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Release of DEFB126 From Macaque Sperm and Completion of Capacitation Are Triggered by Conditions That Simulate Periovulatory Oviductal Fluid

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SUMMARY

Capacitation of macaque sperm in vitro has been achieved efficiently only with the addition of both cyclic nucleotides and methylxanthines. The use of these exogenous sperm activators clouds an understanding of the normal mechanisms underlying capacitation and may slow early embryo development following in vitro fertilization (IVF). We demonstrate that culture medium which simulates periovulatory oviductal fluid with respect to bicarbonate (HCO_3^-) and glucose concentration induces capacitation in a high percentage of macaque sperm as determined by the ability of sperm to undergo both the release of coating protein DEFB126 and the zona pellucida-induced acrosome reaction (AR). Few sperm were able to undergo the AR following 6 hr incubation in medium containing either 35 mM HCO_3^- ($\sim$7.2 pH) or 90 mM HCO_3^- ($\sim$pH 7.8) with 5 mM glucose. When glucose concentration was lowered to 0.5 mM to match levels reported for women at midcycle, the AR rate increased significantly in sperm incubated in both levels of HCO_3^-, indicating that glucose interferes with sperm responsiveness to increasing HCO_3^- concentration observed in the primate oviduct during ovulation. Even greater synchronization of capacitation could be achieved with nonphysiologic extremes of alkalinity or energy substrate deprivation. In the latter case, sperm achieved high rates of IVF. A shift in pH from 7.2 to 7.8 in a HEPES-buffered medium was sufficient to remove DEFB126 from the surface of most sperm after only 3 hr. The loss of DEFB126 from sperm under periovulaory fluid conditions has implications for the timing of release of sperm from the oviductal reservoir.

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INTRODUCTION

Mature sperm released from the male tract at ejaculation must still spend additional time in the female tract before they are competent to fertilize (Austin, 1952; Chang, 1955). This final maturation process, termed capacitation, has been recognized for more than 50 years as an essential prerequisite for fertilization, but how this process takes place in the female tract is still poorly understood (Weinman and Williams, 1961; Bedford, 1963; Florman and Babcock, 1991; Zaneveld et al., 1991; Cross, 1998; Baldi et al., 2000). It is generally accepted from in vitro studies in defined media that a number of changes accompany capacitation which include alterations in membrane composition/distribution,

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the removal of surface coats, and increases in intracellular Ca^{++}, protein tyrosine phosphorylation, intracellular pH and bicarbonate levels (Evans and Florman, 2002; Jaiswal and Eisenbach, 2002; for review). How and when these specific cellular events occur during sperm transport in the female tract remain mysterious, prompting some to question the physiological relevance of data derived from many in vitro capacitation systems (Talevi and Gualtieri, 2004; Barratt and Kirkman-Brown, 2006). Yet most agree that although the timing may vary between species, these events are completed while sperm reside in the oviduct (Yanagimachi, 1994, for review; Hunter and Rodriguez-Martinez, 2004; for review). Release of sperm from the oviductal reservoir appears to signal the completion of sperm capacitation in many species (Suarez, 2002; Suarez and Pacey, 2006; for review).

