Capacitation was done using PAF and the acrosome reaction was induced by progesterone. Multiple regressions were done after the data was log transformed. Intact was identified when the acrosomal content and its membranes were maintained.

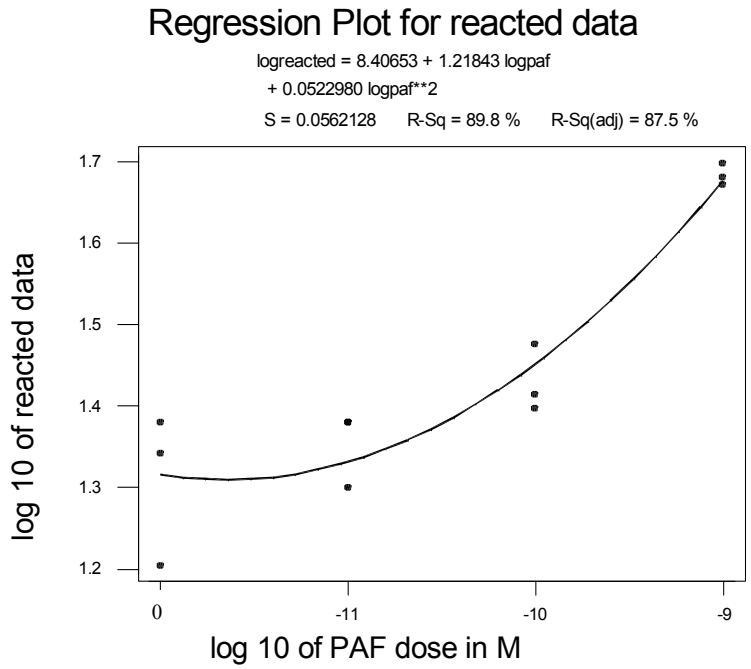


Figure 14: Effect of PAF concentration 10^{-9} to 10^{-11} M on the acrosomal status (reacted) of stallion spermatozoa as detected by TEM. Capacitation was done using PAF; acrosome reaction was induced by progesterone. Multiple regressions were done after data was log transformed. Reacted status was identified as loss of the outer acrosomal and overlying plasma membranes.

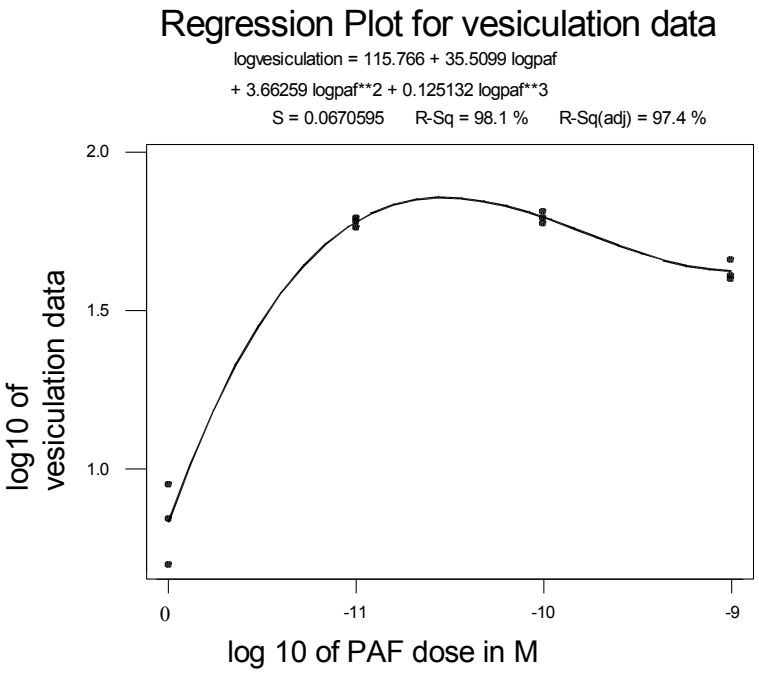


Figure 15: Effect of PAF concentration 10^{-9} to 10^{-11} M on the acrosomal status (vesiculation) of stallion spermatozoa as detected by TEM. Capacitation was done using PAF and the acrosome reaction was induced by progesterone. Multiple regression analysis was done after data was log transformed. Vesiculation was identified upon fusion of the plasma and the outer acrosomal membranes or undergoing acrosomal exocytosis with differing amounts of acrosomal matrix still present.

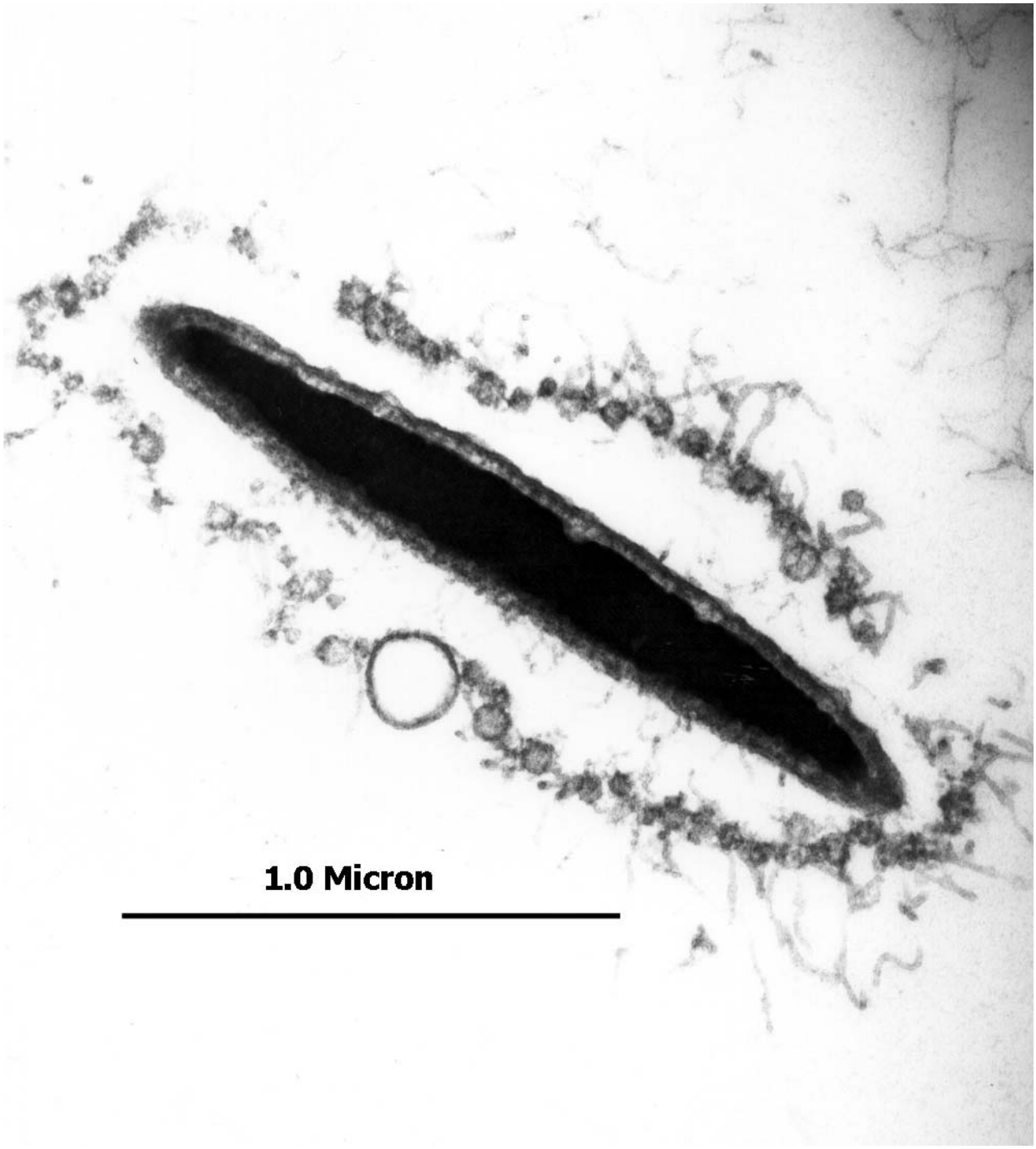


Figure 16: Transmission electron micrograph of stallion spermatozoa during vesiculation of the plasma and the outer acrosomal membranes. Capacitation was done in-vitro by PAF and the acrosomal reaction was induced by progesterone. Magnification: 79,750x.

