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Map Kinases in Cynomolgus Monkey Sperm Hyperactivation

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ABSTRACT

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Mammalian sperm exhibit characteristic motility patterns, termed hyperactivated (HA) motility, associated with completion of capacitation. In cynomolgus monkey (*Macaca fascicularis*) sperm, this HA motility is dependent *in vitro* upon the addition of exogenous cyclic nucleotide mediators, caffeine and dibutyryl-cyclic adenosine monophosphate (dbcAMP). Previous reports have shown protein tyrosine phosphorylation to be an integral component of this caffeine- and cAMP-stimulated HA motility. This study investigated the involvement of the mitogen-activated protein (MAP) kinase-signaling cascade. Semen specimens were collected in Talp-HEPES medium from proven breeders via electroejaculation. After washing, sperm were incubated in the presence and absence of the MAP kinase kinase (MAPKK) inhibitor, PD-98059 for 90 minutes at RT. Sperm were transferred to Talp-bicarbonate medium and incubated with and without the sperm activators, caffeine (1mM) and dbcAMP (1mM) for 0.5h at 37°C and 5% CO_2. Proportion of sperm exhibiting hyperactivated motility was determined by computer assisted motion analysis (HTM-IVOS) using sorting criteria previously established in our laboratory. Tyrosine phosphorylation of sperm tail proteins was determined with the antiphosphotyrosine antibody, PY-20, by immunocytochemistry (ICC) and by immunoblotting to examine total proteins. Inhibition of the phosphorylation of MAP kinase was determined with ICC by assessing

the proportion of sperm exhibiting phosphorylated MAP kinase immunoreactivity and by immunoblotting. Treatment of macaque sperm with PD-98059 resulted in a significant decrease ($p<0.01$) in the amplitude of lateral head displacement (ALH), but no significant difference was noted in the curvilinear velocity (VCL) or linearity (LIN) compared to sperm stimulated with caffeine and dbcAMP. Treatment of macaque sperm with the MAPKK inhibitor, PD-98059, resulted in a dose dependent decrease in phosphorylated MAP kinase immunoreactivity with an inhibitory concentration producing 50% normal activity (IC_{50}) of 0.1μM. Similarly, PY-20 immunoreactivity decreased in a dose dependent manner with an IC_{50} of 0.1 μM and hyperactivated motility decreased in a dose dependent manner with an IC_{50} of 2 μM. However, while PD-98059 decreased MAP kinase phosphorylation from 69% ± 10 to baseline levels (14% ± 1), complete inhibition of tyrosine phosphorylation of sperm tails as evidenced by PY-20 ICC was not observed at those doses. Similarly, the proportion of sperm exhibiting hyperactivated motility did not decrease to baseline levels at the doses of PD-98059 tested. Immunoblotting detected a decrease in tyrosine phosphorylation of total proteins in a range of 40-120 kDa with PD-98059 treatment. This suggests MAP kinase is one, but not the exclusive upstream component in the signaling cascade of caffeine- and cAMP-stimulated hyperactivated motility in macaque sperm.

This thesis is dedicated to my parents for their love and continued support of my educational aspirations. This work is also your accomplishment and proof of your success in rearing me. Thank you for believing in me, motivating me, loving me and showing me that I am the only obstacle preventing me from attaining my goals.

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CHAPTER I

INTRODUCTION

Capacitation

Capacitation is defined as the sum of physiological events conferring fertilization competency on spermatozoa. Austin (1951, 1952) and Chang (1951, 1955) independently documented the first experiments showing capacitation of mammalian spermatozoa fifty years ago, however understanding the molecular basis for capacitation is still in progress. There are many biochemical and molecular changes that occur in the spermatozoa during capacitation.

The alterations occurring in sperm will be discussed briefly. First, the spermatozoa, like other cells in the body, must maintain ionic gradients across the plasma membrane. Currently, the presence of these changes and their functions are controversial, but research supports the following hypotheses. Hyne *et al* (1985) reported a significant intracellular increase in the concentration of sodium (Na^+) and a decrease in the concentration of potassium (K^+) in guinea pig spermatozoa, possibly due to a gradual inactivation of the Na^+/K^+-ATPase pump in an environment deficient in K^+. There is currently no agreement on the intracellular concentration of calcium (Ca^{2+}) during capacitation, but the general thought is there is no change (Yanagimachi, 1994). There are reports of increases in metabolism during capacitation in response to glycolytic activity and oxygen consumption after incubation in medium that supports capacitation *in vitro* or in the female reproductive tract (Fraser *et al*, 1988). Many very significant

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alterations occur in the head and tail plasma membranes. The nuclear membranes of most mature spermatozoa are stable due to extensive disulfide crosslinkages (Calvin, 1971). The stability of the membrane is increased during capacitation due to the loss of sulfhydryl radicals from nuclear proteins (LeLannou *et al*, 1985). The membranes of spermatozoa release and absorb proteins *in vivo* (Voglmayr and Sawyer, 1986) in response to the capacitating environment. Also, intramembranous particles, which are intrinsic proteins, have altered distribution in the head and tail regions during capacitation (Friend, 1974; Suzuki, 1989, 1990). Research has shown that cholesterol exerts significant effects on the characteristics of all biological membranes through regulation of orientation, fluidity and thickness (Chang, 1957). In the spermatozoa, the amount of cholesterol in the membrane is reduced after capacitation (Suzuki, 1989), while the amount of membrane phospholipids are increased, allowing the sperm tail flexibility to bend during movement. Physiological events assessed as endpoints of capacitation are the acrosome reaction, zona penetration, and hyperactivated motility. The action and purpose of the acrosome reaction is fusion of inner and outer acrosomal membranes with simultaneous dispersal allowing release of enzymes to aid in forming an opening in the zona pellucida through which the spermatozoa can swim and connect with the oocyte plasma membrane (Yanagimachi, 1988). Hyperactivation of the sperm tail will be discussed in detail at a later point. Figure 1 shows the proposed relationship between capacitation, acrosome reaction, hyperactivation and other fertilization events (Yanagimachi, 1989).

In Vivo Capacitation vs. *in vitro* Capacitation

In vivo capacitation normally occurs in the female reproductive tract in response

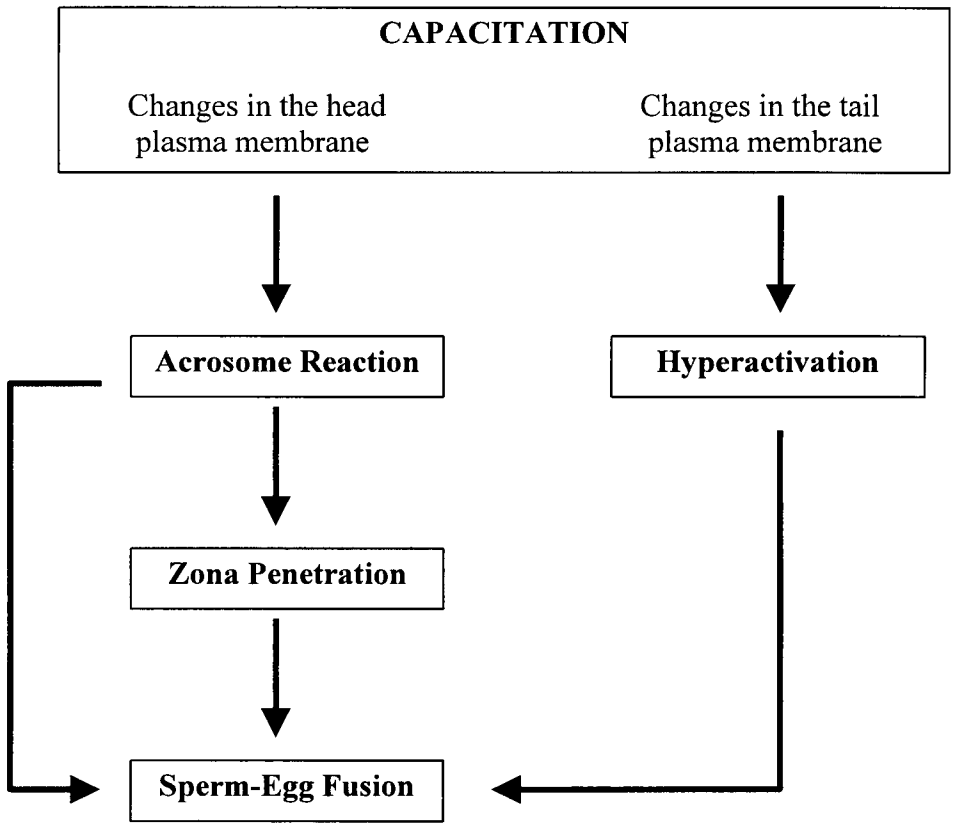

Figure 1. Possible relationship between sperm capacitation, acrosome reaction and hyperactivation. Proposed relationships between capacitation, hyperactivation, the acrosome reaction and other fertilization events in mammalian spermatozoa. (Yanagimachi, 1994, p. 221)

to humoral and cellular components. The actual site of capacitation will vary according to species. In species where spermatozoa are deposited in the uterus during coitus (i.e. hamster, pig, and horse), the spermatozoa complete most physiological components of capacitation in the lower segment of the Fallopian tube isthmus where fertilizing spermatozoa are stored (Smith and Yanagimachi, 1989, 1990; Hunter, 1991). In species where spermatozoa are deposited in the vagina at coitus (i.e., human and rabbit) capacitation may begin while spermatozoa pass through the cervical mucus (Gould *et al*, 1985; Katz *et al*, 1989; Lambert *et al*, 1985).

Early experiments with *in vitro* capacitation included the use of various biological fluids (i.e. oviductal and follicular fluid), but the complexity of the components made it difficult to determine exactly which component was involved in inducing capacitation and fertilization. Toyoda *et al* (1971) published the first successful report of *in vitro* fertilization of mouse oocytes using spermatozoa incubated in a chemically defined medium. Many scientists studying *in vitro* fertilization use fertilization as an indicator of completion of capacitation. The problem in using this endpoint is that while successful fertilization assumes the completion of capacitation, unsuccessful fertilization does not mean that capacitation did not occur (Yanagimachi, 1994). Some researchers use the acrosome reaction as an endpoint of capacitation and while spermatozoa usually capacitate before they acrosome react, there are some reagents that induce the acrosome reaction without capacitation (Yanagimachi, 1994). There is no homogeneity in capacitation among species due to different capacitating environments found *in vivo*, thus necessitating different capacitating environments *in vitro*.

Many factors affect *in vitro* capacitation. Capacitation of sperm is temperature

dependent (Flemming, 1985; Mahi, 1973) with an incubating range of 37°C to 38° depending upon the species. Some species require very specific media components for capacitation, while others do not require these special components. For example, human spermatozoa were once thought to be very difficult to capacitate and are now considered the "easiest" among mammals (Yanagimachi, 1994; Plachot, 1986). Length of incubation also varies among species. The minimum time for capacitation can be an hour or less in some species (mice, cats, humans) or up to several hours in others (rabbits) (Yanagimachi, 1994). Capacitation times vary, since all spermatozoa do not capacitate at the same time, labeled as synchrony, nor do spermatozoa from the same species capacitate in the same amount of time. There is no one defined medium for all species, however, the media commonly used for *in vitro* capacitation studies are modified Tyrode's and Kreb's-Ringer's solutions (Yanagimachi, 1994) supplemented with the appropriate energy sources (i.e. glucose, lactate, and pyruvate) and serum albumin. Although components of the media vary depending upon the species, it is suggested that serum albumin, calcium and bicarbonate play important regulatory roles in promoting capacitation in most, if not all mammalian species (Visconti and Kopf, 1998). Capacitation *in vitro* may occur without the addition of special reagents (termed spontaneous) in some species' spermatozoa (i.e., human) as evidenced by the functional changes that include hyperactivated motility and completion of the acrosome reaction (Yanagimachi, 1994).

Hyperactivation

Hyperactivation of spermatozoa was first recognized and documented in the golden hamster by Yanagimachi (1969). This hyperactivated motility is characterized by

vigorous, wide-amplitude flagellar beats with nonprogressive movement. The size, structure, beat frequency and response to surrounding medium vary depending upon the species. The physiological role of hyperactivation is not well defined, but there are many hypotheses. Katz (1989) reported that there are several main functions of hyperactivated motility that have been correlated through other documented findings. Hyperactivated motility enables the sperm to cover a greater area of the oviductal lumen, which will increase the probability of contact with the egg. The nondirectional motion seen in hyperactivated motility may assist the spermatozoa in detaching from the epithelium of the inner folds and crypts of the oviductal isthmus where some spermatozoa attach during *in vivo* capacitation (also documented by Demott, 1992; Smith, 1990). Others have reported that there is a correlation between the ability of the spermatozoa to undergo hyperactivated motility and fertilize zona-intact eggs (Yanagimachi, 1994). Suarez (1991 and 1992) also documented that the thrusting power generated by vigorous tail movements may facilitate the spermatozoa swimming through the viscous fluid of the oviduct and egg vestments, like the zona pellucida. The zona pellucida is a glycoprotein coat that surrounds and protects the oocyte and embryo from damage (Nichols, 1989) and assists in the provision of a special microenvironment for the developing embryo (Yanagimachi, 1994). The zona is the last barrier that spermatozoa must pass through in order to fertilize the egg, therefore, the vigorous movement and strong thrusting power of hyperactivation is paramount to successful fertilization.