What stimulates the release of sperm in a fully capacitated state from oviductal reservoirs is not known. Sperm appear to be recruited out of the oviductal isthmus due to exposure to periovulatory hormones such that the appearance of capacitated sperm in the ampulla is well coordinated with the time of ovulation (Hunter et al., 1999; Hunter and Rodriguez-Martinez, 2004). In vitro, a number of compounds including steroid hormones and glycosaminoglycans associated with the cumulus complex surrounding the egg have been shown to induce capacitation and it has been proposed that the trigger for capacitation of sperm in the oviduct may be these egg-associated factors (Yanagimachi, 1994). Recently, it has been purported that alkalinization of the oviduct could potentially trigger capacitation. Bull sperm respond to elevations in extracellular pH with a rise in pH_i that results in capacitation (Galantino-Homer et al., 2004). This event is dominated by the activity of a Na^+-dependent HCO_3^-/Cl^- exchanger in sperm in the presence of extracellular HCO_3^- (Zeng et al., 1996) which is known to climb dramatically in the oviduct of several species just prior to ovulation (Maas et al., 1977; Leese, 1988; Nichol et al., 1997; for review). The HCO_3^- concentration of oviductal fluid is key to fertility, affecting the function and development of sperm, ova and zygotes (Boatman, 1997; for review; Boatman and Robbins, 1991). A mid-cycle peak in HCO_3^- secretion by the oviductal epithelium markedly increases both the $[HCO_3^-]$ and pH of oviductal fluid in several species (Leese, 1988) including primates (Maas et al., 1977). In rhesus monkeys, for instance, in preovulatory, anovulatory and castrated individuals, luminal pH was stable in the range of 7.1–7.3 and showed no variation between days 3 and 14 of the cycle (Maas et al., 1977). However, as soon as a day after ovulation (Days 15–16), pH had increased to 7.5–8.0 and remained constant around 7.6 throughout the luteal phase of the cycle. From measurements of P_{CO_2}, it was estimated that ovulation increased oviductal HCO_3^- from around 35 mM to approximately 90 mM (Maas et al., 1977). The mid-cycle change in HCO_3^- concentration was independent of the presence of ova or sperm, and therefore must be under hormonal control.

Simultaneous with these periovulatory events, glucose concentration in oviductal fluid drops precipitously in several mammalian species (Gardner and Leese, 1990; Nichol et al., 1992; Edwards and Leese, 1993), including humans (Gardner et al., 1996). The reason for the drop in glucose is unclear but is likely due to a combination of factors that include decreased carrier-mediated transport across oviductal epithelium (Edwards and Leese, 1993), increased utilization of glucose by oviductal tissues (Nichol et al., 1992), and increased oviductal fluid secretion (Leese et al., 2001) resulting in the dilution of luminal glucose. In any case, the drop in glucose in oviductal fluid does not appear to be related to the presence of gametes or embryos (Nichol et al., 1998). Probably not coincidentally, culture media that either lack or have minimal glucose appear to be beneficial for sperm capacitation (Parrish et al., 1994; Albarracin et al., 2004) and early embryo development (Quinn et al., 1995).

We have demonstrated in the cynomolgus monkey that a single epididymis-derived protein, DEFB126 is the major coating protein on sperm and plays a critical role in the process of capacitation. DEFB126 is the dominant component of the macaque sperm glycocalyx (Yudin et al., 2005a,b) which is released from the sperm surface during in vitro capacitation (Yudin et al., 2003; Tollner et al., 2004). The loss or removal of DEFB126 from over the head of macaque sperm is required for sperm to recognize and bind to the extracellular coat of the egg, the zona pellucida (Tollner et al., 2004). Viable sperm recovered from the cervices and uteri of mated female macaques are evenly coated with DEFB126 suggesting that DEFB126 is retained on sperm in the upper female reproductive tract (Tollner et al., 2008). We have found that both removal of DEFB126 and treatment with anti-DEFB126 Ig result in significantly reduced binding of sperm to oviductal epithelium (Tollner et al., 2008). Addition of soluble DEFB126 back to sperm that had previously shed DEFB126 from their surface (Tollner et al., 2004) resulted in recovery of sperm-epithelial binding (Tollner et al., 2008). The ability of DEFB126 to tightly tether sperm to the apical surfaces of oviductal cells suggests that this coating protein is important in the formation of a reservoir of sperm in the oviduct. Collectively, these findings imply that DEFB126 must be released from the sperm surface as sperm undergo completion of capacitation in the oviduct. We speculate that the loss of DEFB126 results in the release of sperm from the oviductal reservoir; subsequently the released sperm are enabled to recognize and bind to the zona pellucida of the egg.

In this report, we investigate the effects of changing HCO_3^- and glucose concentrations, as observed in the primate oviduct during ovulation, on the capacitation of macaque sperm as determined by the zona-induced acrosome reaction and in vitro fertilization (IVF). We also evaluate if these conditions are sufficient to trigger the release of DEFB126 from sperm, as the loss of DEFB126 is critical for sperm–zona recognition and is potentially responsible for the release of sperm from oviductal reservoirs. The development of more physiologic in vitro capacitation conditions in the macaque has significant implications for understanding the mechanisms underlying capacitation as well as for refinement of IVF procedures which have relied exclusively on the use of exogenous activator compounds to promote capacitation.