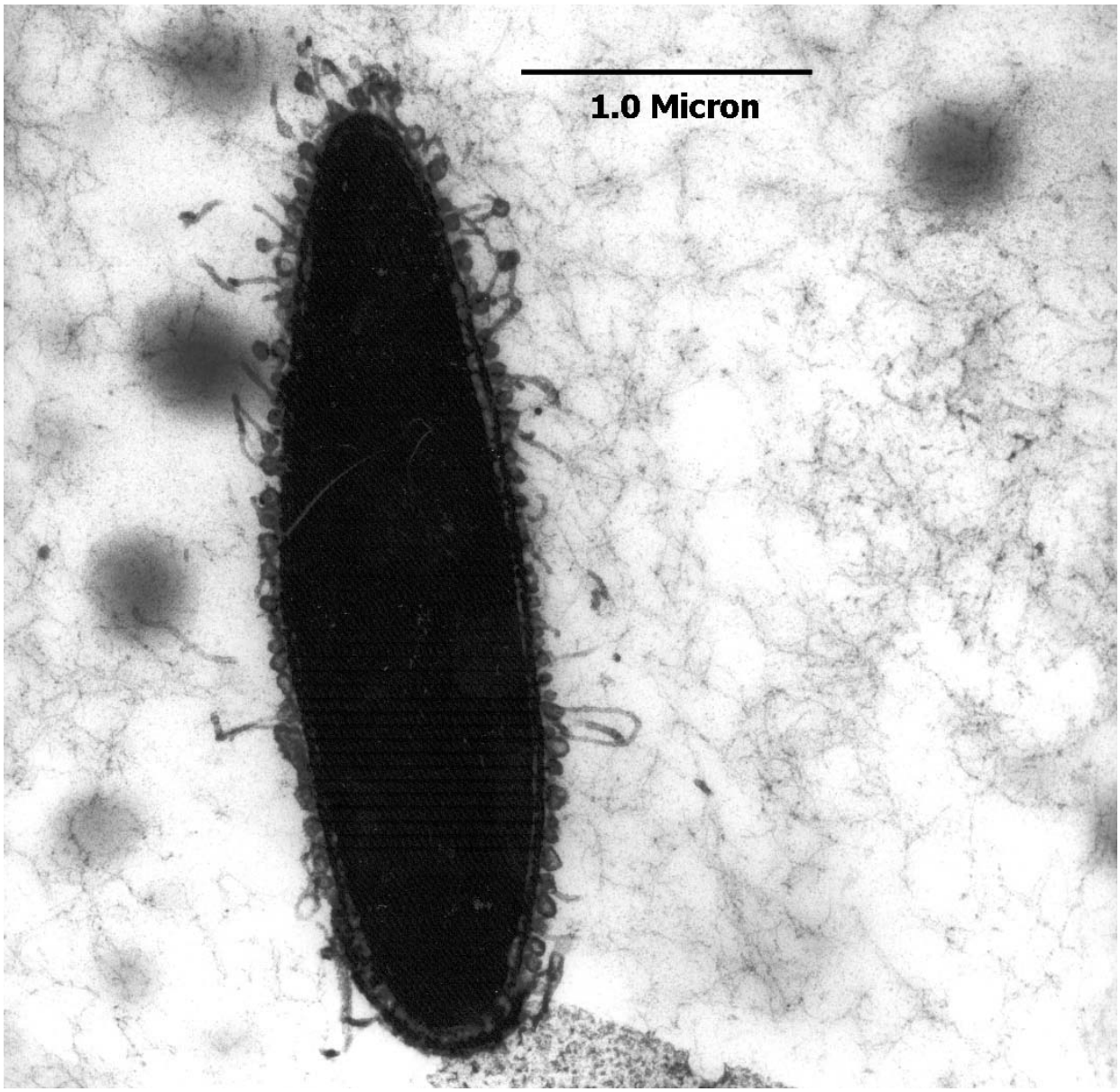


Figure 17: Transmission electron micrograph of stallion sperm during vesiculation of the plasma and the outer acrosomal membrane. Capacitation was carried out in-vitro by PAF and acrosomal reaction is induced by progesterone. Magnification: 52,250x.

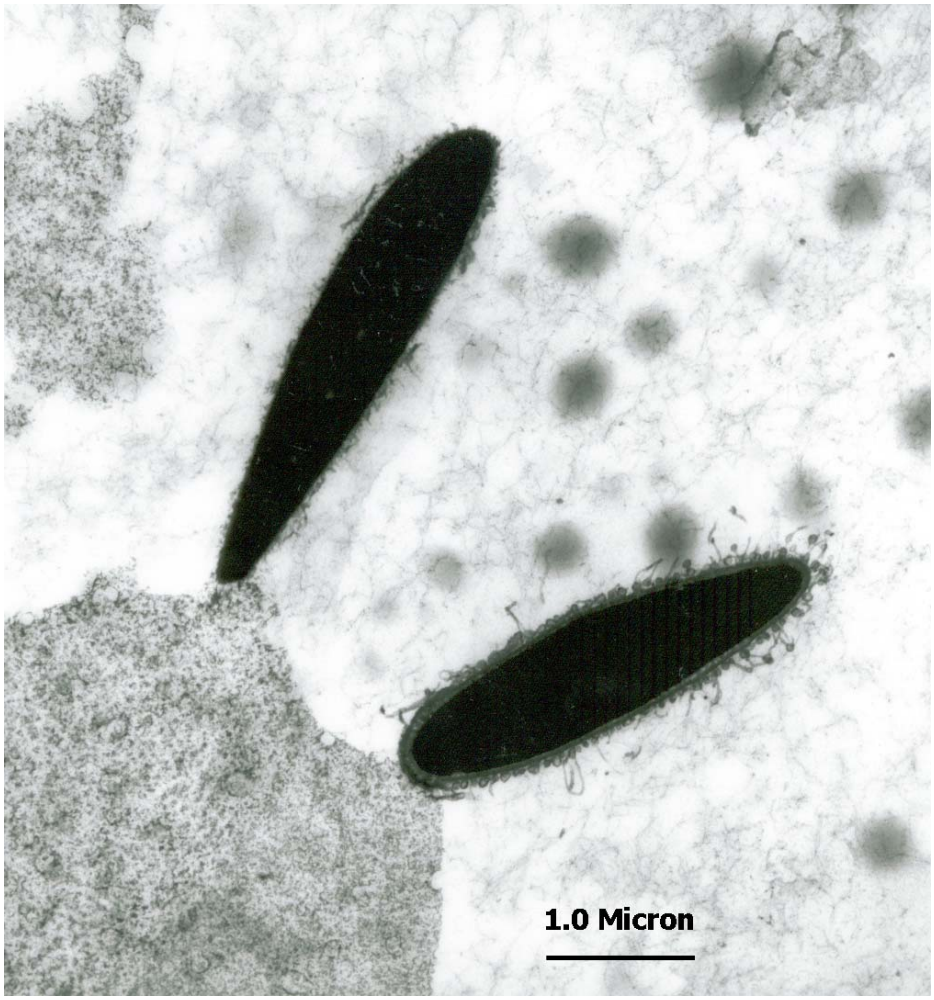


Figure 18: Transmission electron micrograph of stallion sperm capacitated in vitro (showing acrosome reacted spermatozoa). Capacitation was done in-vitro by PAF and acrosomal reaction was induced by progesterone. Notice an acrosome-reacted (upper left) and vesiculated (lower right) spermatozoa. Magnification: 27,500x.