The measurable parameters indicating hyperactivated motility in most species are an increase in curvilinear velocity, an increase in the amplitude of lateral head displacement, a decrease in linear movement and an increase in flagellar bending

amplitude (Burkman, 1990). These characteristics are measured from data of the sperm head positions using computer-assisted analysis, commonly performed with the Hamilton-Thorne Motion Analyzer. Amplitude of lateral head displacement (ALH) is the measure of side-to-side movement of the head, curvilinear velocity (VCL) measures the speed of the path traveled by the sperm (i.e. track speed), and linearity (LIN) is a ratio of progressive velocity (also referred to as straight-line velocity, VSL) divided by VCL (Aitken, 1990). The amplitude of lateral head displacement is the single most important criterion that determines the success of sperm-cervical mucus interaction (Aitken, 1990), thus proving the importance of this measurement in any fertility study involving sperm hyperactivation. Hyperactivation is a reversible event both *in vivo* and *in vitro*, meaning that once it has been achieved, it is possible to stop and return to a nonhyperactivated state. This phenomenon may be dependent upon the levels of calcium in the sperm intracellular milieu (Suarez and Dai, 1995).

Role of cAMP in Capacitation and Hyperactivation

Cyclic adenosine 3',5'-monophosphate (cAMP) is an intracellular second messenger normally activated in response to an extracellular stimulus. The term second messenger means an external stimulus (i.e. hormonal, nervous, etc.) causes an increase or decrease in the messenger, which in turn, stimulates a target enzyme (Fruton, 1999; Price and Stevens, 1999). Elevation of intracellular cAMP causes a cascade of events through stimulation of a cAMP-dependent kinase and phosphorylation of a specific target protein to alter the behavior of a specific intracellular protein. Kinases are a class of enzymes that phosphorylate specific amino acid residues within proteins (Copeland, 2000). In spermatozoa, cAMP appears to regulate the initiation and maintenance of sperm motility

with subsequent stimulation of protein phosphorylation. The plasma membrane of spermatozoa experience many changes in intracellular pH and ionic concentrations (Tash, 1990), which are believed to be catalysts for a chain of events leading to increased activity of adenylate cyclase, an enzyme that catalyzes the formation of cAMP using ATP as a co-factor (Fruton, 1999). The increased levels of cAMP could also be caused by decreased enzyme activity of phosphodiesterase, which inactivates cAMP by catalyzing its hydrolysis to 5'-AMP (Fruton, 1999). Receptors on the surface of the membrane chemically react with ligands stimulating G-protein, a plasma membrane component made of three subunits linking seven transmembrane domain receptors to proteins that induce changes in cellular functions (Fruton, 1999; Greenspan and Strewler, 1997). The inactive form of this receptor protein is guanosine diphosphate (GDP), which when stimulated by a specific membrane receptor becomes the more highly energetic guanosine triphosphate (GTP), stimulating a number of effector proteins and mechanisms like adenylyl cyclase, calcium channels and potassium channels (Greenspan and Strewler, 1997). This activation opens intramembrane calcium channels allowing an influx of calcium believed to stimulate adenylate cyclase to increase intracellular levels of cAMP. The changes believed to mediate the stimulation of mammalian sperm adenylate cyclase are movement of calcium and bicarbonate (Visconti, 1990; Garty, 1987; Hyne, 1979; Gross, 1987; Kopf 1984 and Okamura, 1991). Although the exact mechanism and function of calcium continues to elude researchers, Suarez and Dai (1995) documented that the sperm tail is the main or only site of calcium influx for hyperactivation. The increased level of cAMP stimulates the enzyme, protein kinase A, which activates a tyrosine kinase to phosphorylate tyrosine residues of an axonemal protein, allowing the

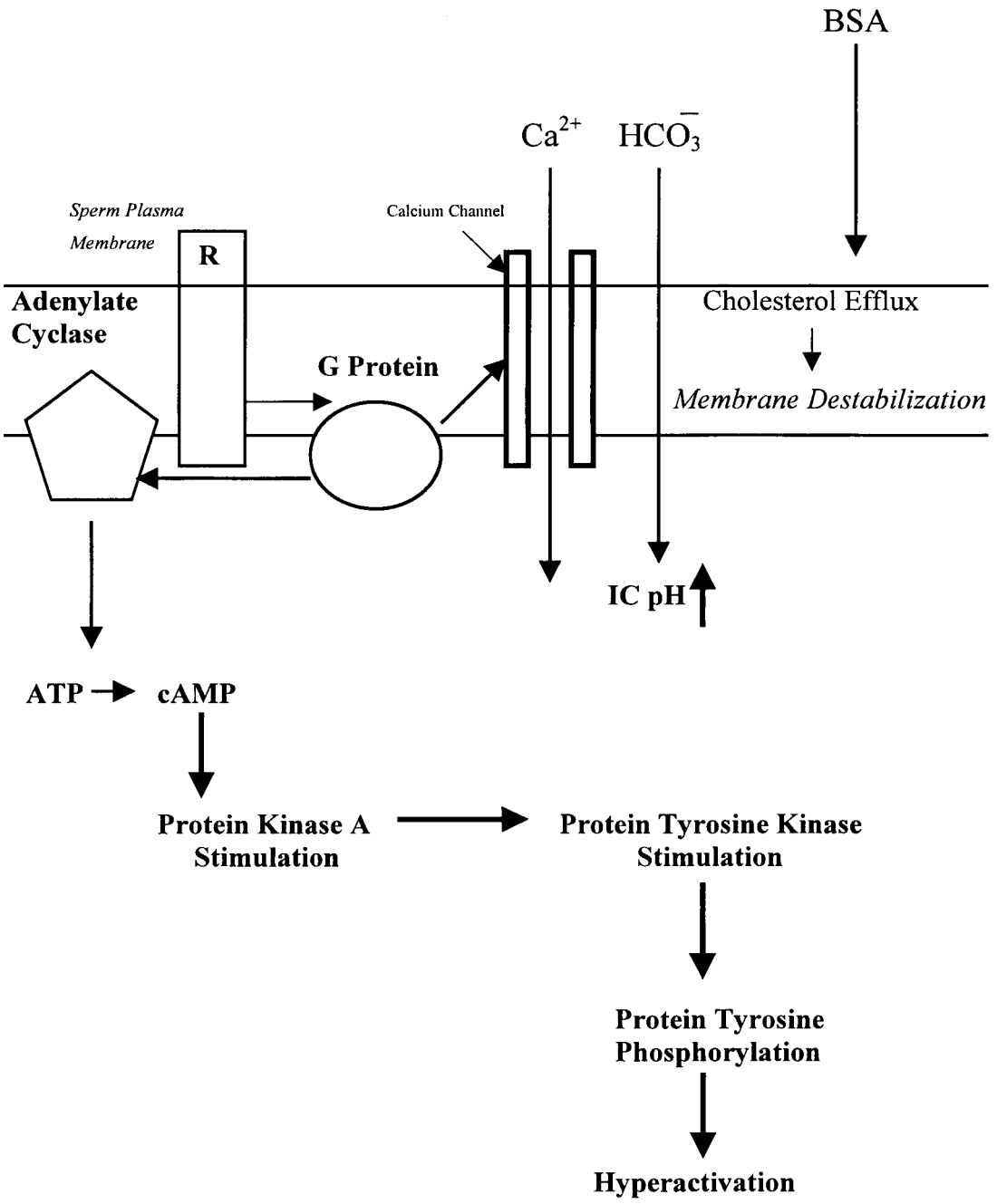

Figure 2. Pathway Leading to Capacitation and Tyrosine Phosphorylation via a cAMP-Stimulated Pathway. Proposed intracellular events in the spermatozoa depicting the cAMP-stimulated pathway leading to Protein tyrosine phosphorylation and hyperactivation of the sperm tail. Shows stimulation of the calcium channel and adenylate cyclase via the G-protein. R = Receptor; IC = Intracellular

axoneme to slide and bend (Morisawa, 1990) as depicted in Figure 2. Protein kinase A (PKA) is an enzyme activated by extracellular signals that cause changes in the concentration of intracellular second messengers (i.e. cAMP) (Cooper, 1999) and phosphorylates a variety of serine/threonine residues in proteins (Greenspan, 1997). PKA is inactive in the absence of second messenger activity (Cooper, 1999).

Protein Tyrosine Phosphorylation in Sperm Motility

Tyrosine phosphorylation is another intracellular regulatory component of sperm hyperactivated motility. The phosphorylation of tyrosine residues of proteins in the fibrous sheath, along with changes in membrane flexibility leads to decreased stiffness enabling the flagella to generate greater bends producing hyperactivated motility (Si and Okuno, 1999). The composition of capacitating media is important in achieving tyrosine phosphorylation and hyperactivation *in vitro*. Serum albumin, thought to remove cholesterol from the plasma membrane (Cross, 1998; Langlais, 1985; Davis, 1979; Go, 1985), is an integral component. Addition of albumin leads to a change in the fluidity of the membrane allowing bicarbonate and potassium molecules to move across the membrane, stimulating G-protein and eventually activating the cAMP pathway (Figure 2). Also of importance is the addition of calcium, which has not been well defined, but studies have shown that it has a role in signal transduction, as previously mentioned, through stimulation of adenylyl cyclase and inhibition of phosphodiesterase, the enzyme responsible for the breakdown of cAMP to 5'AMP. The presence of bicarbonate leads to an increase in intracellular pH in the sperm cell (Cross, 1997; Uguz, 1994; Zeng, 1996) and may also assist in regulation of cAMP metabolism in the sperm cell through the stimulation of adenylyl cyclase (Visconti, 1990; Okamura, 1985; Garty 1983). Tyrosine-

phosphorylated proteins are localized to the principal region of the tail and a small region in the neck of the sperm. Previous studies of tyrosine phosphorylation in other species have shown these proteins to range from 40-120 kD (reviewed by Visconti and Kopf, 1998).

Mitogen-Activated Protein (MAP) Kinases

Mitogen activated protein (MAP) kinases are a group of serine-threonine kinases activated in response to extracellular stimuli through a G-protein coupled receptor (Lopez-Ilasaca, 1998) to activate MAP kinase cascade (shown in Figure 3). The MAP kinase cascade can also be activated through protein kinase C with the phosphorylation of Raf-1 (member of Raf family of serine/threonine kinases) or through nucleotide exchange on p21Ras (member of the Ras family of proteins). Both are members of the GTP-binding proteins (Busca, 2000). The cascade of events in somatic cells begins with the activation of Ras, recently linked to cAMP-dependent activation of MAP kinases in melanocytes (Busca *et al*, 2000). Raf protein is normally activated in response to Ras, but is inhibited by a substance, possibly cAMP or PKA, and will not activate the MAP kinase cascade. This finding was correlated by Vossler *et al* (1997) who showed the initiation of the MAP kinase-signaling cascade through activation of Ras followed by stimulation of B-Raf (Lange-Carter *et al*, 1993; Vaillancourt *et al*, 1994; Bokemeyer *et al*, 1996). It is important to note that MAP kinases are highly selective activators of their targeted proteins by phosphorylation of both threonine and tyrosine regulatory sites (Bokemeyer *et al*, 1996).