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RESULTS

In *series 1* experiments, macaque sperm were incubated in media that varied in the concentration of bicarbonate ion and energy substrates, components that have been shown to change dramatically in oviductal fluid in primates around the time of ovulation. We evaluated the ability of these changes to induce capacitation in macaque sperm using physiological ranges of glucose, lactate, and HCO_3^-. In women, the concentration of glucose during the follicular phase is 5.5–3.04 mM, depending on the report (Lippes et al., 1972; David et al., 1973; Casslen and Nilsson, 1984; Gardner et al., 1996). At midcycle, the concentration of glucose in the oviduct drops considerably, to 2.4 mM (Lippes et al., 1972) or as low as 0.5 mM (Gardner et al., 1996). We opted to work with the extreme values to represent the changes in periovulatory oviductal glucose concentration. Reports of the follicular phase levels of lactate in oviductal fluid vary from as high as high as 21.4 mM (Lippes et al., 1972) to as low as 4.87 mM (Gardner et al., 1996). Similarly, a broad range of values has been reported in ampullary fluid around the time of ovulation (15 mM, Lopata et al., 1976; 10.5 mM, Gardner et al., 1996). As with glucose, we selected extreme values of lactate, assuming that lactate flux across oviductal epithelia declines during periovulatory period as has been described in other mammalian species (Leese et al., 2001; for review). During ovulation, the pH of oviductal fluid of rhesus macaques has been reported to increase on average from 7.2 to 7.8, corresponding to an increase in HCO_3^- concentration from 35 to 90 mM (Maas et al., 1977). We evaluated the effects of changing levels of energy substrates on sperm responses at these two extremes of bicarbonate concentration.

Following 6 hr of incubation (after the long pre-incubation), very few sperm incubated in medium resembling follicular phase levels of energy substrates (medium A: 5.5 mM glucose/21 mM lactate) were able to undergo the acrosome reaction after binding to zonae fixed to glass slides (Fig. 1). The small change in acrosome reaction rate with elevated bicarbonate (35 vs. 90 mM) was insignificant (1.6 ± 1.3 vs. 2.5 ± 1.0). Following incubation in media with periovulatory levels of energy substrates (medium C and D: 0.5 mM glucose/10.5 mM lactate), significantly more sperm underwent the zona-induced acrosome reaction. This effect was not independent of bicarbonate concentration. Sperm incubated in 90 mM HCO_3^- had double the rate of zona-induced acrosome reactions of sperm incubated in 35 mM HCO_3^- (31.3 ± 4.4 vs. $14.5 \pm 3.1\%$, respectively). Two-factor analysis indicated that both changing concentrations of HCO_3^- and energy substrates acted independently on sperm responses ($P \leq 0.002$ and $P \leq 0.001$, respectively). These factors also had significant interactive effects ($P \leq 0.006$). The average number of sperm that bound to the zonae ranged from 21.2 ± 2.9 to 28.6 ± 4.4 with no significant differences in binding due to treatment. No differences in percent motility or progression were observed with type of incubation medium. Sperm % motility always exceeded 60% with the majority of sperm exhibiting vigorous forward progression. Following the long pre-incubation, no sperm incubated in any medium were observed to undergo the zona-induced acrosome reaction after 1 and 3 hr (data not shown). No sperm were observed to undergo the zona-induced acrosome reaction at any time following the short pre-incubation period.