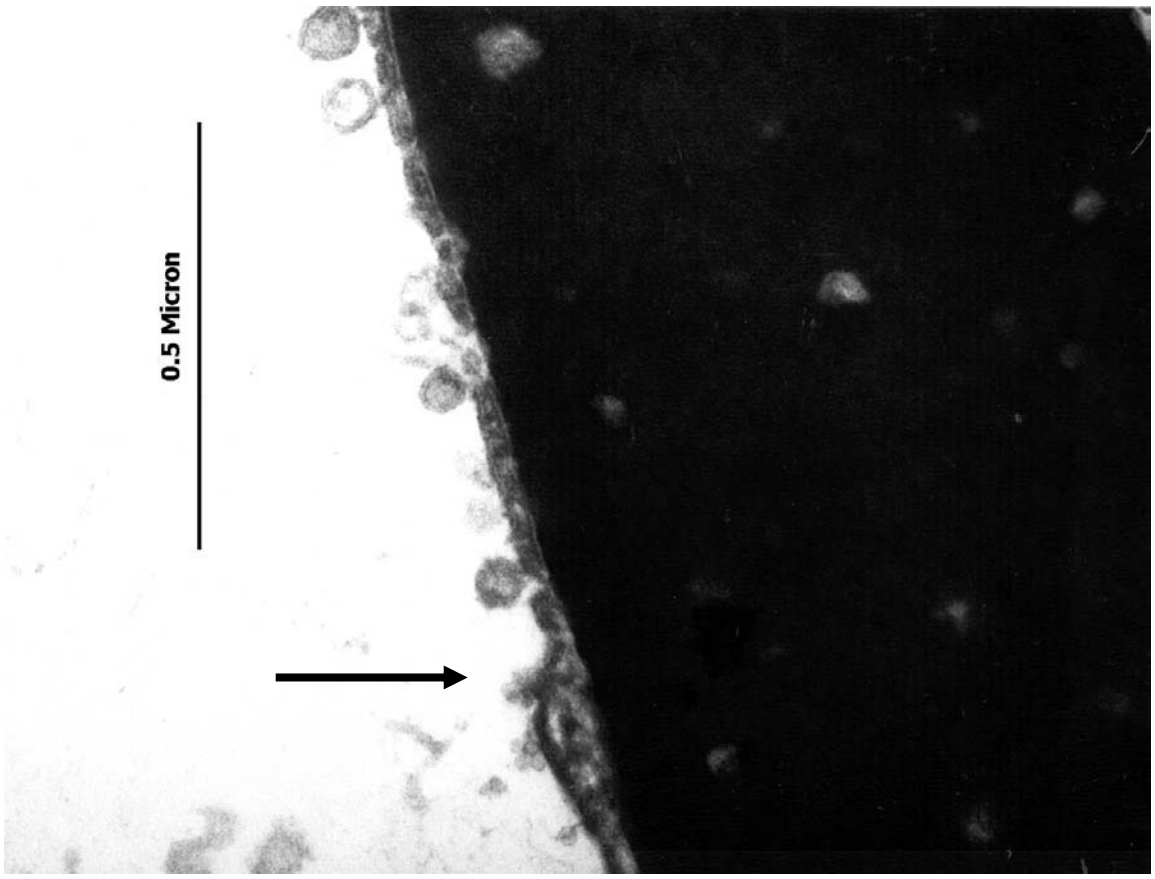


Figure 19**:** Transmission electron micrograph showing vesiculation around the equatorial segment. The plasma membrane of the equatorial segment (arrow) does not vesiculate, but it fuses with the remaining part of the outer acrosomal membrane which then becomes continuous with the inner acrosomal membrane during the AR. Magnification: 201,600x

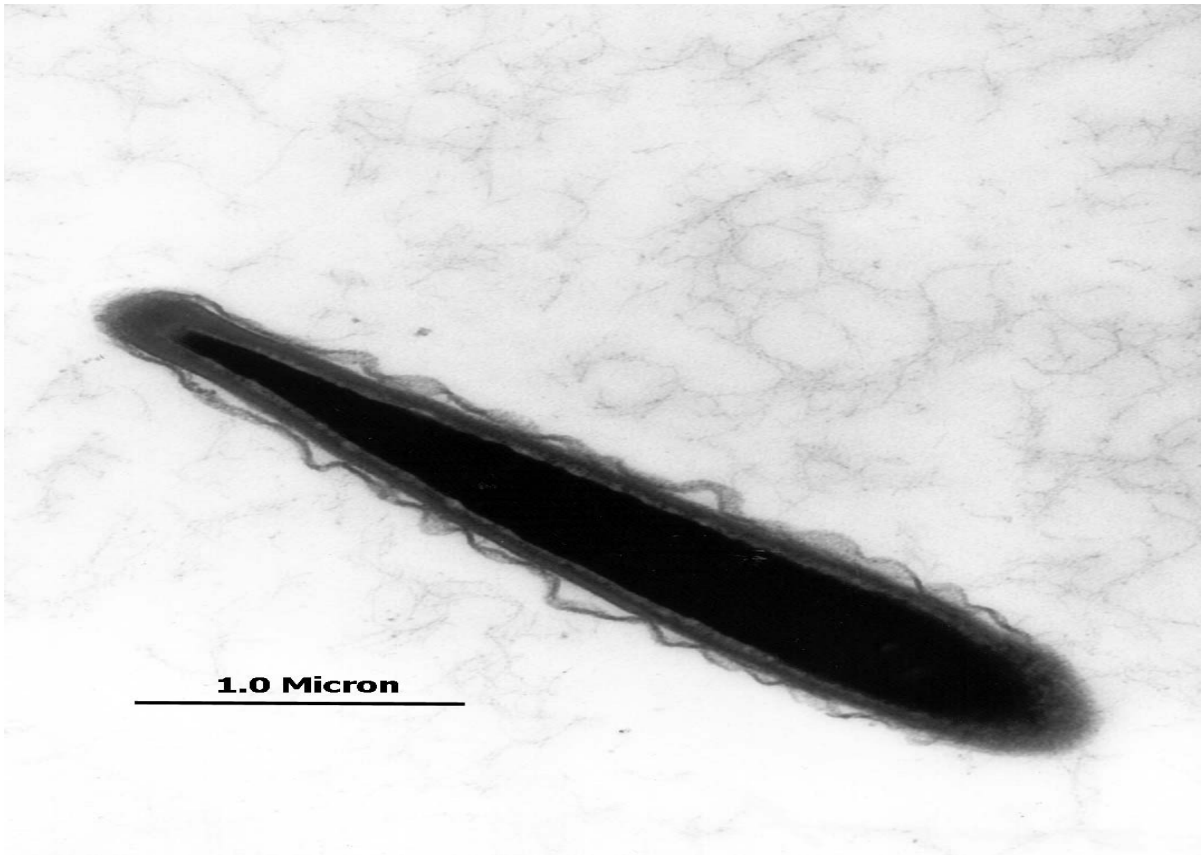


Figure 20: Transmission electron micrograph of stallion sperm capacitated in vitro (showing membrane-intact spermatozoa). Capacitation was carried out in-vitro by PAF and the acrosomal reaction was induced by progesterone. Non-reacted (intact) sperm maintains the acrosomal membrane and its content. Magnification: 52,250x.