MAP kinases function in protein kinase cascades to control cellular responses such as DNA synthesis through the cell cycle, cell proliferation and differentiation (Cano,

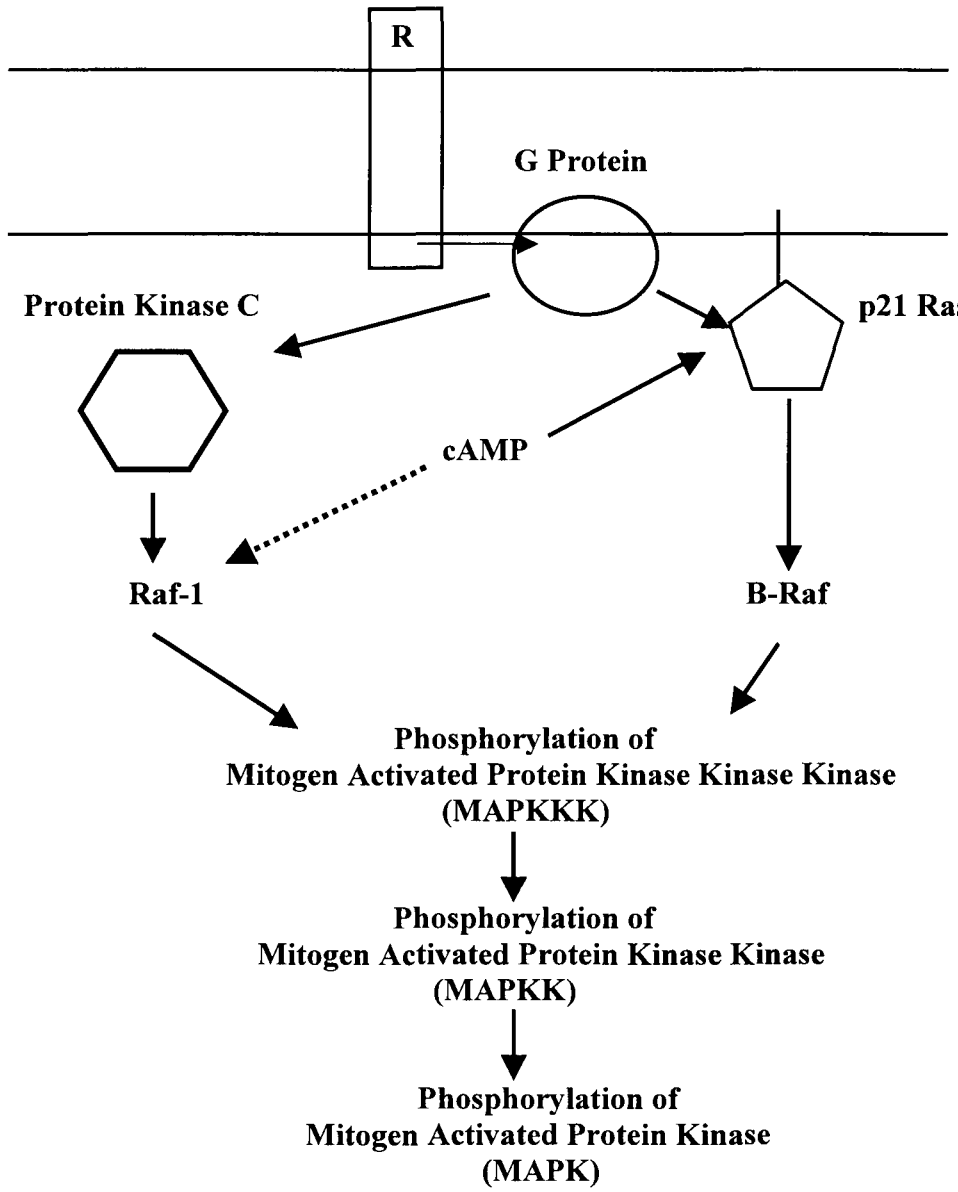

Figure 3. Events in the MAP Kinase-Signaling Cascade. Initiation of cascade at stimulation of G-protein showing at least two possible sites of activation through B-Raf and Raf-1. Positive relationships are denoted by solid lines, while negative relationships are denote by the dashed line.

1995, Cohen, 1997; Bokemeyer *et al*, 1996). There are three subfamilies of MAP kinases: (1) extracellular signal-regulated kinases (ERK), (2) SAPK (stress-activated protein kinase, also called c-jun N-terminal kinase: JNK), and (3) p38 kinase. MAP kinases were shown to combine with cAMP as a link to regulate biological responses (Houslay and Kolch, 2000) and Camarillo (1997) documented the tyrosine phosphorylation of a MAP kinase, ERK, in the rat. The significance of the MAP kinase cascade relates to the spermatozoa since the presence and biological activity of p21 Ras was shown in human spermatozoa (Naz, 1992). Also, recent research showed MAP kinases in human spermatozoa as a mediator of the acrosome reaction (Luconi *et* al, 1998).

Capacitation of Macaque Sperm

With the close phylogenetic relationships to man, nonhuman primates provide a unique model to study many areas of human reproductive physiology. There was a need to show the success of *in vitro* fertilization in a species closely related to humans using the nonhuman primate cynomolgus monkey of the genus *Macaca*. Boatman and Bavister (1984) chose this nonhuman primate model for several reasons. Its overall reproductive physiology is comparable to humans and the social behavior and mental development are similar. The cynomolgus monkey was previously studied for embryology and postnatal development (Harlow and Mears, 1979) followed by the discovery that spermatozoa from this species have unique requirements for capacitation *in vitro* (Marston and Kelly, 1968). Spermatozoa from the cynomolgus monkey (*Macaca fascicularis*) and other species of the genus *Macaca* exhibit unique requirements for the completion of capacitation, capacitation-related hyperactivated motility to complete *in vitro* fertilization. Although

the exact mechanism for the induction of capacitation-related hyperactivation *in vivo* has not been determined, *in vitro* cynomolgus monkey sperm must receive exogenous stimulation with the sperm activators, caffeine and dibutyryl (db)-cAMP (Boatman and Bavister, 1984). Dibutyryl cAMP is an exogenous cyclic nucleotide that is unresponsive to endogenous phosphodiesterase actions. Caffeine is a phosphodiesterase inhibitor used to further increase endogenous cAMP. Zona pellucida interaction is another endpoint utilized to demonstrate successful completion of capacitation, and Mahony *et al* (1996) documented that the presence of these activators is a requirement for zona penetration *in vitro*. Protein tyrosine phosphorylation is an integral component in macaque sperm caffeine- and dbcAMP-stimulated hyperactivation (Mahony and Gwathmey, 1999). Also, in cynomolgus monkey sperm, hyperactivation occurs under different stimulating conditions than induction of the acrosome reaction (VandeVoort *et al*, 1992, 1994). Since these capacitation-dependent events are separate, the intracellular signaling pathways can be studied independently.

In this present study, the objective was to further define the signaling cascades integral to hyperactivation. Specifically, the involvement of MAP kinase in cAMP-driven hyperactivated motility and protein tyrosine phosphorylation was examined. In other words, we examined the interrelationship between the cAMP-stimulated pathway and the MAP kinase pathway (Figure 4). The cynomolgus monkey sperm model was chosen due to the unique requirement of exogenous stimulation with caffeine and dbcAMP. The MAP kinase kinase inhibitor, PD-98059, was utilized since it blocks the phosphorylation of MAP kinase. PD-98059 is a noncompetitive, light-sensitive inhibitor *in vitro* and is believed to act as a highly specific inhibitor of the activation of MAP

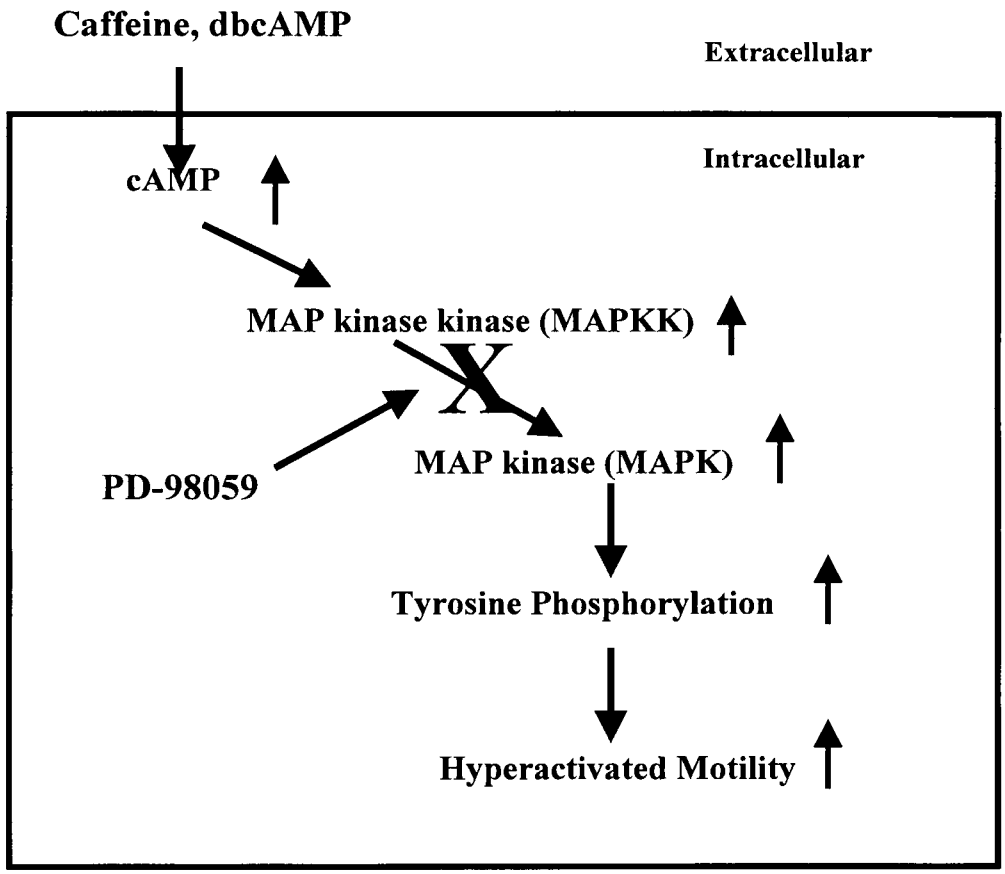

Figure 4. Proposed Intracellular Signaling Components in a Sperm Cell Leading to Hyperactivated Motility. Proposed events in the sperm cell for this experiment beginning with the introduction of the sperm activators, caffeine and dbcAMP. This pathway includes the point of action for the MAPKK inhibitor, PD-98059, the initiation of tyrosine phosphorylation and ends in hyperactivation of the sperm tail.

kinase *in vivo* at an IC_{50} of 4 μM (Alessi *et al*, 1995). The dose dependent effect of PD-98059 was examined on capacitation-dependent caffeine- and dbcAMP-stimulated hyperactivated motility, tyrosine phosphorylation and phosphorylation of MAP kinase in sperm tail proteins by immunocytochemistry and of total proteins by immunoblotting.

CHAPTER II

MATERIALS AND METHODS

Animals

Adult proven breeder male cynomolgus monkeys (*Macaca fascicularis)* (n = 15) weighing 5-8 kg were used in this study. Monkeys were housed in individual cages with a room temperature of 22°C and 12 light:12 dark, and fed a diet of commercially available monkey chow and water *ad libitum.* Before these studies were initiated, protocols were approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School and were in accordance with the *Guiding Principles for the Care and Use of Research Animals.*

Sperm Processing

Semen specimens were collected by electroejaculation directly into 1.0 mL Tyrode's albumin lactate pyruvate (Talp)-HEPES media supplemented with 0.3 % bovine serum albumin (BSA) pre-equilibrated to room temperature (RT) (Bavister *et al,* 1983). Sperm were washed twice with Talp-HEPES medium supplemented with 0.3% BSA at 300 x g for five minutes. After the second wash, each specimen was split and washed in increasing doses (0-3μM) of the MAPKK inhibitor, PD-98059, in Talp-HEPES. The sperm pellet was loosely dislodged and overlaid with Talp-HEPES with and without PD-98059 to allow for the collection of the motile sperm fraction. Following a 90-minute incubation at room temperature in the dark since PD-98059 is light sensitive. The motile sperm fraction was collected in the overlaid medium and motion characteristics of each control or treated specimen assessed by computer-assisted motion analysis.

Sperm Activation with Caffeine and dbcAMP

The motile fraction of sperm was washed by centrifugation and the supernatant replaced with Talp-bicarbonate medium supplemented with 0.3% BSA (in increasing doses of PD-98059), pre-equilibrated overnight at 37°C and 5% CO_2 in water-saturated air. Sperm were induced to hyperactivate with a combination of caffeine (1mM) and dbcAMP (1mM) (Boatman and Bavister, 1984) at 37°C and 5% CO_2 in water-saturated air for 0.5 hr after which assessments of motion characteristics for control and treated sperm groups were again completed.

Assessment of Sperm Motility

Sperm samples were analyzed for motility by the HTM-IVOS (Hamilton-Thorne Research, Danvers, MA) as described previously for human sperm (Mahony *et* al, 1988) and modified for assessment of macaque sperm (Mahony *et al*, 1993 and Mahony *et al*, 1999). The changes take into account the increase in velocity observed in monkey sperm compared to that of human sperm. All samples were equilibrated to and assessed at 37°C to allow for standardization between treatments. The pertinent settings used during this assessment were: frames acquired = 30; frame rate = 60 Hz; minimum contrast = 80; minimum cell size = 5 pixels; "slow cells" were accepted as motile. At the outset of each experiment, the verified settings permitted accurate differentiation of motile sperm vs. nonmotile sperm or debris by utilizing the "playback" option. During "playback", the motions of sperm in the previous field were replayed; a green dot was located over the head of all motile sperm for each frame and a red dot was positioned over the head of nonmotile sperm. When errors were detected, the settings were adjusted until the problem was corrected. Using computerized motion analysis, the following motion

parameters were compared among the different sperm groups: % motility; VCL (derived from all 30 head positions); VSL (based on the first and last head positions only); ALH; and LIN.