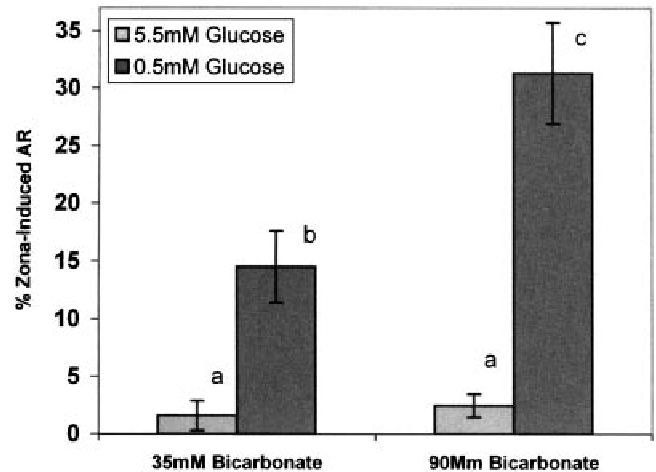

Figure 1. Capacitation assay with sperm incubated for 6 hr (following long pre-incubation) in media that varies in both HCO_3^- and energy substrates. Bars represent the mean percentage of zona-bound sperm that underwent the acrosome reaction. Acrosomal status of zona-bound sperm was determined with acrosomal probe Alexa-SBTI as described in Tollner et al. (2003). Experiment was conducted with sperm from 5 different males and a total of 10 zona per treatment. Error bars represent the standard error of means. Different letters indicate significant differences between treatment means ($P \leq 0.01$).

In additional sperm capacitation experiments, levels of glucose and lactate were varied independently with HCO_3^- concentration maintained at 90 mM (levels that supported zona-induced acrosome reactions in Fig. 1). As observed above at 6 hr following the long pre-incubation, the higher level of glucose (5.5 mM) was associated with significantly lower zona-induced acrosome reaction rates ($P \leq 0.01$; Fig. 2). This response was independent of the level of lactate. Within each level of glucose, differences between lactate concentrations were not significant, although 21 mM lactate appeared to enhance capacitation at low levels (0.5 mM) of glucose (Fig. 2). The average number of sperm that bound to the zonae ranged from 17.6 ± 2.8 to 24.8 ± 3.54 with no significant differences in binding due to treatment. Percent motility of sperm across all treatments exceeded 60% but forward progression of sperm incubated in medium with 10 mM lactate and 0.5 mM glucose was notably less vigorous.

Loss of the sperm surface glycocalyx DEFB126 is associated with capacitation in the macaque (Yudin et al., 2003; Tollner et al., 2004). In *series 1* experiments, we labeled sperm for the presence of DEFB126 at 1, 3, and 6 hr of incubation in media A–D (Fig. 3). The levels of glucose/lactate appeared to have no bearing on either the labeling intensity or distribution of DEFB126 on sperm. Notable differences were seen between the 35 and 90 mM HCO_3^- media independent of level of glucose/lactate (Fig. 3). At 90 mM HCO_3^-, sperm started to lose DEFB126 after 1 hr

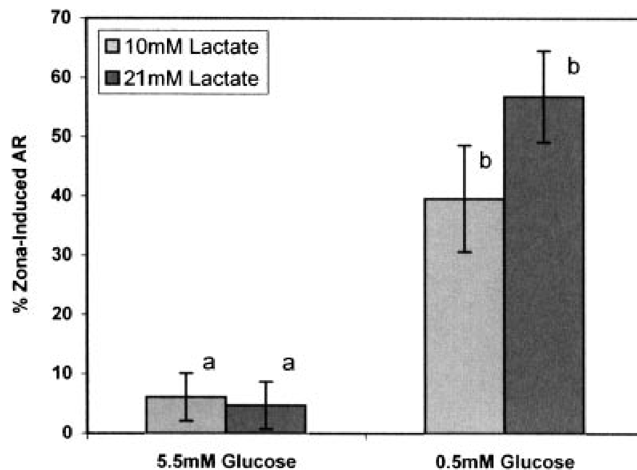

Figure 2. Capacitation assay with sperm incubated for 6 hr (following long pre-incubation) in media that varies in both lactate and glucose. Bars represent the mean percentage of zona-bound sperm that underwent the acrosome reaction. Acrosomal status of zona-bound sperm was determined with acrosomal probe Alexa-SBTI as described in Tollner et al. (2003). Experiment was conducted with sperm from four different males and a total of eight zona per treatment. Error bars represent the standard error of means. Different letters indicate significant differences between treatment means ($P \leq 0.01$).

with near complete loss of the surface coat at 3 hr. Sperm incubated in 35 mM HCO_3 had only a slight loss of DEFB126 at 3 hr. By 6 hr, much of DEFB126 had been released from sperm incubated in 35 mM HCO_3^-. In both 35 and 90 mM HCO_3^--buffered media, sperm tend to initially lose DEFB126 completely from over the flagellum while retaining a faint "dusting" of DEFB126 over the head. After 6 hr in 90 mM HCO_3 media, $47.8 \pm 2.5\%$ ($n = 4$) sperm had complete absence of DEFB126 while only $4.8 \pm 2\%$ of sperm incubated in media with 35 mM had a complete loss of DEFB126.