Experiment 4: The reduced heterogeneity of Fluo-3/Fura Red ratios of stimulated and resting cells can be observed graphically by comparing the pre-stimulated (resting) region and stimulated region of figure 21. Stimulation was done using ionomycin. The ratio was measured in channels and the difference between resting stage and stimulated stage indicate the amount of calcium that was released under the effect of PAF. The Fluo-3 increases in intensity when bound to calcium while Fura Red increases intensity when not bound. When the ratio of Fluo-3/Fura Red is calculated, an increase intracellular free calcium concentration is accompanied by an increase in Fluo-3 intensity (as the dye becomes calcium bound) and a decrease in Fura Red intensity (as the dye becomes calcium bound), thus making a larger positive shift in this ratio than would be present for Fluo-3 alone.

As can be seen in table 6 and figure 22, there was a linear relationship between the doses of PAF that ranged from 10^{-7} and 10^{-12} M and the change in intracellular calcium response. The concentration of PAF (see figure 22) that was able to induce half the change in the intracellular calcium (effective concentration; EC_{50}) was 10^{-10} M PAF (r^2=0.36.6, P<0.01).

Treatment of spermatozoa with the PAF antagonist FR-49175 inhibited the release of calcium by PAF with a median inhibitory concentration (IC_{50}) of $10^{-7.5}$ M. The R-Square value is 0.82 (P<0.01, figure 23). The values indicated in figure 23 are adjusted values were Y = calcium values for each antagonist concentration – control / calcium value at 10^{-7}M PAF – control. The differences between the upper and lower means were used to generate a predictive equation to calculate EC_{50} and IC_{50}.

Table 6: Flow cytometry results measuring intracellular calcium using Fluo3/Fura Red ratio (n=4).

Stallion	MPAF					
	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
01	299.7	266.7	264.2	250.9	245.7	181.8
02	192.3	191.6	185.3	180.9	156.7	135.7
03	277.4	242.7	230.0	204.3	147.6	112.9
04	187.8	185.9	185.4	183.6	183.8	183.4

The differences between the resting and stimulated cells (figure 21) are the net value that indicate amount of calcium released

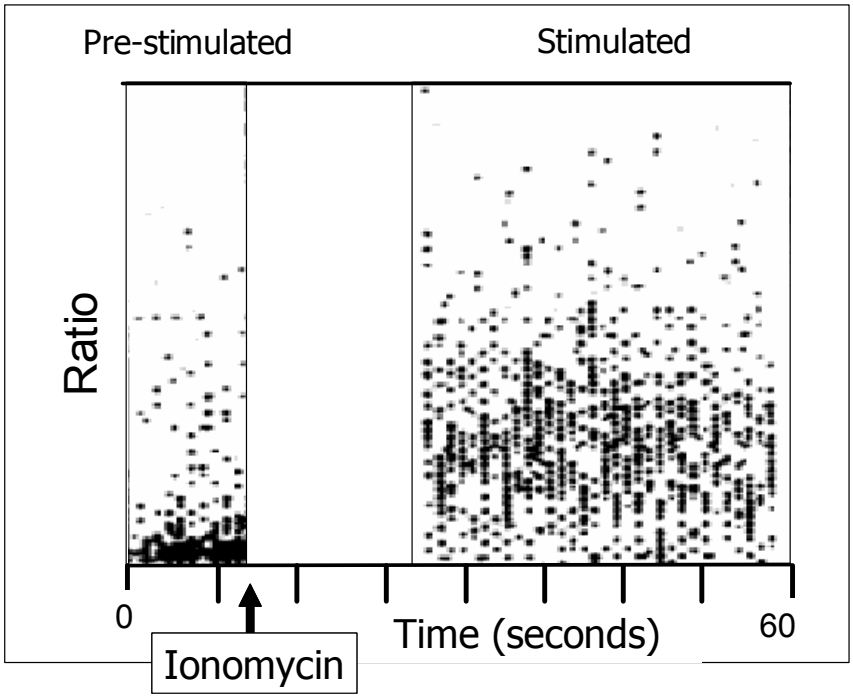


Figure 21: Representative kinetic display using Fluo-3/Fura Red ratio to measure intracellular calcium changes after PAF treatment. Ratio was measured in channels and the difference between resting stage (pre-stimulated) and stimulated stage indicate the amount of calcium that was released under the effect of PAF. Cells were stimulated with ionomycin (arrow).

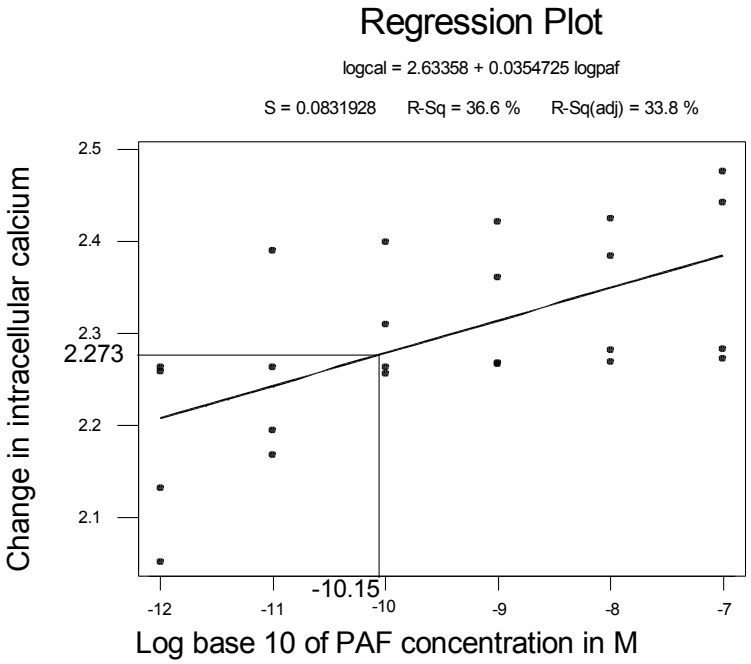


Figure 22: Effect of 10^{-7} to 10^{-12} M PAF on the intracellular calcium concentration of Stallion spermatozoa. The ratio of Fluo-3/Fura Red was used to detect the change in Ca^{+2} Mobilization when spermatozoa stimulated with ionomycin. By taking the difference between the upper and lower means, the calculated effective concentration (EC_{50}) was10^{-10} M PAF.

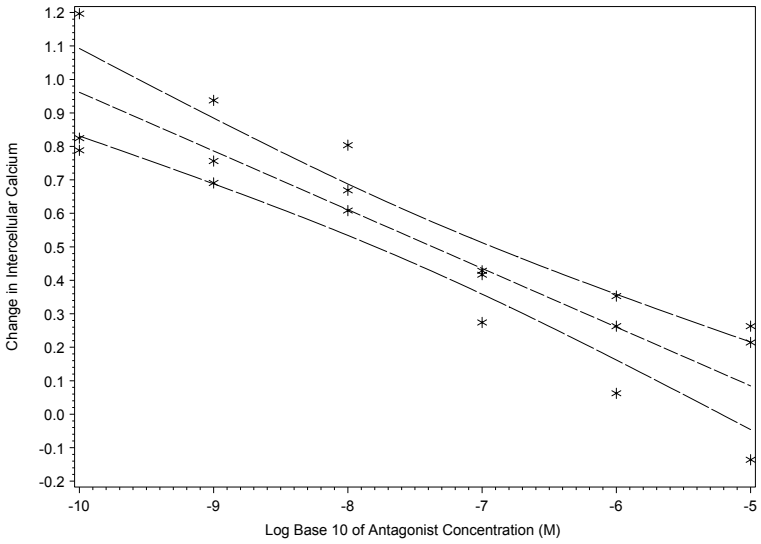


Figure 23: Effect of PAF antagonist FR-49175 on the intracellular calcium of stallion spermatozoa. By taking the difference between the upper and lower means the calculated median inhibitory concentration (IC_{50}) value of antagonist was $10^{-7.5}$ M.