Tyrosine-phosphorylated Protein Immunoreactivity

For the assessment of phosphorylated tyrosine residues in macaque spermatozoa, control and treated groups of sperm were washed by centrifugation with sperm fixative containing 2% aprotinin, 2% leupeptin, 2% pepstatin, 0.39% benzamidine, 1μM sodium orthovanadate and 0.5% sodium azide in Dulbecco's phosphate-buffered saline (PBS) (GibcoBRL, Life Technologies, Grand Island, NY). Sperm were washed twice by centrifugation for 5 minutes at 300 × g. Washed sperm were air-dried onto Teflon coated spot slides, methanol-fixed, and stored at -70°C until evaluated. When immunocytochemistry was complete, sperm were equilibrated to room temperature and washed with PBS. Nonspecific binding sites were blocked with PBS supplemented with 1% BSA. Control and treated sperm were next incubated for 1 hour with the antibody PY-20 (a monoclonal antibody raised against phosphorylated tyrosine residues on proteins) conjugated with fluorescein isothiocyanate (FITC; 1μg/mL) (Calbiochem, LaJolla, CA). After washing, antiquench solution (Mahony and Gwathmey, 1999) was placed on each spot and a cover slip placed on the slide. Sperm were visualized at a magnification of 600× by epifluorescence microscopy. A minimum of 100 sperm per control or treated group was evaluated.

MAP Kinase Protein Immunoreactivity

Control and treated sperm were also examined for localization of phosphorylated MAP kinase immunoreactivity. The same procedure previously utilized for preparation

of spot slides in evaluation of tyrosine phosphorylation was followed. Once nonspecific sites were blocked with SuperBlock® Blocking Buffer in TBS (Pierce, Rockford, Ill) supplemented with 0.05 % Tween-20, according to manufacturer's guidelines, sperm were incubated for 24h at 4°C with an antibody raised against phosphorylated MAP kinase and immunoreactive with a 42-44kD protein, Phospho-p44/42-MAPK antibody (50 μg/mL) (Cell Signaling Technology, Beverly, MA). After rinsing unbound antibody, the sperm were incubated with FITC-conjugated goat α-rabbit IgG for 1h at RT. Sperm were again rinsed free of unbound antibody, antiquench solution was added, and a cover slip placed on the slide. Sperm were visualized at a magnification of 600× by epifluorescence microscopy. A minimum of 100 sperm per control or treated group was evaluated.

Western Blot Analysis

Following assessment of sperm motion characteristics, control and treated sperm were diluted in Talp medium supplemented with 1mM sodium orthovanadate that had been pre-equilibrated overnight at 37°C and 5% CO_2. Each sperm group was sonicated for 30 sec at a dial setting of 2.5 (Fisher Model 60 Sonic Dismembrator, Pittsburgh, PA), washed by centrifugation and placed in protein extraction buffer (0.5M Tris, glycerol, 10% SDS, and 0.01% bromophenol blue) without mercaptoethanol at a concentration of 5 million sperm/10 μL for one hour at RT. Following protein extraction, the samples were centrifuged at 13,000 × g and the proteins in each supernatant recovered. Each supernatant was diluted with an equal volume of extraction buffer with 2× mercaptoethanol. Control and treated sperm groups and rainbow molecular weight markers (Amersham Life Science) were incubated at 95°C for 5 minutes prior to loading

on a 10% SDS-PAGE mini gel. The presence of protein was confirmed by staining gels with GELCODE® Blue Stain Reagent (Pierce; Rockford, Illinois). Proteins were transferred to a nitrocellulose membrane (Micron Separations, Inc., Westborough, MA) and nonspecific sites blocked overnight at 4°C in Pierce SuperBlock® in PBS (1:1, v/v) supplemented with 0.5% Tween-20.

After washing, transferred proteins were immunostained for the presence of phosphorylated tyrosine residues with horseradish peroxidase (HRP) conjugated PY-20 (Zymed, San Francisco, CA) followed by treatment with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The immunoreactive protein bands were visualized by exposure to x-ray film for 30 sec to 1 minute. For detection of MAP kinase immunoreactivity, each membrane was stripped of PY-20 using ImmunoPure® Elution Buffer (Pierce, Rockford, Ill) at RT for 2h. After washing, the nonspecific sites were blocked for this portion of the experiment with SuperBlock® Blocking Buffer in TBS according to manufacturer's guidelines (Pierce, Rockford, Ill) supplemented with 0.1% Tween-20 overnight at 4°C. Sperm proteins were reprobed with Phospho-p44/42-MAPK, then HRP-goat α-rabbit IgG (Zymed, San Francisco, CA), visualized by chemiluminescence and detected via exposure to x-ray film.

Statistical Evaluations

The data was analyzed by an analysis of variance (ANOVA) followed by Bonferroni's post-test or chi-square analysis. $P \leq 0.05$ was considered significant. The results are expressed as the mean ± standard error of the mean.

CHAPTER III

RESULTS

Preliminary Experiment

Initial experiments were conducted to determine potential deleterious effects of the MAPKK inhibitor, PD-98059 on baseline (no caffeine and dbcAMP) levels of sperm motion characteristics, including hyperactivated (HA) motility and protein tyrosine phosphorylation, and the phosphorylation of MAP kinase proteins. There was no significant difference between baseline levels of hyperactivated motility (11 ± 6), tyrosine phosphorylation (11 ± 4) or the phosphorylation of MAP kinases (18.6 ± 6) when compared to the same values in sperm pretreated with PD-98059 (3 μM): HA motility (12 ± 3), tyrosine phosphorylation (21 ± 4), and phosphorylation of MAP kinases (14 ± 1). Therefore, in order to have a maximal amount of sperm available in each control and test group, the remaining experiments were conducted using the following three sperm groups: 1) no treatment with PD-98059, stimulation with caffeine and dbcAMP, 2) treatment with PD-98059 and stimulation with caffeine and dbcAMP, and 3) treatment with PD-98059 and no stimulation with caffeine and dbcAMP. Subsequent experiments did not include the experimental group in which there was no treatment and no caffeine and dbcAMP stimulation. The results from these experiments will be presented according to these guidelines, except in the dose response experiments where there are more than three treatment groups.

Determination of Hyperactivated Motility Using Sorted Criteria

We first determined that, in our hands, macaque sperm achieved the motion characteristics associated with HA motility. Macaque sperm were induced to

hyperactivation with caffeine and dbcAMP and compared against the baseline values (no stimulation with caffeine or dbcAMP). These motion characteristics compared between treated and control (baseline) groups included ALH, VCL, LIN, and the resulting HA motility values. Sperm treated with caffeine and dbcAMP resulted in a significant increase in ALH (7 ± 0.5) compared to baseline sperm (5 ± 0.4) ($p<0.01$). The same effect was noted in VCL for sperm treated with the activators (253 ± 11) compared to baseline levels (180 ± 13) ($p< 0.01$). Sperm treated with the activators also experienced a significant decrease in LIN (44 ± 2) compared to baseline sperm (63 ± 3) ($p<0.01$). Finally, sperm treated with the activators experienced a significant increase in HA motility (51 ± 4) compared to baseline sperm (12 ± 3) ($p<0.01$).

Effect of PD-98059 on Motion Characteristics

Treatment with PD-98059 (3 μM), resulted in a significant decrease in ALH compared to control sperm treated with caffeine and dbcAMP ($p<0.05$) (Figure 5) and there was a significant increase in ALH in sperm treated with PD-98059 (8 ± 0.5) compared to baseline levels. There was no significant difference in VCL in sperm pretreated with PD-98059 and caffeine and dbcAMP (205 ± 14) compared to baseline levels (180 ± 13) ($p<0.01$) (Figure 6). There was also no significant difference in VCL in sperm treated with PD-98059 compared to the sperm group treated with caffeine and dbcAMP (Figure 6). There was no significant difference in LIN in sperm treated with PD-98059 (47 ± 2) compared to the sperm group stimulated with caffeine and dbcAMP (Figure 7).

Effect of PD-98059 on Hyperactivated Motility

We next examined macaque sperm for the involvement of the MAP kinase

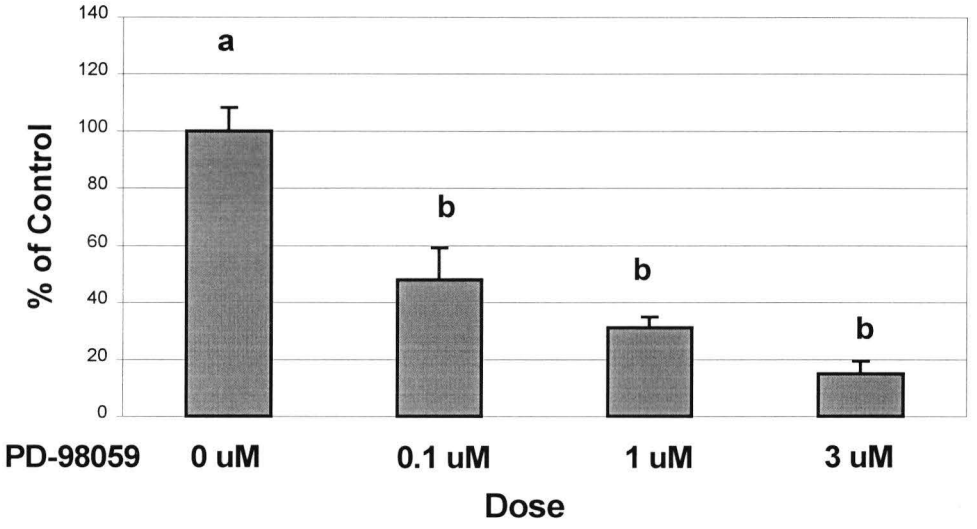

Figure 5. Effect of the MAPKK Inhibitor, PD-98059, on the amplitude of lateral head displacement (ALH). There was a significant increase in ALH in sperm stimulated with caffeine and dbcAMP compared to baseline sperm ($p<0.01$). Treatment with the MAPKK inhibitor resulted in a significant decrease in ALH compared to sperm stimulated with caffeine and dbcAMP ($p<0.01$), however it did not decrease ALH to baseline levels. (Note: a, b, and c denote significantly different groups).

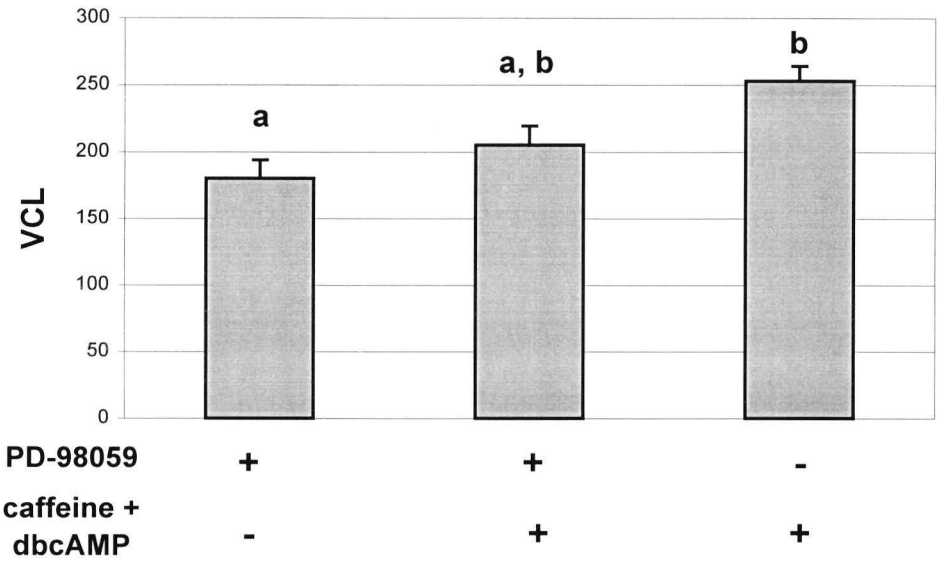

Figure 6. The effect of the MAPKK Inhibitor, PD-98059, on the curvilinear velocity (VCL). Sperm stimulated with caffeine and dbcAMP experienced a significant increase in VCL compared to baseline sperm ($p<0.01$). There was no significant difference between sperm treated with PD-98059 and these caffeine and dbcAMP stimulated sperm groups. There also was no significant difference between sperm treated with PD-98059 and baseline sperm groups. (Note: a and b denote significantly different groups).