In *series 2* experiments, sperm were incubated in non-physiologic media that induced capacitation in a large proportion of sperm. It was evident from *series 1* that glucose, and potentially other energy substrates interfered with the process of capacitation. Thus, we evaluated the effects of removing energy substrates from incubation medium. Sperm resuspended into MES medium became capacitated after only 2 hr of incubation as determined by significant increases in both sperm–zona binding and zona-induced acrosome reactions as compared to sperm resuspended in normal BWW medium (Fig. 4). Sperm incubated in MES medium also displayed a near complete loss of DEFB126 from over both the head and flagellum (Fig. 5). No differences in percent motility were observed in sperm incubated in either normal or MES medium, but many sperm in MES medium exhibited vigorous hyperactivated motility, which was not observed in normal medium. Sperm from six different males, all resuspended in MES medium, were co-incubated with viable eggs. Sperm from each male was able to achieve fertilization with a mean fertilization rate of 47.3% (Table 1). In five of the males, fertilization resulted in advanced early embryo development.

We had noticed in preliminary studies that extremes in pH could accelerate acrosome reaction rates of zona-bound sperm (data not shown). We evaluated the effect of adjusting the pH of media with 90 mM HCO_3^- (and periovulatory levels of energy substrates) to 8.0. Sperm exhibited an enhanced response to the elevated pH. At 6 hr of incubation,

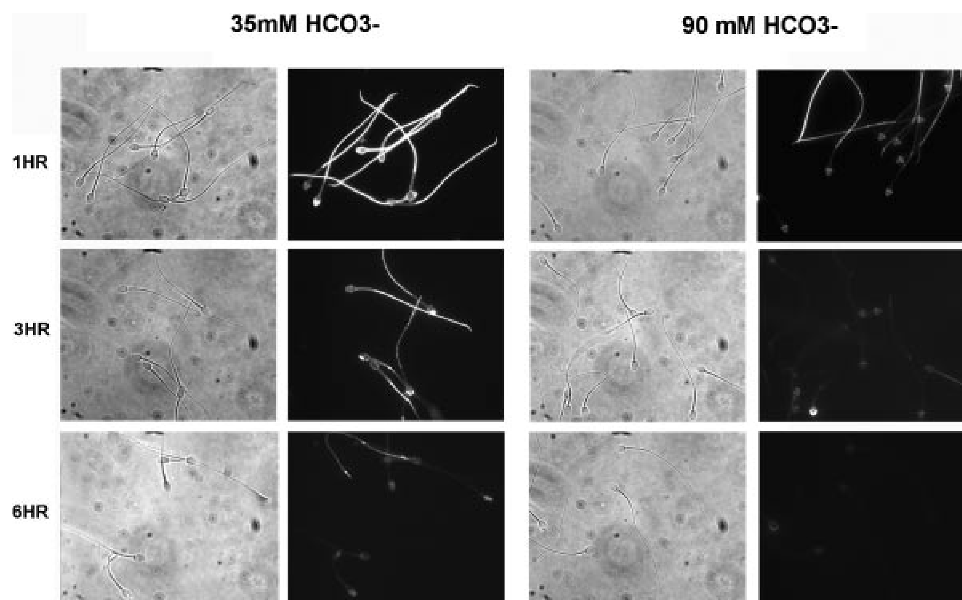

Figure 3. Micrographs of immunofluorescent labeling of DEFB126 on sperm. Pictures are of sperm incubated in either BWW medium with either 35 or 90 Mm HCO_3^- following 1, 3, and 6 hr. Both media contained 5.5 mM glucose/21 mM lactate, but labeling patterns and intensity were nearly identical to that observed of sperm incubated in 0.5 mM glucose/10.5 mM lactate.