Experiment 5: Table 7 shows the net fluorescence intensity (FI) of PAF receptor localization levels among different regions of equine spermatozoa. As can be seen in table 7 and figure 24, the highest FI was found in the post-acrosomal region of the head, with a mean of 2.60 (95% CI = 2.26 to 2.93). Analysis of variance (ANOVA) was applied to determine whether a statistically significant difference exists somewhere among the different regions of equine spermatozoa. The regions comparisons were identified using one-way ANOVA; the alpha level at which statistical significance was declared is given as 0.05. There was a statistically significant difference among the spermatozoa regions ($P< 0.01$). Follow-up for the multiple comparisons were made with Duncan's procedure to further examine if there were any differences among spermatozoal regions. The test revealed that the mean for the post-acrosomal region (2.60+/- 0.148; expressed as mean+/- SEM) was the same as ANOVA analysis revealed previously; additionally, it was significantly higher than all other regions. There was no significant difference between the neck (1.88+/- 0.221) and the acrosomal region (1.57+/- 0.058); however, the midpiece FI is higher (1.15+/- 0.067) than the principal piece (0.40+/- 0.089) and the latter is higher than the end piece (0.05+/- 0.050) of the tail. For the boar spermatozoa, FI was found in the distal region of the head, while with human spermatozoa, the FI was found in the post-acrosomal region of the head and the midpiece.

Figure 24 shows distribution of FI in the boar, stallion and human spermatozoa. The antagonist results show that 10^{-5} M of PAF antagonist FR-49175 completely inhibited spermatozoal fluorescence intensity (r^2=0.918, $P<0.01$; figure 25). The calculated median inhibitory concentration (IC_{50}) of FR-49175 was 10^{-8} M at a PAF concentration of 10^{-7} M (figure 26).

Table 7: Net fluorescent intensity (FI) levels in Equine spermatozoa (n=10).

Stallion	Acrosomal region	Post-Acrosomal	Neck region	Midpiece	Principal region/tail	End region/tail
01	1.60	2.20	1.80	1.00	0.50	0.00
02	1.30	2.70	1.40	1.50	0.60	0.00
03	1.50	2.80	1.50	1.10	0.10	0.00
04	1.70	3.00	3.00	1.20	0.80	0.50
05	1.90	1.50	1.50	1.40	0.70	0.00
06	1.50	3.00	3.00	1.00	0.00	0.00
07	1.40	2.80	1.50	1.30	0.40	0.00
08	1.50	2.60	1.00	1.00	0.50	0.00
09	1.50	2.40	1.60	1.20	0.40	0.00
10	1.80	3.00	2.50	0.80	0.00	0.00
*Mean+/-SEM	[b]1.57+/-0.06	[a]2.60+/-0.15	[b]1.88+/-0.22	[c]1.15+/-0.07	[d]0.40+/-0.09	[e]0.05+/-0.05

Values expressed as Mean+/-SEM

* Means with different letters were significantly different

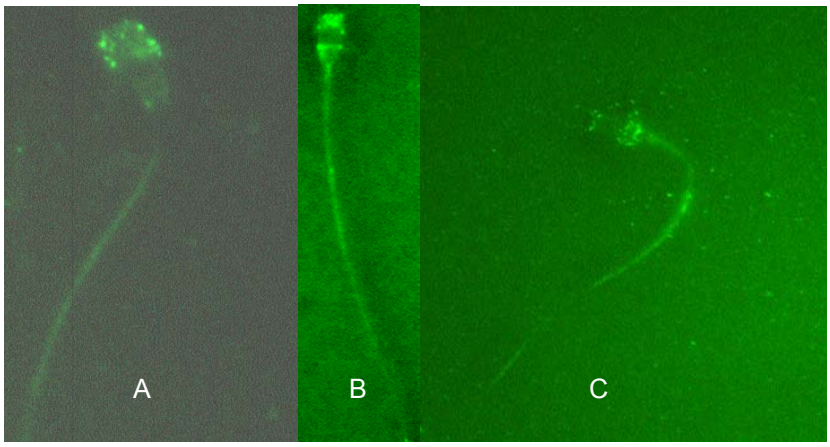


Figure 24: Immunofluorescent assay of boar (A), stallion (B) and human (C) spermatozoa showing localization of PAF receptor.

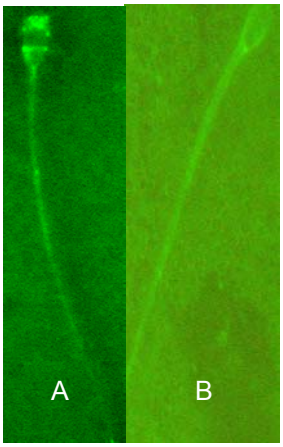


Figure 25: Immunoflourescent assay of stallion spermatozoa showing localization of PAF receptor in the spermatozoa (A) and inhibition of fluorescence intensity by PAF antagonist FR-49175 in the spermatozoa (B).

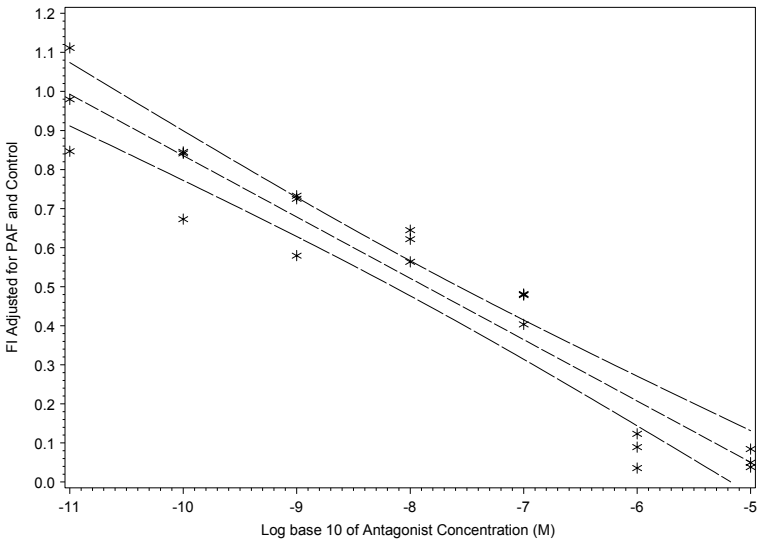


Figure 26: Effect of PAF antagonist FR-49175 on fluorescence intensity of spermatozoa from 3 stallions. The median inhibitory concentration (IC_{50}) of FR-49175 was 10^{-8}M.

DISCUSION

Male fertility requires the production of an adequate concentration of normal mature spermatozoa with sufficient motility and the ability to undergo capacitation/acrosome reaction so as to bind and penetrate the zona pellucida for fertilization. Defects in any of these necessary characteristics can lead to male factor infertility. Routine semen analysis parameters such as concentration, motility and morphology are helpful in the evaluation of male factor infertility, but fall short of predicting the fertilization potential of any given semen sample. None of the parameters obtained during routine semen analysis actually predict the fertilizing ability of the spermatozoa. This ability is influenced by a large number of variables such as age, season, collection procedure and handling of semen. There are a lot of molecular events that mediate sperm activation, and even a larger number of parameters that impact the sperm-egg interaction.