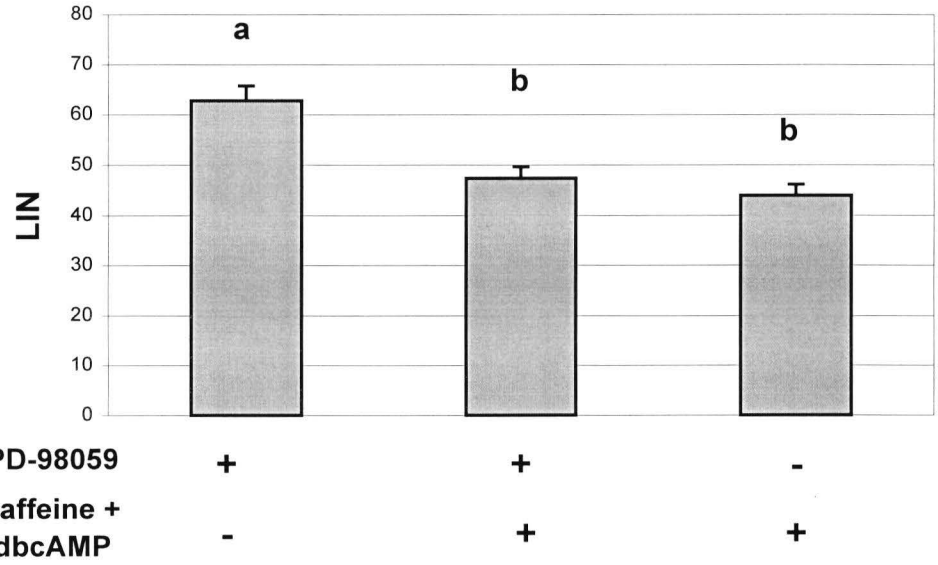

Figure 7. The effect of the MAPKK inhibitor, PD-98059, on linearity (LIN). Sperm groups stimulated with caffeine and dbcAMP experienced a significant decrease in linearity ($p<0.01$). There was no significant difference between sperm stimulated with caffeine and dbcAMP and sperm treated with PD-98059 and caffeine and dbcAMP. There also was no significant difference between the sperm group treated with PD-98059 and the baseline sperm group. (Note: a and b denote significantly different groups).

systems by determining the effect of PD-98059 on HA motility. In the first experiments, the effect of PD-98059 on caffeine and dbcAMP stimulated macaque sperm hyperactivated motility was examined. The addition of PD-98059 (3μM) to caffeine and dbcAMP treated sperm resulted in a significant decrease in HA motility from 51% ± 4 to 29% ± 4 (Figure 8). This inhibition of HA motility by PD-98059 was not to baseline levels (12 ± 3).

The next set of experiments examined the dose dependent effects of PD-98059 on macaque sperm HA motility. Increasing doses of PD-98059 (0 μM to 3 μM) were added to baseline and caffeine and dbcAMP groups. There was a significant dose-dependent effect of the MAPKK inhibitor on sperm HA ($p<0.01$) with an inhibitory concentration producing 50% normal activity (IC_{50}) of 2μM (extrapolated from dosage data) (Figure 9).

Effect of PD-98059 on Tyrosine Phosphorylation of Macaque Sperm Proteins

We next examined the role of the MAP kinase system on protein tyrosine phosphorylation, an integral intracellular signaling component of macaque sperm HA. Immunoctyochemical analysis of tyrosine phosphorylation of sperm proteins with the anti-phosphotyrosine antibody, PY-20, localized tyrosine labeling to the principal piece of the tail region and a small region of the neck in methanol-fixed sperm groups (Figure 10). As expected, there was a significant increase in phosphotyrosine immunoreactivity observed in sperm treated with caffeine and dbcAMP compared to baseline levels ($p<0.01$) (Figure 11). Treatment with PD-98059 (3 μM) resulted in a significant decrease in tyrosine phosphorylation of sperm tail proteins from 70% ± 6 to 42% ± 7 ($p<0.01$) (Figure 11).

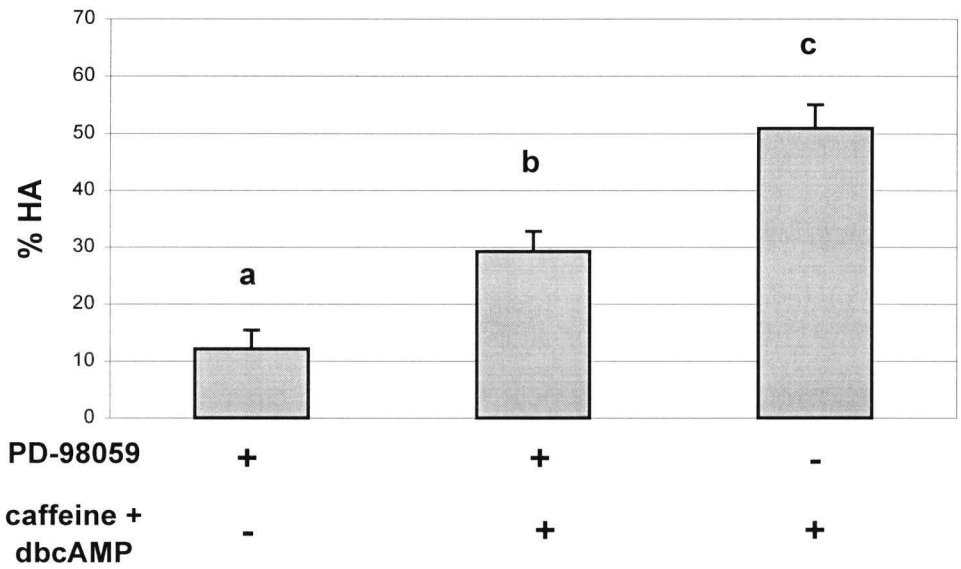

Figure 8. The effect of the MAPKK Inhibitor, PD-98059, on caffeine and dbcAMP-stimulated hyperactivated (HA) motility. There was a significant increase in caffeine and dbcAMP stimulated hyperactivation compared to baseline sperm ($p<0.01$). The addition of PD-98059 resulted in a significant decrease in hyperactivation, but this decrease was not to baseline levels. (Note: a, b, and c denote significantly different groups).

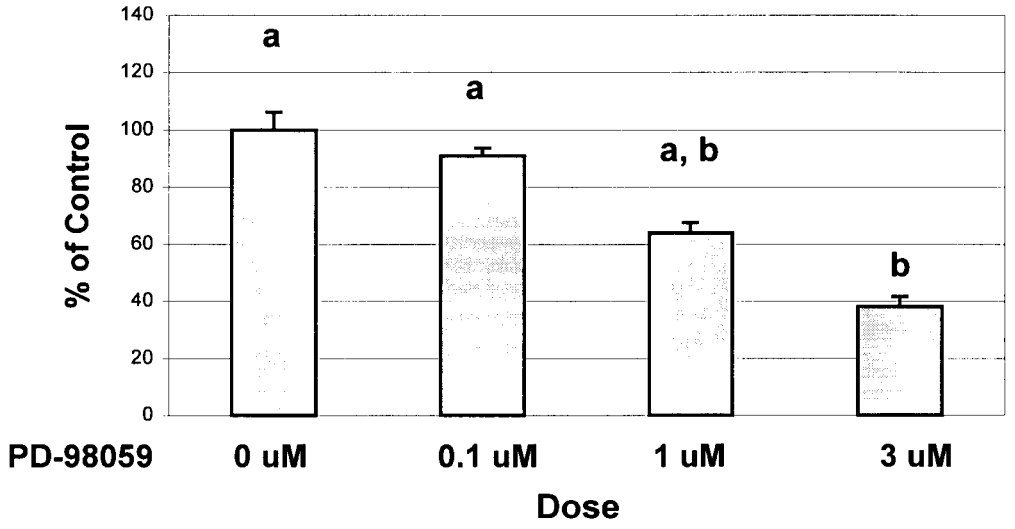

Figure 9. Dose Dependent Effects of the MAPKK inhibitor, PD-98059, on caffeine and dbcAMP-stimulated macaque sperm HA motility. There was a significant decrease in sperm HA motility when treated with 1-3 μM PD-98059 ($p<0.01$) with an $IC_{50} = 2$ μM (Determined by extrapolation of data). (Note: a and b denote significantly different groups).

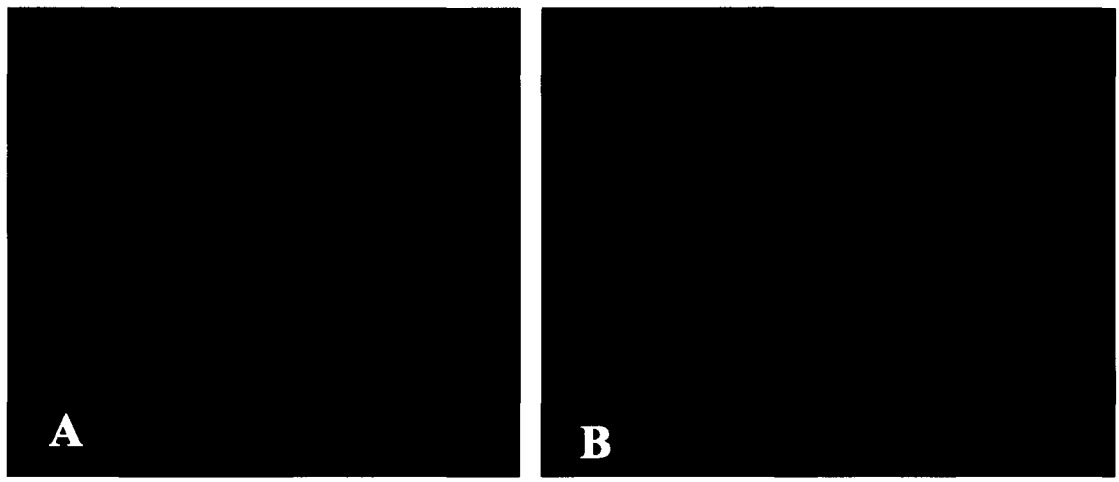

Figure 10. Effect of Macaque Sperm Treatment with the Activators, Caffeine and dbcAMP, on Protein Tyrosine Phosphorylation of Sperm Tail Proteins. Immunostaining was completed with PY-20 antiserum against tyrosine-phosphorylation proteins by epifluorescent microscopy. Localization of tyrosine-phosphorylated proteins in methanol-fixed cynomolgus monkey spermatozoa showed staining in the principal piece region of the flagellum. A) Baseline (without caffeine and dbcAMP) and B) with caffeine and dbcAMP). Pretreatment of PY-20 antiserum with an excess of unlabeled phosphotyrosine resulted in an absence of immunoreactivity, indicating the specificity of the reaction. (Data not pictured).

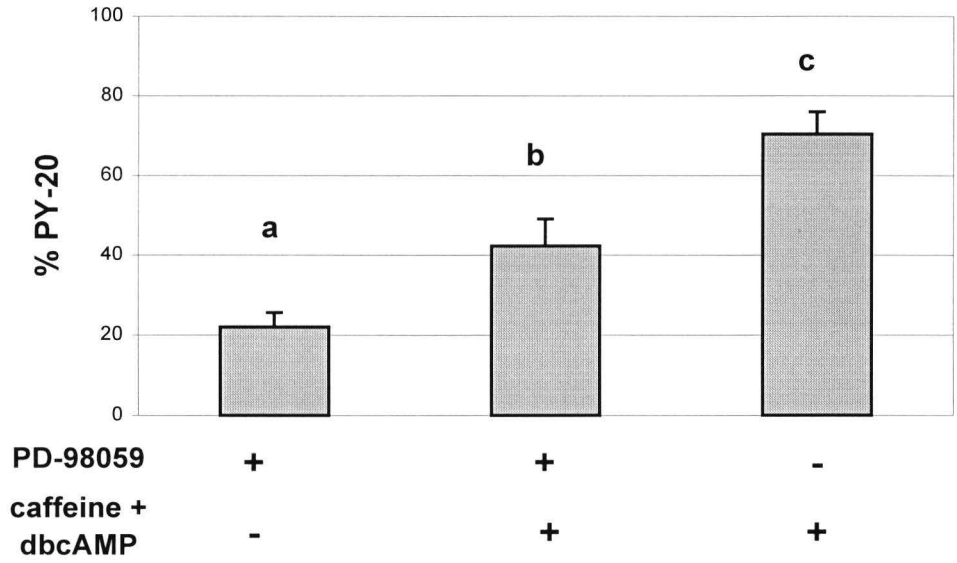

Figure 11. The effect of the MAPKK inhibitor, PD-98059, on caffeine and dbcAMP-stimulated tyrosine phosphorylation of macaque sperm tail proteins. There was a significant increase in tyrosine phosphorylation of sperm tails in sperm stimulated with caffeine and dbcAMP ($p<0.01$). Treatment with PD-98059 and stimulation with caffeine and dbcAMP resulted in a significant decrease in tyrosine phosphorylation compared to sperm stimulated with caffeine and dbcAMP ($p<0.01$). Percentages determined through the evaluation of at least 100 sperm per treatment group. (Note: a, b and c denote significantly different groups).