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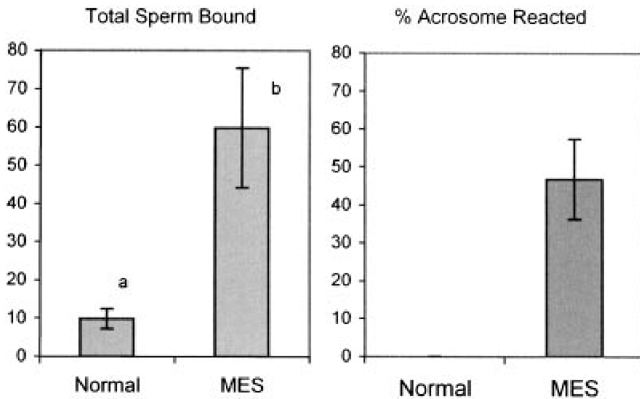

Figure 4. Sperm incubated for 2 hr in medium with minimal energy substrates (MES) were compared to sperm incubated in normal energy substrate supplemented BWW (normal) in the ability to bind to intact zona pellucidae and undergo the acrosome reaction. Bars represent (A) the mean numbers of sperm bound to the zona and (B) the mean percentage of zona-bound sperm that underwent the acrosome reaction. Experiment was conducted with sperm from four different males and a total of eight zona per treatment. Error bars represent the standard error of means. Different letters indicate significant differences between treatment means ($P \leq 0.01$). Sperm incubated in Normal medium did not undergo the zona-induced acrosome reaction and therefore could not be analyzed statistically, however the difference in sperm response from the different media is clearly significant.

$78.5 \pm 9.4\%$ of sperm bound to the zona underwent the acrosome reaction ($N = 3$). Unlike at pH of 7.8, sperm could be induced to acrosome react ($30.7 \pm 13.2\%$) after only 3 hr of incubation at $pH = 8.0$. These sperm, however, were not capable of achieving IVF (attempted with 26 eggs from 2 cycles). Failure in IVF likely was due to poor survival of sperm in insemination drops. No sperm capacitated in medium D displayed motility after the 12-hr co-incubation with eggs, probably owing to the requisite long (overnight) pre-incubation period.

It was clear from *series 1* experiments that the levels of energy substrates had little if any impact on the release of DEFB126 from sperm. In *series 3* experiments, we evaluated the role of pH on the shedding of DEFB126. Mirroring experiments in *series 1*, sperm were washed into media resembling A and B, with the exception that media were primarily buffered with HEPES (both media contained 4 mM HCO_3^-) and adjusted to a pH of either 7.2 or 7.8. Sperm incubated in pH 7.8 media displayed a loss of DEFB126 after 1 hr that was near complete by 3 and 6 hr (Fig. 6). Sperm incubated in pH 7.2 media, on the other hand, displayed only a modest loss of DEFB126 over 6 hr (Fig. 6). Results were very similar to those achieved with media that were buffered with HCO_3^- (Fig. 3), suggesting that absolute media pH, and not bicarbonate concentration, triggers the release of DEFB126. Sperm incubated in HEPES-buffered media did not undergo the zona-induced acrosome reaction, even though numbers of sperm bound to the zona resembled numbers observed with bicarbonate-buffered media (data not shown).

DISCUSSION

In this report, we evaluated formulations of capacitation media in light of what is known about oviductal conditions during the time of ovulation in primates. Our data show that a medium reflecting oviductal fluid conditions of HCO_3^- and glucose at the time of ovulation was optimal for inducing capacitation in macaque sperm. Medium D, with 90 mM HCO_3^- and 0.5 mM glucose induced a 20-fold increase in levels of capacitated sperm as compared to medium A, which contains follicular phase levels of glucose and lactate (Fig. 1). Incidentally, medium A contains levels of HCO_3^- and energy substrates frequently used in human sperm capacitation systems and has been used by our lab and others for processing of macaque sperm for IVF. Significant induction of capacitation was also observed with 35 mM HCO_3^- (medium C) but only if glucose concentration was lowered to 0.5 mM (Fig. 1). The level of lactate appeared to have no significant influence on sperm responsiveness to extremes of glucose concentration in the presence of 90 mM