The types of interactions, and the molecules involved in such interactions, are dictated in a species-specific manner (Gadella et al., 2001). Figure 27 illustrates some of the major stages of the sperm-egg interaction.

Other studies have demonstrated that treating mammalian spermatozoa with PAF affects motility (human: Ricker et al., 1989 and 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 was found to be present in squirrel monkey spermatozoa (Roudebush and Mathur, 1998) and PAF concentrations in rhesus monkey spermatozoa was found to be directly correlated with spermatozoal motility and progression (Roudebush et al., 2002). Treatment of human spermatozoa for 5 minutes with synthetic

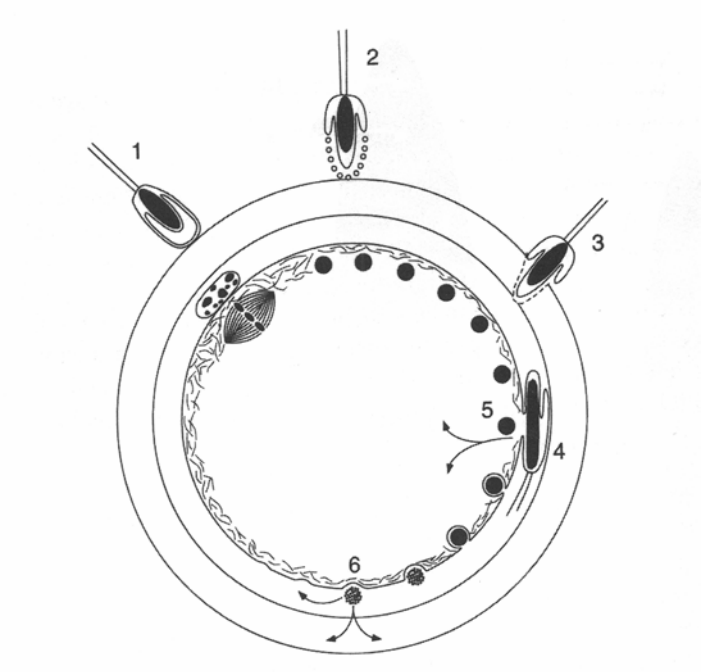


Figure 27: Diagrammatic representation of the sequences involved in the sperm-egg interactions (Gadella et al., 2001)

1) Sperm binding to the ZP of oocyte with its apical subdomain
2) The acrosome reaction, a multiple fusion event between the outer- acrosomal membrane and the plasma membrane of the sperm
3) The penetration of the sperm cell through the ZP
4) Sperm binding and fusion with the oolemma (fertilization) at the equatorial plasma membrane
5) Activation of the oocyte by cytosolic factors and fast polyspermy block
6) Secretion of cortical granules (cortical reaction) causing a slow polyspermy block

PAF at concentrations ranging from 10^{-7} to 10^{-13} M, exhibits a statistically significant ($P<0.05$) increase in motility while treatment with $\geq 10^{-5}$ M caused immediate cell death (Ricker et al, 1989). Human spermatozoa exposed to exogenous PAF concentrations of 0.5-100 nM resulted in a significantly increased linear velocity and the greatest increase in linear motion was observed at 50 nM PAF (Jarvi et al., 1993). A threefold improvement in motility of human spermatozoa was reported after exposure to 10 nM PAF for 4 hours (Krausz et al., 1994). For assessment of human and mouse spermatozoal hyperactivated motility after treatment with PAF, Wu et al (2001) used a chemotaxis assay where changes in motility of spermatozoa were assessed by their ability to migrate through a polycarbonate filter containing 8-μm pores. In their study, human spermatozoa showed a significant quadratic dose response ($P<0.001$) with a significant enhancement reaching a peak of 18 nmol PAF/liter, but declining at 18 μmol/liter PAF. While in mice, PAF enhanced spermatozoa migration under the same conditions, with a concentration ranging from 18 nmol/liter to 18 μmole/liter ($P<0.001$). The effect of PAF on mouse epididymal spermatozoa indicated that 10^{-4} M PAF reduced spermatozoal motility and decreased ($P<0.05$) the fertilization rate (Kuzan et al., 1990).

Motility result conducted in this research indicated that PAF effects on motility of stallion spermatozoa were 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 ($P <0.01$). Motility was best maintained at 120 minutes, at a PAF concentrations ranging from 10^{-10} to 10^{-13} M, however there was no significant differences among this range of concentrations at 120 minutes. These concentrations were lower than that was reported for human, the time of

incubation may be a factor as in human spermatozoa were incubated for 5 minutes while in stallion spermatozoa incubated for 120 minutes, species differences and the medium conditions also of consideration.

Since spermatozoal motility is an important parameter of a semen analysis, and motility was best maintained at 120 minutes, this time was chosen for the capacitation/AR experiments. The final stages of capacitation are associated with hyperactivated motility and it has been suggested that this increase in motility is necessary for fertilization both in vivo and in vitro (Ricker et al., 1989). Wu et al (2001) indicates that their results on motility confirm that exogenous PAF acts in specific fashion to induce spermatozoa motility changes associated with capacitation.

The CTC stain has been used to study the effect of different agents on capacitation and the acrosome reaction on different species (human: DasGupta et al., 1993; bull: Fraser et al., 1995; mouse: Huo and Yang, 2000). CTC was highly correlated with TEM for estimation of acrosome reacted spermatozoa when the effect of heparin on capacitation/AR of stallion spermatozoa was investigated (Varner et al., 1993). The Ca^{2+} related changes in the capacitation state of human spermatozoa were evaluated using CTC. The results of CTC were comparable with fluorescein-conjugated Pisum sativum agglutinin (PSA) staining that was verified by TEM (DasGupta et al., 1993). Huo and Yang (2000) investigated the effects of PAF on capacitation and acrosome reaction of mouse spermatozoa by the CTC and Coomassie blue staining respectively. Their results showed that the percentage of capacitated mouse spermatozoa was increased by incubation with 50 ng/ml for 90 minutes and the AR was increased at 5000 ng/ml at the same time. The effects of PAF on the AR of human spermatozoa as detected by FITC-

PSA assay indicates that PAF concentrations ranging from 10^{-9} to 10^{-11} M affects AR in a dose dependent manner (Sengoku et al.,1996). In bovine spermatozoa PAF concentration of 100 μM was observed to be most optimal, as at this level, the AR detected by Giemsa stain, improved significantly without much loss of motility (Aravindakshan and Sharma, 1995).

In this research, the CTC was conducted to study the effect of PAF on capacitation and the acrosome reaction of stallion spermatozoa. Three main patterns of CTC fluorescence staining were identified. Since these patterns were the same as described in other species, the same nomenclature was used. For determining viability, spermatozoa were treated with H-33258 before CTC staining. Statistical analysis for capacitation indicated that 83% of sperm were characterized as live-capacitated after treatment with PAF (r^2= 0.81, P<0.01). Statistical analysis for the AR indicated that 20% of spermatozoa were characterized as live and acrosome reacted following treatment with PAF (r^2=0.20, P<0.01). Enhancement of spermatozoal motility at a PAF concentration ranging from 10^{-10} t0 10^{-13} M at 120 minutes was compatible with the CTC result for capacitation at the same time and levels of PAF. It will be of a benefit for future experiments to look at the lateral head displacement parameter that is indicative of hyperactivity, one of the characteristics of capacitation.

Progesterone induces the acrosome reaction in human (Wistrom and Meizel, 1993) and in porcine (Melendrez et al., 1994) spermatozoa acting through elevation of intracellular levels of calcium. Because the equine preovulatory follicle has been reported to secrete progesterone prior to 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. So as a follow up to the CTC results that was obtained in this research on capacitation, and the previous conclusion that suggest the ability of PAF at lower concentrations to induce capacitation, TEM was conducted to find the true acrosome reaction on spermatozoa that were capacitated by PAF and induced for acrosome reaction by progesterone. The spermatozoa acrosome reacted in response to progesterone, leading to the conclusion that capacitation of stallion spermatozoa had occurred by treatment with PAF.