The next set of experiments examined the dose dependent effects of PD-98059 on protein tyrosine phosphorylation. PD-98059 was administered in increasing doses (0 μM to 3 μM). There was a significant dose dependent effect of PD-98059, on the proportionof sperm exhibiting PY-20 immunoreactivity of sperm tail proteins ($p<0.01$) with an $IC_{50} = 0.1$ μM (Figure 12).

Effect of PD-98059 on Phosphorylation of MAP Kinase of Macaque Sperm Proteins

The next set of experiments was conducted to determine the specificity of the MAPKK inhibitor. PD-98059 should act to prevent the phosphorylation of MAP kinase proteins; therefore, we examined immunoreactivity patterns of phosphorylated MAP kinase proteins in macaque sperm. Immunocytochemical analysis of the phosphorylation of sperm proteins with the phosphorylated MAP kinase antibody, Phospho-p44/42 MAP Kinase, resulted in labeling in four patterns: control (absence of immunoreactivity); postacrosomal region through the principal piece; the neckpiece and principal piece; and the principal piece only (Figure 13). Immunoreactivity in the postacrosomal region appeared to be specimen dependent. There was a significant increase from 15% ± 1 to 69% ± 10 ($p<0.01$) in phosphorylation of MAP kinase immunoreactivity of sperm tail proteins observed in sperm treated with caffeine and dbcAMP (Figure 14). Pretreatment of sperm with PD-98059 prior to stimulation with caffeine and dbcAMP resulted in a significant decrease in phosphorylated MAP kinase immunoreactivity to baseline levels ($p<0.01$).

Macaque sperm were next treated with PD-98059 in increasing doses (0 μM to 3 μM) to determine if there was a dose dependent effect of the MAP kinase inhibitor on the phosphorylation of MAP kinase in sperm proteins. There was a significant decrease in

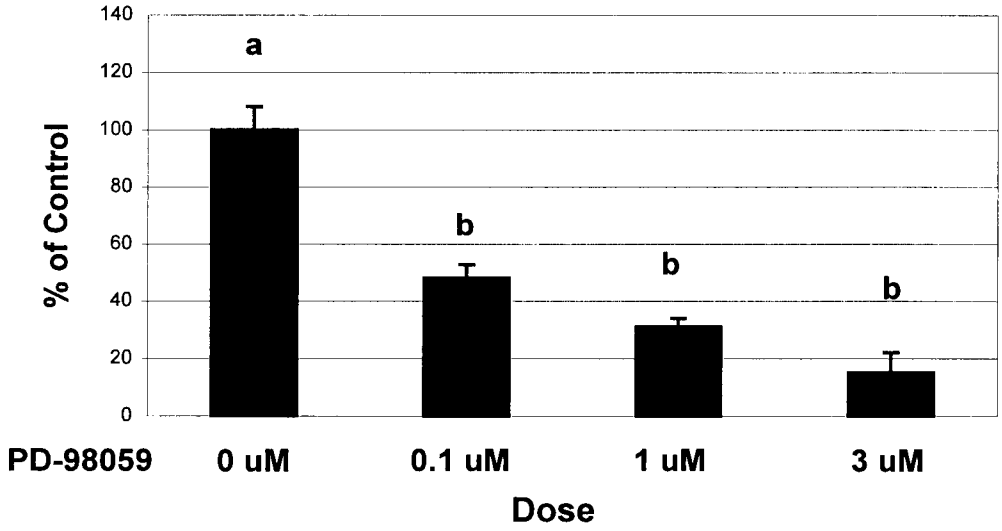

Figure 12. Dose dependent effect of the MAPKK inhibitor, PD-98059, on caffeine and dbcAMP-stimulated tyrosine phosphorylation of sperm tail proteins. There was a significant decrease in tyrosine phosphorylation of sperm tail proteins in sperm groups treated with 0.1-3 μM of PD-98059 ($p<0.01$) with an IC_{50} = 0.1 μM. (Note: a and b denote significantly different groups).

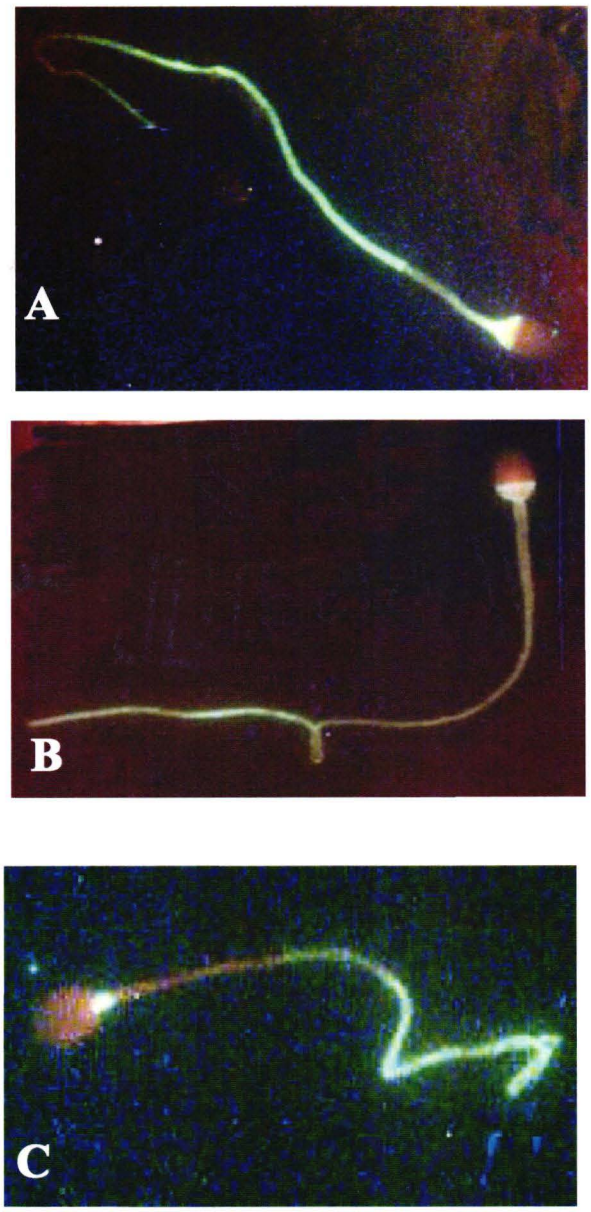

Figure 13. Effect of the MAPKK Inhibitor, PD-98059, on Caffeine and dbcAMP-stimulated phosphorylation of MAP kinase in Macaque Sperm. Immunocytochemical analysis of phosphorylation of MAP kinase sperm proteins resulted in four labeling patterns: A) postacrosomal region, midpiece and principal piece; B) principal piece only; and C) neck region and principal piece. The control (absence of immunoreactivity) is not pictured. Staining in the postacrosomal region appeared to be specimen-dependent.

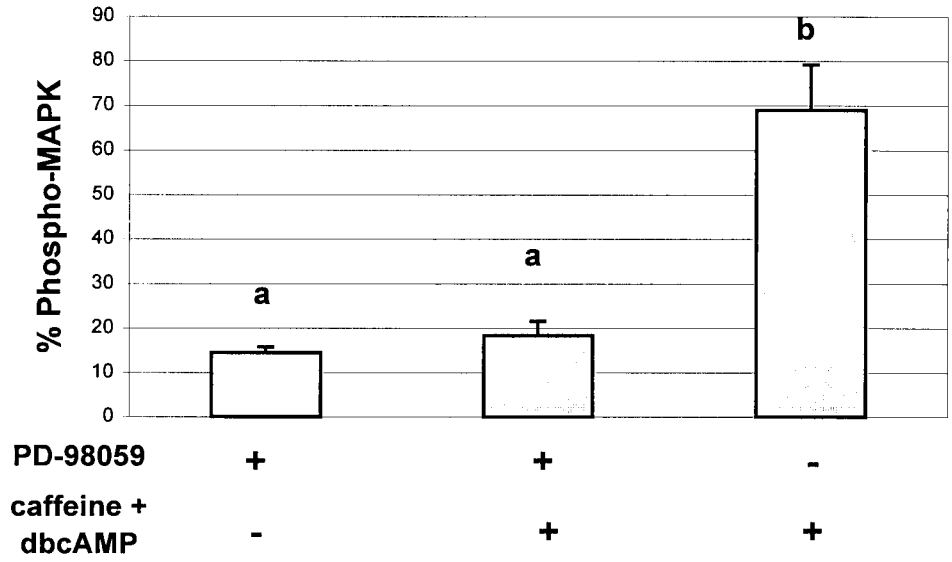

Figure 14. Effect of the MAPKK inhibitor, PD-98059, on caffeine and dbcAMP-stimulated phosphorylation of MAP kinase proteins in macaque sperm. There was a significant increase in phosphorylation of MAP kinase proteins when sperm were stimulated with caffeine and dbcAMP ($p<0.01$). The addition of PD-98059 to these caffeine and dbcAMP stimulated sperm groups resulted in a significant decrease ($p<0.01$) in tyrosine-phosphorylated sperm tail proteins and this decrease was to baseline levels. Percentages obtained through the evaluation of at least 100 sperm per treatment group. (Note: a and b denote significantly different groups).

phosphorylation with increasing doses of PD-98059 ($p<0.01$) with an $IC_{50} = 0.1\mu M$ (Figure 15).

Effects of MAPKK Inhibitor, PD-98059, on the Migration of Sperm Proteins

Immunoblotting was performed to further determine the effect of the MAP kinase inhibitor, PD-98059, on tyrosine phosphorylation and phosphorylation of MAP kinase in sperm proteins. When baseline sperm were stimulated with caffeine and dbcAMP, there was a visual increase in the range of proteins known to have tyrosine-phosphorylated residues (40-120 kDa) (Lane 1, Figure 16). With the addition of PD-98059 (3μM), phosphorylation of these PY-20 immunoreactive proteins was essentially absent (Lane 2, Figure 16).

Activation of macaque sperm groups with caffeine and dbcAMP exhibited increased tyrosine phosphorylation and phosphorylation of the MAP kinase proteins in the 42-44kDa range compared to baseline levels (Top picture, Figure 17). Since PD-98059 is an inhibitor of the phosphorylation of these proteins, there should be no immunoreactivity in the treated sperm groups. Treatment of these stimulated sperm with PD-98059 resulted in decreased MAP kinase immunoreactivity to baseline levels (Figure 16).

The equal loading of sperm protein was confirmed by staining gels with GELCODE® Blue Stain Reagent (Pierce; Rockford, Illinois). There were similarities of banding patterns observed in control and treated groups (Data not shown).

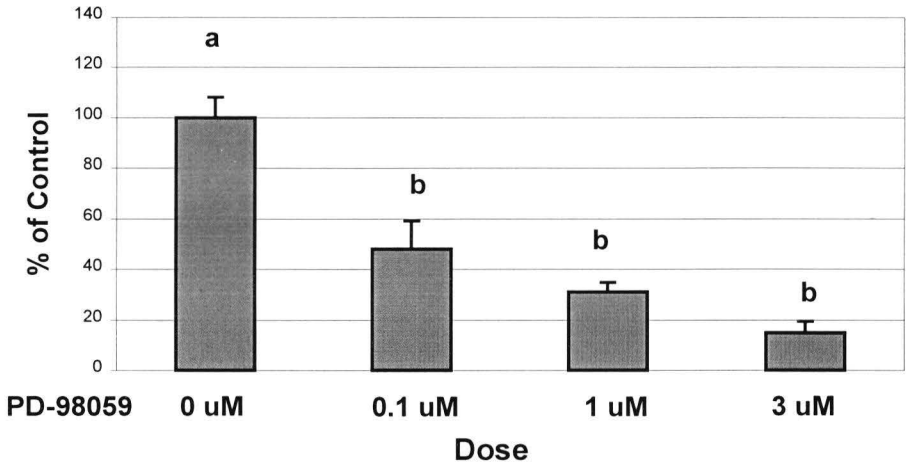

Figure 15. Dose dependent effect of the MAPKK inhibitor, PD-98059, on the phosphorylation of MAP kinase macaque sperm proteins. Stimulation of sperm with caffeine and dbcAMP resulted in a significant increase in the phosphorylation of MAP kinase proteins. There a significant dose dependent effect of PD-98059 on the phosphorylation of MAP kinase with an IC_{50} = ~0.1μM. (Note: a and b denote significantly different groups).