PAF receptors were localized in the midpiece and proximal head of human spermatozoa by immunofluorescence (Reinhardt et al., 1999). The amount of PAF receptor mRNA was found to be higher in abnormal bull spermatozoa than that was found in normal spermatozoa (Roudebush et al., 2001a). 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 displayed a significantly reduced rate of capacitation as assessed by the spontaneous onset of the acrosome reaction in vitro (Wu et al., 2001).

PAF antagonists have been reported to inhibit sperm motility, acrosome reaction and fertilization, thus suggesting the presence of receptors for PAF (Roudebush, 2001). The immunofluorescence results in this research indicated that PAF receptors do exist in stallion spermatozoa, and Duncan's method indicated that their localization was more prominant in the post-acrosomal region as compared to the other regions of spermatozoa.

The post-acrosomal region contains the proximal centriol which plays a role in embryo development which was shown to be enhanced after fertilization of an oocyte with PAF treated spermatozoa (Sathanathan et al., 1996). The spermatozoal mitochondria

that are essential for spermatozoa motility are located in the midpiece. The flouresence intensity was more prominent at the post-acrosomal region, the neck and the midpiece of stallion spermatozoa suggesting a role for PAF at these locations.

Over all results in this research, suggest that exogenous PAF has the potential to binds to a specific receptor of stallion spermatozoa and induces signal transduction and the molecular changes of capacitation. Calcium influx is a requirement for the mammalian spermatozoa acrosome reaction, which is an exocytotic event occurring in the spermatozoal head prior to fertilization. During capacitation, the $[Ca^{2+}]_i$ is enhanced in the acrosomal region of the head and in the tail (Florman, 1994). The biological properties of PAF have indicated that the PAF molecule is involved in intracellular signaling in a variety of pathophysiological situations (Chao and Olson, 1993). Results in this study showed that PAF at lower concentrations was able to induce capacitation in stallion spermatozoa.

Travers et al indicated that the dose of PAF that was able to achieve half maximal calcium mobilization response in Raji lymphoblasts was 6.3 nM and they also indicated that PAF receptor antagonists CV-3988 and BN-52021 inhibited the PAF-induced calcium changes at doses that competed with PAF binding assays. The PAF antagonist FR-49175, also known as bisdethiobis (methylthio) gliotoxin, was shown to inhibit PAF induced rabbit platelets aggregation at IC_{50} of 8.4μM (Okamoto et al., 1986). Treatment of mouse spermatozoa with 10^{-5}M of PAF antagonist CV-3988 inhibited capacitation and the acrosome reaction completely (Sengoku et al, 1992). The involvement of PAF in ovulation in rats was tested by injection of a specific PAF antagonist BN52021 (isolated

from the Chinese tree Ginkgo biloba) and the reported median inhibitory concentration (IC_{50}) was 10^{-7} M (Akinwunmi et al, 1989).

Since differences in intracellular dye concentrations affect emission intensity, making accurate $[Ca^{2+}]_i$ calibration difficult, the flow cytometric approach using Fluo-3/Fura Red ratio developed by Novak and Rabinovitch (1994) was shown to exhibit much less variation with regard to these factors. This was the technique that was used in this research to investigate if the action of PAF involves changes in $[Ca^{2+}]_i$. The results indicated that the changes in $[Ca^{2+}]_i$ in response to PAF was dose dependent. Data in this study show that PAF induces capacitation and that PAF treatment induces changes in $[Ca^{2+}]_i$, therefore suggesting that the capacitation mechanism may involve an increase in $[Ca^{2+}]_i$. The PAF dose that was able to achieve half maximal calcium mobilization was 10^{-10} M PAF and this value is in agreement with Prescott et al (1990) suggesting that concentration of 10^{-9} M PAF almost always acts as an intracellular messenger. The PAF antagonist FR-49175 inhibited calcium release by PAF with an IC_{50} of $10^{-7.5}$M. Since elevation of the intracellular calcium was suppressed dose dependently by PAF-receptor antagonist, this may indicate that the elevation of the intracellular calcium induced by PAF is a receptor mediated process. Inhibition of fluorescence intensity with PAF antagonists FR-49175 (IC_{50}= 10^{-8}M) supports the above hypothesis.

Cloning and expression of the human leukocyte PAF receptor gene has shown the PAF receptor to be a member of the family of G-protein coupled receptors with phospholipase C as the effector (Kunz et al, 1992). Actions of PAF through binding to these receptors have been reported previously in other cells and tissues (Snyder, 1990; Gordon et al., 1989). Coupling of PAF to these receptors will activate GTPase activity

and also intracellular pathways that include turnover of inositole phosphate and modulation on intracellular calcium concentrations (Gordon et al., 1989). This work, combined with the results of the literature, supports the previously proposed mechanism of action of PAF: PAF binds to a specific receptor on the plasma membrane of the spermatozoa, causing the activation of a guanine nucleotide-binding (G) protein, which in turn activates phospholipase C. Phospholipase C causes the hydrolysis of a membrane phospholipid, phosphatidyl-inositol 4,5-bisphosphate (PIP2), which yields a water-soluble product, inositol-1, 4,5-trisphosphate (IP3), and a lipid, 1,2-diacylgycerol (DAG). IP3, and perhaps additional products of IP3 metabolism, then causes the release of calcium from intracellular stores, while DAG in conjunction with calcium ions activates protein kinase C (Valone, 1987). Calcium itself has a broad range of effects, activating a variety of enzyme systems, both as a cofactor and in conjunction with the calcium binding protein calmodulin (Rabinovitch and June, 1990). This pathway (phosphoinositide pathway) is also one of the pathways that are involved in the acrosome reaction (Figure 28). A role for kinase C in the acrosome reaction has been suggested and kinase C activity has been found in human spermatozoa (Zaneveld et al., 1991). Hydrolysis of PIP2 was found to take place in human spermatozoa when treated with acrosome reaction stimulators (Thomas and Meizel, 1989). Nevertheless, purification or characterization of PAF receptor at the molecular level would provide more information and hopefully fill the gaps and complete the suggested picture of PAF mechanism of action on spermatozoa and other cells as well.

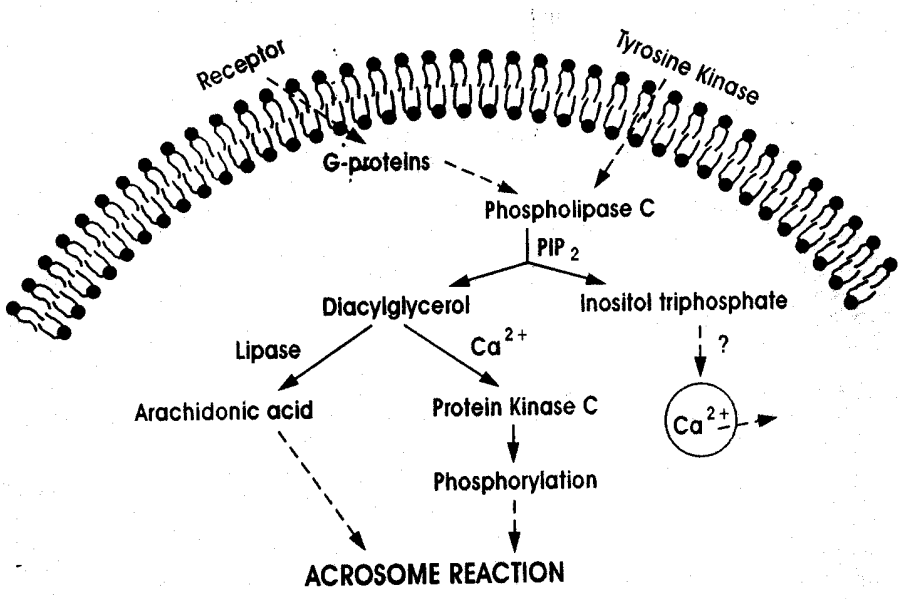


Figure 28: Proposed phosphoinositide second messenger pathway in human spermatozoa acrosome reaction (Zaneveld et al., 1991).

PLANS for FUTUR RESEARCH

In this research the effects of the PAF on stallion spermatozoal motility was evaluated, it will be beneficial for future works to evaluate the effect of the PAF on other motility parameters such as progressive velocity and lateral head displacement which is more indicative of hyperactivation that is associated with capacitation. The actual PAF concentration and stallion spermatozoal motility can also be investigated for any correlation. The concentration of PAF in motile and nonmotile spermatozoa may be different, and if nonmotile spermatozoa have more PAF that may suggest their inability to utilize it for motility.

The process of capacitation is not completely defined, therefore assays for capacitation rely upon the assessment of changes in membrane binding pattern of Ca^{+2} and the CTC was one of those assays and it was used by others and in this research. However treatment of stallion spermatozoa with PAF prior to fertilization will be more indicative to the PAF effect on capacitation, the acrosome reaction processes and embryo development as well. PAF may have a role in AI in mares that are bred close to ovulation by accelerating capacitation then fertilization. The changes in the PAF level in stallion spermatozoa incubated under capacitation conditions that lead to the AR can also be evaluated and correlated with capacitation. PAF acetylhydrolase (PAF - AH) was found in the seminal plasma and associated with sperm. PAF is degraded to lyso-PAF which is the inactive form of PAF by PAF-AH. For the future, it will be good to measure the amount of endogenous PAF and PAH-AH in stallion spermatozoa upon capacitation.

Determination of the presence/absence of the PAF receptor gene and measurement of the PAF content in spermatozoa from fertile and infertile stallions may be another

parameter that can be examined for predicting fertility. The PAF receptor belongs to the G-protein coupled receptor, but the signal transduction system remained unclear; thus, cloning, expression, and fuctional characterization of stallion spermatozoa PAF receptor cDNA will provide direct evidence to its role and the signal transduction events.

CONCLUSION

In vitro fertilization has had limited investigation in the stallion, the first paper reporting IVF of equine oocyte was published more than 12 years ago and only 11 additional studies involving standard IVF in the stallion have subsequently been reported (Hinrichs et al., 2002). As stated previously, one of the major obstacles to IVF in the horse has been the poor capacitation and induction of the AR, which seems to be a complex series of events rather than a discrete individual biochemical reaction. The major 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 spermatozoal penetration of equine oocyte is related to the difficulty to capacitate spermatozoa and then their inability to undergo the AR at the surface of the zona pellucida. Since the limited success in the 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. Without proper capacitation, spermatozoa are unable to initiate the acrosome reaction which is a prerequisite for fertilization. To add more insight about the effect of PAF on stallion spermatozoa, it will be also beneficial to treat spermatozoa with PAF prior to ART 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.

SUMMARY

Platelet activating factor (PAF) is an ether phospholipid. The ether phospholipids are ubiquitous and relatively minor components of animal cells; nevertheless they have been implicated in many if not all of the previously mentioned steps of the sperm-egg interaction. The exact mechanism of actions of PAF remain unclear, nevertheless its importance for normal reproductive functions has been investigated extensively by different researchers indicating that PAF has a significant role in reproductive physiology. In this research immunofluorescence demonstrated the presence of the PAF receptor on stallion spermatozoa to be most prevalent at the post-acrosomal region of the head followed by the midpiece and the neck. Exogenous PAF affects stallion spermatozoal motility, capacitation and the acrosome reaction (AR). Treatment of 10 stallion semen samples with 10^{-4} to 10^{-13} M PAF resulted in statistically significant differences in motility and capacitation respectively. The PAF concentrations ranging from 10^{-9} to 10^{-11} M was able to induce capacitation and at these concentrations the true AR was conducted by transmission electron microscopy (TEM) on the spermatozoa from 3 stallions. Incubation of spermatozoa with 10^{-7} to 10^{-12} M PAF for 2 minutes caused an increase in intracellular calcium in a dose dependent manner. PAF is thought to act via a specific receptor and specific receptor antagonists can inhibit PAF effects. Treatment of three stallion's spermatozoa with the PAF antagonist FR-49175 completely inhibited fluorescence intensity. The results indicated that equine sperm posses PAF receptors and

once PAF binds to these receptors then its effects on motility, capacitation and the AR is dose and time dependent.

Roudebush and colleagues (1990-2002) have documented the presence of PAF in human, boar, bull and rhesus monkey spermatozoa and further reported enhanced fertilization potential and embryo development of rabbit oocytes fertilized in vitro with PAF-treated spermatozoa, but little work has been done on equine spermatozoa.

FACILITIES and BUDGET

The work of this research has been conducted in the research laboratories of the Virginia-Maryland College of Veterinary Medicine, Blacksburg, Virginia. The majority of the work was conducted in the Center for Reproduction Excellence using Assisted Technology and Endocrinology (C. R. E. A. T. E). The financial support for this research has been provided by the Patricia Bonsall Stuart Awards for Equine Studies.

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APPENDIX 1

Composition of 0.2M Sodium Cacodylate (NaCaco) Stock

Use 42.8 gm of sodium cacodylate-trihydrate (cacodylic acid) with molecular weight of 214.02 in 1000 ml distilled water ($D.H_2O$).

Composition of 100ml fixative (5% Glut/3%Sucrose/0.1M NaCaco.,pH 7.6)

Step 1: To prepare 5% Glutaraldehyde (Glut). In 0.1M NaCaco. Take 10 ml of 50% Glut +40 ml $D.H_2O$+ 50 ml of 0.2 M NaCaco. Stock then mix solution

Step 2: Measure 3gm of sucrose and add above solution to 100 ml

Step 3: adjust pH to 7.6

Composition of 0.1M NaCaco.

Use equal parts of 0.2 M NaCaco stock and $D.H_2O$.

Composition of 0.1% Tannic acid in 0.1 M NaCaco. Buffer

Weight 0.1 gm tannic acid then add up to 100 ml of 0.1M NaCaco.

Adjust pH to 7.4

Composition of 100 ml of 1% Osmium tetroxide in 0.1 M NaCaco.

25 ml of 4% OsO_4 (4gm in 100 ml $D.H_2O$) + 25 ml $D.H_2O$ + 50 ml of 0.2 M NaCaco.

Composition of 0.1 M Phosphate Buffer

100 ml of 0.2 M phosphate buffer, 100 ml of $D.H_2O$, and 20 drops of 1% $CaCl_2$.

Composition of Hat media in 1000 ml $D.H_2O$

10.472 gm of Hat media, 0.2 gm of $CaCl_2$, 0.3 gm bovine serum albumin heat inactivated and 1.176 gm of $NaHCo_3$

APPENDIX 2

Content of PAF in 3 stallions as detected by RIA. Values are calculated as pM concentration per 10^6 spermatozoa (personal communication with Dr. W. Roudebush).

Stallion	pg/100 µl
A	< lowstd
B	125.75
C	> 3000
D	975.75

VITA

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- Pentoxyfillin treatment: High gravity centrifugation with cushion protection method for sperm selection and cryo-accumulation of severely oligo-spermic semen. Presented at the 48th annual meeting of the American Society for Reproductive Medicine, 1992.

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