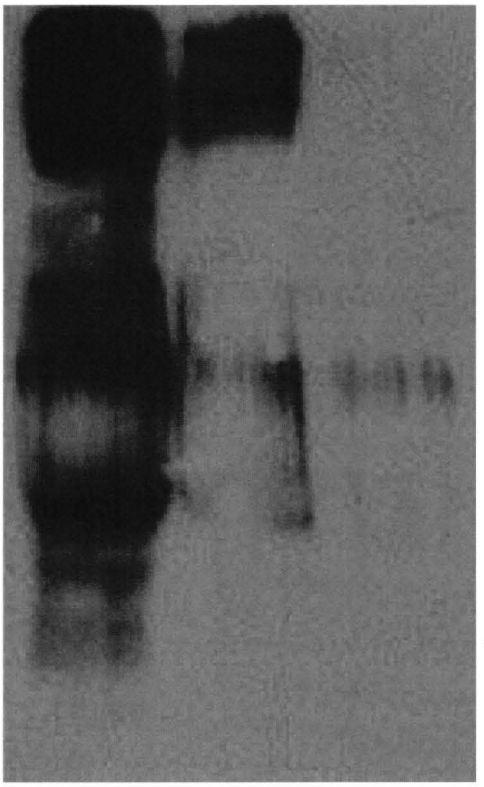

Figure 16. Effect of the MAPKK Inhibitor, PD-98059, on Caffeine and dbcAMP-stimulated Sperm Tyrosine Phosphorylated Proteins Labeled with the PY-20 antiserum. Immunoblotting was performed on extracted macaque sperm proteins to determine the effect of the MAPKK inhibitor on protein tyrosine phosphorylation of sperm proteins. Those proteins migrating to 55, 80, 95 and 105 kDa exhibited increased levels of protein tyrosine phosphorylation with caffeine and dbcAMP stimulation. Phosphorylation of these proteins was essentially absent with PD-98059 (3μM) treatment.

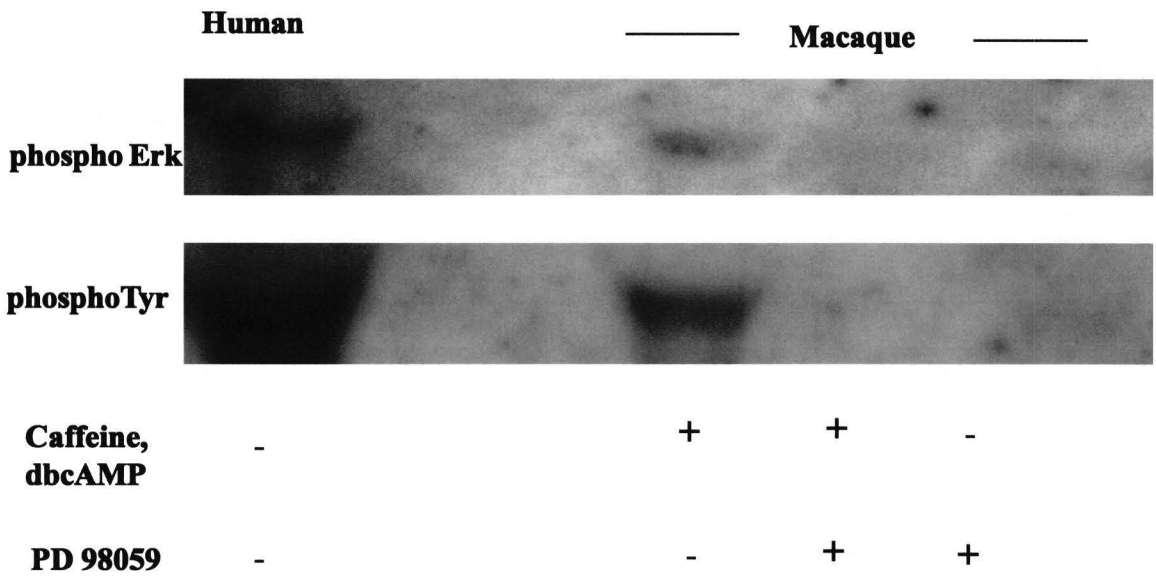

Figure 17. Effect of the MAPKK Inhibitor, PD-98059, on Caffeine and dbcAMP-stimulated phosphorylation of MAP Kinase and Tyrosine Residues on Macaque Sperm Proteins. Immunoblotting was performed on sonicated and extracted macaque sperm proteins to determine the effect of the MAPKK inhibitor on protein tyrosine phosphorylation and phosphorylation of MAP kinase sperm proteins migrating to 42 to 44 kDa. Phosphorylation of tyrosine and MAP kinase increased with caffeine and dbcAMP stimulation. Phosphorylation of these proteins was decreased to baseline levels with PD-98059 treatment.

CHAPTER IV

DISCUSSION

Capacitation, first described fifty years ago, is defined as physiological and molecular events that confer upon spermatozoa the ability to fertilize an oocyte (Austin, 1951, 1952; Chang, 1951, 1955). Various alterations occur within spermatozoa and their membranes during capacitation, but many of the intracellular signaling pathways causing these alterations have yet to be elucidated. Capacitation *in vivo* occurs in the female reproductive tract in response to cellular and humoral components and is species dependent. Capacitation *in vitro* is also species dependent with some species requiring specific media constituents (i.e. glucose, lactate and pyruvate). The completion of capacitation is often measured not only by the fertilization of an oocyte, but also through several upstream endpoints, including the acrosome reaction, zona penetration and hyperactivation.

Cynomolgus monkey sperm provide an excellent model for studying capacitation-related events. They exhibit an absolute requirement of exogenous stimulation with the cyclic nucleotide cAMP in the membrane permeable form of dbcAMP, and the phosphodiesterase inhibitor, caffeine (Boatman and Bavister 1984; Mahony *et al* 1996; VandeVoort *et al* 1992, 1994) to complete the pathway leading to *in vitro* fertilization, including hyperactivated motility, zona binding and penetration. These requirements for capacitation provide a controlled system to study these cellular events.

Hyperactivated motility is an endpoint often used to determine the successful completion of capacitation (Yanagimachi, 1994). After an incubation period either *in vivo* or *in vitro* to allow the spermatozoa to capacitate, a special motility pattern is noted,

namely, hyperactivation. First described in golden hamster spermatozoa (Yanagimachi, 1969), hyperactivated motility is characterized by vigorous, wide-amplitude, flagellar beats with nonprogressive movement. cAMP is an important regulatory component of the sperm intracellular milieu that regulates the initiation and maintenance of sperm motility and one major action of this cyclic nucleotide is the subsequent phosphorylation of tyrosine residues on sperm proteins. Protein tyrosine phosphorylation is considered to be a significant indicator of intracellular changes preceding and/or associated with capacitation (Visconti *et al*, 1995) and capacitation-dependent hyperactivated motility (Mahony and Gwathmey, 1999). One known intracellular signaling pathway involved in the hyperactivation of the sperm tail is initiated through an increase in intracellular cAMP leading to stimulation of protein kinase A and subsequent tyrosine phosphorylation of sperm tail proteins (Morisawa, 1990; Si and Okuno, 1999). The tyrosine phosphorylation of fibrous sheath proteins allows the flagella to generate greater bends (Si and Okuno, 1999), while the membrane fluidity changes caused by the efflux of cholesterol and increase in membrane phospholipids allow the tail to become more flexible. The phosphorylation of these tyrosine residues is mainly localized to the principal region of the tail since that is the region containing the fibrous sheath and a small region of the neck in macaque sperm. These labeling patterns are similar to those noted in human sperm (Carrera *et al*, 1996). In macaque sperm, there is a distinct capacitating environment required *in vitro* to attain hyperactivated motility that does not support the acrosome reaction and vice versa. This difference between capacitating environments allows the use of macaque sperm to study the intracellular events related specifically to hyperactivation.

VandeVoort *et al* (1992, 1994) previously reported that the main distinguishing feature of macaque sperm hyperactivation stimulated with caffeine and dbcAMP is an increase in flagellar bending amplitude which is detected by computer assisted motion analysis as an increase in the amplitude of lateral head displacement (ALH). ALH is the most important indicator of spermatozoal ability to fertilize an oocyte (Aitken, 1990). In this present study, a significant increase in ALH (Figure 5) in sperm stimulated with caffeine and dbcAMP was observed as compared to baseline sperm. There was a significant decrease in linear movement or linearity (LIN) in caffeine and dbcAMP-stimulated sperm (Figure 7). LIN, the ratio of the straight line velocity over the curvilinear velocity, decreased with caffeine and dbcAMP stimulation, while VCL (curvilinear velocity) increased significantly (Figure 6). These results support previous reports indicating that ALH and VCL are increased with stimulation by caffeine and dbcAMP. Stimulation with caffeine and dbcAMP also significantly increased hyperactivated motility of sperm tails as previously reported (Mahony and Gwathmey, 1999). Thus, stimulation of macaque sperm with the cyclic nucleotides, caffeine and dbcAMP have positive effects on motility characteristics leading to successful capacitation and capacitation-related hyperactivation.

Studies of several mammalian species have indicated unique relationships between protein tyrosine kinase and cAMP signaling pathways during capacitation (Visconti *et al*, 1995; Leclerc *et al*, 1996; Galantino-Homer *et al*, 1997). The results of this current research confirm previously published results (Mahony and Gwathmey, 1999) that treatment of macaque sperm with the exogenous cyclic nucleotide analogue dbcAMP and caffeine, a phosphodiesterase inhibitor, significantly increased tyrosine

phosphorylation of sperm tail proteins, as determined through immunocytochemistry. Along with the increase in tyrosine phosphorylation of sperm tail proteins was a concomitant increase in hyperactivated motility in sperm stimulated with caffeine and dbcAMP. Mahony and Gwathmey (1999) previously demonstrated that tyrosine phosphorylation is an integral component of the caffeine and cAMP-stimulated hyperactivation and these results fully support that information. Also, the labeling patterns for tyrosine phosphorylation with the antibody PY-20 observed in these macaque sperm experiments were similar to those reported for mouse and human sperm (Carrera, 1994, 1996). In those species, the major phosphotyrosine proteins identified were, a major fibrous sheath protein, A kinase anchoring protein (AKAP) and its precursor polypeptide, proAKAP82.

A number of experiments utilizing immunoblotting have also documented proteins in the sperm tail that undergo tyrosine phosphorylation in cAMP-stimulated hyperactivation. Leclerc *et al* (1996) reported two major proteins involved in tyrosine phosphorylation in human sperm in response to intracellular increases in cAMP, one with a molecular weight of 81 kDa and the other of molecular weight 105 kDa. The protein bands (81 and 105 kDa) obtained in the aforementioned experiment were absent with no incubation, but increased once a longer incubation was achieved in capacitation-supported medium. Si and Okuno (1999) reported a fibrous sheath protein at an approximate weight of 80kDa in response to cAMP and tyrosine phosphorylation in hamster sperm in a time-dependent manner. As in the first experiment, there was an increase in protein bands with a longer duration of incubation. Mandal *et al* (1999) documented a fibrous sheath phosphotyrosine 95 kDa protein that is tyrosine

phosphorylated with cAMP-stimulated hyperactivation in human sperm using two dimensional gel analysis. Vijayaraghavan *et al* (1997) documented a 55kDa protein regulated through a cAMP and calcium signaling system involving protein kinase A in caput and caudal epididymal bovine sperm. Sperm stimulated in this current study with caffeine and dbcAMP experienced visual increases in magnification of protein bands in the range of tyrosine phosphorylated proteins (40-120 kDa) and resulted in the greatest magnification of phosphotyrosine immunoreactivity. Most of these bands were absent in baseline sperm (no stimulation with caffeine and dbcAMP) due to the requirement of the sperm activators for capacitation and hyperactivated motility in macaque sperm. As discussed previously, human sperm are able to achieve capacitation and subsequent hyperactivation without the addition of stimulators as seen with macaque sperm. The media requirements for most mammalian sperm to spontaneously capacitate are serum albumin, bicarbonate and calcium. Macaque sperm, as shown in these results, must have stimulation with caffeine and dbcAMP to achieve capacitation and capacitation-related hyperactivation (Figure 8). To support the results obtained from immunoblotting, staining of gels with GELCODE® Blue Stain Reagent (Pierce, Rockford, Il) was completed to determine that equivalent amounts of proteins were present in each lane. The similarities of banding in control and treated groups confirmed the equal loading of sperm on the gels used for immunoblotting. These results were also confirmed through immunocytochemistry in the proportion of sperm exhibiting antiphosphotyrosine immunoreactivity. Results of the current study indicate that tyrosine phosphorylation is indeed an important component of cAMP-stimulated sperm hyperactivated motility.

Mitogen activated protein kinases are important mediators in the intracellular network of proteins that respond to extracellular stimuli. The function of mitogen-activated kinase in somatic cells includes cellular proliferation and differentiation (Bokemeyer et al, 1996), while the role of these kinases in the capacitation of spermatozoa remains to be determined. The activation of these kinases can be split into two distinct phases, membranous and cytoplasmic. Although there are several types of receptors which when bound with the appropriate ligand will stimulate this process (i.e. tyrosine kinase receptors, cytokine receptors) (Bokemeyer et al, 1996), the receptor thought to be important in spermatozoa is a G-protein coupled receptor. Other receptors important to the spermatozoa *in vivo* have been studied including a ZP3 receptor for interaction with the oocyte and a progesterone receptor for interactions with the female reproductive tract. One pathway in the MAP kinase signaling cascade is stimulated by the activation of the Ras signaling component through the Raf family of kinases (Raf-1 and B-Raf, specifically) and subsequent phosphorylation of MAP kinase. Raf activity is modulated through upstream kinases such as protein kinase A (Morrison *et al*, 1993; Vaillancourt *et al*, 1994), a well-characterized kinase whose enzyme activity is sensitive to intracellular increases in cAMP. Several studies have documented relationships between the levels of intracellular cAMP and the MAP kinase-signaling cascade. The "traditional" pathway of cAMP action is through activation of protein kinase A and its A kinase anchoring proteins (AKAP). However, it is now clear that this system is not the only effector of cAMP events in the cell. Ras has been linked to an increase in intracellular cAMP in melanocytes (Busca *et al*, 2000), while the increase in intracellular

cAMP and its stimulation of protein kinase A activity had negative effects on Raf-1, but not B-Raf in neuronal cells (Vossler *et al*, 1997).

MAP kinases are known to translocate from one cell compartment to another in response to extracellular stimuli (Bokemeyer *et al*, 1996). This translocation was noted in the nucleus and the cell surface membrane. Recently, the presence of MAP kinase was documented in the human sperm acrosome reaction (Luconi, *et al*, 1998) and there was selective localization of these kinases to the postacrosomal region of the head. Another set of data revealed the translocation of the extracellular regulated kinases to the equatorial region of the human sperm head after stimulation with progesterone (Luconi *et al*, 1998) and the calcium ionophore, A23187, to induce the acrosome reaction. The four labeling patterns noted in the immunocytochemical analysis of the phosphorylation of MAP kinase sperm proteins were 1) in the postacrosomal region, midpiece, and principal piece, 2) in the principal piece only, 3) in the neck region and the principal piece, and 4) no staining (baseline/control sperm). There were similarities in labeling between the two species (i.e. the postacrosomal region), but differences were observed between these two species of sperm in the tail region. This should not be unexpected since different experimental conditions were utilized. These labeling patterns were specimen dependent and may be indicative of a difference among semen samples from different macaques. The patterns observed in this study of macaque sperm were different from labeling patterns noted previously in human sperm, possibly due to differences between the two species. Sperm in current experiments were stimulated with caffeine and dbcAMP for the purpose of studying hyperactivation-related events in macaque, while human sperm capacitation and the acrosome reaction were the endpoints of the previous experiment

(Luconi *et al*, 1998). Two different antibodies were used in the two experiments, suggesting another reason for the differences in the labeling patterns of the capacitated spermatozoa. Lastly, macaque sperm do not capacitate *in vitro* without the addition of caffeine and dbcAMP to the media, while human sperm are able to capacitate spontaneously. This may be another explanation for differences in the labeling patterns noted between these experiments. Additionally, there were alterations of these labeling patterns with the addition of the exogenous activators. Stimulation of control sperm with caffeine and dbcAMP resulted in a significant increase in the proportion of sperm exhibiting phosphorylation of MAP kinase proteins. Pretreatment of caffeine and dbcAMP-stimulated sperm with PD-98059 decreased the proportion of sperm exhibiting phosphorylation of MAP kinase proteins to baseline (no treatment with caffeine and dbcAMP). The increase in the phosphorylation of MAP kinase proteins experienced a concomitant increase in protein tyrosine phosphorylation and subsequent hyperactivation with stimulation of sperm by caffeine and dbcAMP. This was expected since previous reports indicated the cross talk between cAMP signaling and MAP kinase signaling systems. Therefore, the cAMP pathway is involved in the phosphorylation of these MAP kinase proteins leading to protein tyrosine phosphorylation and subsequent hyperactivation of spermatozoa. Also, the MAPKK inhibitor, PD-98059, did not inhibit the acrosome reaction (Luconi *et al*, 1998), so the inhibition of caffeine and cAMP-stimulated hyperactivated motility is further support for the involvement of this kinase and separates this pathway from the pathway leading to the acrosome reaction.

Luconi *et al* (1998) studied two specific MAP kinase proteins, the extracellular regulated signal kinases (ERK), p44 and p42 these proteins migrate to 44 and 42 kDa,

respectively). Protein bands in this molecular weight range were immunoreactive to antibodies against tyrosine-phosphorylated proteins as well as MAP kinase phosphorylated proteins in human sperm. MAP kinases are activated by phosphorylation on threonine and tyrosine residues (Bokemeyer *et al*, 1996). Sperm proteins were probed with the antiphosphotyrosine antibody, PY-20, and the phosphorylated MAP kinase antibody, Phospho-p44/42-MAP kinase, and bands were detected in the molecular weight range of 42-44kDa. Sperm stimulated with caffeine and dbcAMP experienced a visual increase in the protein bands in this molecular weight range when probed with PY-20 as well as Phospho-p44/42-MAP Kinase. Pretreatment with PD-98059 of sperm stimulated with caffeine and dbcAMP resulted in a decrease in magnification of protein bands in this molecular weight range for both protein tyrosine phosphorylation and phosphorylation of MAP kinase proteins. There were similar staining patterns with both antibodies, but the immunoreactivity was greater with the antiphosphotyrosine antibody. These results suggest two theories: 1) MAP kinase phosphorylation is increased with stimulation by caffeine and dbcAMP in macaque sperm, but tyrosine phosphorylation is the major recipient of action through the cAMP-stimulated pathway or 2) the concentration of MAP kinase proteins in the sperm is much smaller than the concentration of tyrosine phosphorylated proteins.

PD-98059 is a specific inhibitor of MAP kinase kinase, which normally phosphorylates MAP kinase, both *in vitro* and in intact cells (Dudley *et al*, 1995). In this present study, the MAP kinase kinase inhibitor was used to examine the inter-relationship between cAMP-dependent hyperactivation processes and the MAP kinase system. This was performed using four sperm groups; 1) control sperm, no stimulation with caffeine

and dbcAMP (baseline), 2) control sperm stimulated with caffeine and dbcAMP, 3) pretreatment with PD-98059 and stimulation with caffeine and dbcAMP, and 4) pretreatment with PD-98059 and no stimulation with caffeine and dbcAMP. Initial testing was completed in this laboratory to determine the potential effects of this inhibitor on these cAMP-stimulated events including hyperactivated motility, tyrosine phosphorylation, and the phosphorylation of MAP kinase proteins. There was no significant difference in the cAMP-stimulated events between sperm pretreated with PD-98059 and stimulated with caffeine and dbcAMP and the baseline sperm group. This group was eliminated once these preliminary tests were complete, although samples were kept frozen for usage in the immunoblotting and immunocytochemical analyses. The elimination of this group was also to ensure that maximum amount of sperm in the remaining control and experimental groups for use in the immunoblotting and immunocytochemical portions of these experiments. The sperm groups pretreated with PD-98059 in the absence of stimulation to hyperactivated motility with caffeine and dbcAMP did not alter motion characteristics in a deleterious manner, indicating that this inhibitor has no effect on the basic physiology of macaque sperm. Pretreatment of sperm stimulated with caffeine and dbcAMP with PD-98059 did in fact decrease the amplitude of lateral head displacement (ALH) significantly. There was a significant decrease in linearity (LIN) with PD-98059 treatment in sperm stimulated with caffeine and dbcAMP compared to baseline, but this decrease was not significantly different from sperm stimulated with caffeine and dbcAMP. There was no increase in curvilinear velocity (VCL) in sperm pretreated with PD-98059 and stimulated with caffeine and dbcAMP compared to baseline. When this PD-98059 pretreated group was compared to the sperm

treated with caffeine and dbcAMP, there was no significant difference. Finally, pretreatment with PD-98059 significantly decreased hyperactivated motility compared to sperm stimulated with caffeine and dbcAMP, although this decrease was not to baseline levels. The motion characteristics of macaque sperm most affected by treatment with PD-98059 in this experiment were hyperactivated motility, linearity and the amplitude of lateral head displacement. These results agree with the theory that ALH is a significant indicator of the spermatozoa's ability to interact with the zona pellucida, thereby fertilizing the egg, since decreased linearity and increased lateral head displacement are characteristics of hyperactivated motility (Aitken, 1990). All of these motion characteristics were significantly affected by PD-98059, showing the involvement of the MAP kinase-signaling cascade in these cAMP stimulated events.

Treatment of macaque sperm with PD-98059 significantly blocked hyperactivation, tyrosine phosphorylation of sperm tail proteins and phosphorylation of MAP kinase sperm proteins in a dose-dependent manner. Pretreatment with PD-98059 did not completely decrease motion characteristics including hyperactivated motility to baseline levels. This inhibitor also did not block completely the protein tyrosine phosphorylation of sperm tail proteins to baseline levels. Pretreatment of macaque sperm with PD-98059 resulted in decreased levels of phosphorylation of MAP kinase proteins. This decrease was to baseline levels, proving that PD-98059 does in fact inhibit the enzyme, MAP kinase kinase, and prevents the phosphorylation of MAP kinase and this pathway is able to interact with cAMP, as previously noted in somatic cells. To conclude, the results provide evidence that the MAP kinase-signaling pathway is stimulated in macaque sperm in response to an intracellular increase in cAMP. These

results also support the hypothesis that the MAP kinase pathway is involved in these caffeine and cAMP-stimulated events but also shows that it may be only one pathway involved in hyperactivation of the sperm tail. The exact mechanism of this stimulation is not known, but this study has given more insight to the stimulation of this pathway in macaque spermatozoa.

There were difficulties encountered in this experiment that warrant further study. The amount of MAP kinase protein in spermatozoa is very small, so it is necessary to determine either a protein concentration or sperm number that will allow an adequate amount of protein to be obtained through sonication, immunoprecipitation and immunoblotting. Also, Luconi *et al* (1998) reported the presence of both MAP kinase proteins (p44 and p42) in the acrosome reaction, but there was only one band noted in these hyperactivation studies. It is possible that only one of these proteins is phosphorylated in the intracellular events leading to caffeine and dbcAMP stimulated hyperactivation or there may be a species related difference as previous studies were performed with human sperm.

There was some variability among the samples obtained from the monkeys as well as the individual sperm studied in this experiment shown by several measurements utilized. Macaque sperm reportedly display more synchrony since a higher proportion of sperm exhibit hyperactivated motility. Mahony and Gwathmey (1999) attributed this to the requirement of exogenous stimulation with caffeine and dbcAMP. In contrast, human sperm capacitate spontaneously and do not hyperactivate at the same time, evidenced by the low proportion exhibiting hyperactivated motility (Oehninger *et al*, 1997). There was also some variability among sperm exhibiting protein tyrosine phosphorylation, most

likely due to the same reasons noted above regarding hyperactivation. Protein tyrosine phosphorylation occurs upstream of hyperactivation, so it is not unexpected to see variation among sperm in this analysis. There was a large amount of variation among sperm analyzed for phosphorylation of MAP kinase proteins. Since there were four labeling patterns and these patterns were specimen dependent, it was expected that there would be variation in this analysis. These differences noted in the measured values of this experiment are expected when comparing samples from different rhesus monkeys and individual spermatozoa from these monkeys.

This study was significant to the current reproductive studies, since scientists are still learning the pathways involved not only in capacitation, but also hyperactivation and other capacitation-related events. The information obtained from this study may assist in understanding clinical problems encountered by men, such as male factor infertility and may be one of several first steps toward formulating a male contraception. The ultimate goal of these studies is to understand the biochemical pathways involved in the capacitation of sperm.

CHAPTER V

CONCLUSIONS

The highly defined criteria for achievement of capacitation and capacitation-related hyperactivated motility in macaque sperm continue to provide a unique model for delineating the cellular events involved in capacitation and *in vitro* fertilization. Previous experiments with macaque sperm have reported that stimulation with caffeine and dbcAMP result in a significant increase in both macaque sperm hyperactivation and tyrosine phosphorylation of sperm tail proteins. The results of these experiments agree with the finding.

The MAP kinase pathway is involved in macaque sperm hyperactivation as evidenced by enzyme studies with the MAPKK inhibitor, PD-98059. This was confirmed through immunocytochemistry and immunoblotting studies to localize phosphorylated MAP kinases in macaque sperm. Treatment with PD-98059 resulted in a significant dose dependent decrease in sperm hyperactivation, tyrosine phosphorylation and phosphorylation of MAP kinase. Although the MAPKK inhibitor, PD-98059, decreased levels of phosphorylated MAP kinase to baseline levels, neither hyperactivation nor tyrosine phosphorylation of sperm tail proteins was completely blocked. These results suggest the MAP kinase-signaling cascade may be only one potential pathway; alternate pathways may also be involved. This system has great potential to provide information that will be useful in studying fertility enhancement and intervention in humans, as well as conservation of endangered nonhuman primates.

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