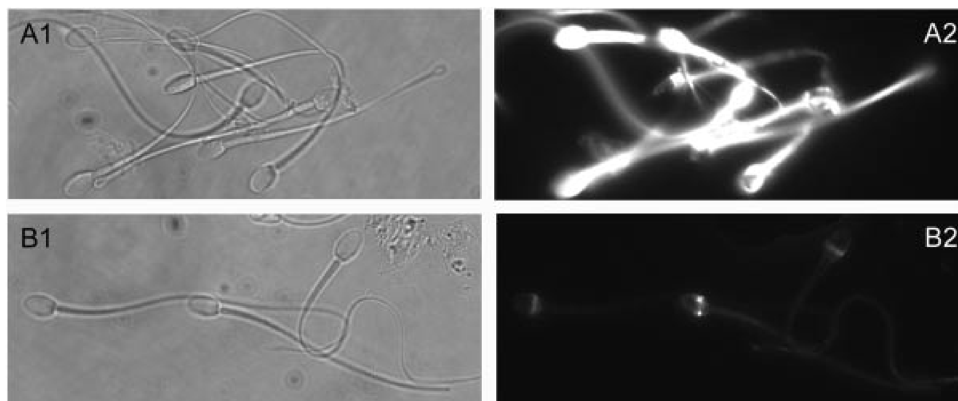

Figure 5. Micrographs of immunofluorescent labeling of DEFB126 on sperm. Pictures are of sperm incubated for 2 hr in either BWW medium with minimal energy substrates (B1, B2) or in BWW medium supplemented with normal levels of energy substrates (A1, A2).

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TABLE 1. IVF Results With Sperm Incubated in Medium Lacking Energy Substrates

Cycle	Male	Total retrieved	Total w/PB's	Total cleaved	% of PB Fertilized	Morula	Blastocyst
1	893	9	7	2	28.6	1	1
2	637	10	8	6	75	2	2
	74	10	5	1	20	1	
3	711	13	6	2	33.3		
	70	13	5	3	60		1
	173	13	6	4	66.6		2
Mean		11.33	6.17		47.25		
SD		1.9	1.2		22.8		

HCO_3^- (Fig. 2). In short, the response of sperm to HCO_3^- at either 35 or 90 mM was effectively blocked by the higher concentration of glucose. These data suggest that the levels of glucose in the lumen of the primate oviduct must drop before sperm can respond to the increased secretion of HCO_3^- at the time of ovulation.

The inhibitory effect of glucose on sperm capacitation has been reported in other species. In guinea pig sperm, the presence of glucose either alone or in combination with lactate and pyruvate inhibited the spontaneous acrosome reaction and delayed IVF (Rogers and Yanagimachi, 1975). In the bull, glucose inhibited the lysophosphatidylcholine (LC)-induced acrosome reaction and the penetration of oocytes of sperm incubated under capacitating conditions with heparin (Parrish et al., 1985, 1989). The anti-fertility effect of glucose coincided with an inhibition of alkalinization of the sperm cytosol (Parrish et al., 1989). Normally, sperm intracellular pH (pH_i) increases by 0.2–0.3 units with heparin-induced capacitation (Parrish et al., 1989; Vredenburgh-Wilberg and Parrish, 1995). From recently derived estimates of rates of glycolysis and pH change in capacitated sperm, Galantino-Homer et al. (2004) calculated that the rate of lactic acid production from aerobic glycolysis is sufficient to provoke acidification of the sperm cytosol by 0.2 pH units, countering the heparin-induced increase in pH_i.

A critical consequence of increased pH_i during capacitation is that under constant CO_2, the carbonic anhydrase buffer equilibrium $[CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-]$ would necessarily shift to the right resulting in increased HCO_3^-, as given by the Henderson–Hasselbalch equation (Boron and Bboulpaep, 2003). The elevation in intracellular HCO_3^- ($[HCO_3^-]_i$) leads to an increase in sperm adenylate cyclase (spAC) activity (Esposito et al., 2004; Hess et al., 2005) and subsequently, the production of intracellular cAMP ($[cAMP]_i$). Elevated $[cAMP]_i$ triggers a number of capacitation-associated events including hyperactivated motility and increased protein kinase A activity leading to protein tyrosine phosphorylation (Jaiswal and Eisenbach, 2002) and changes in plasma membrane lipid composition

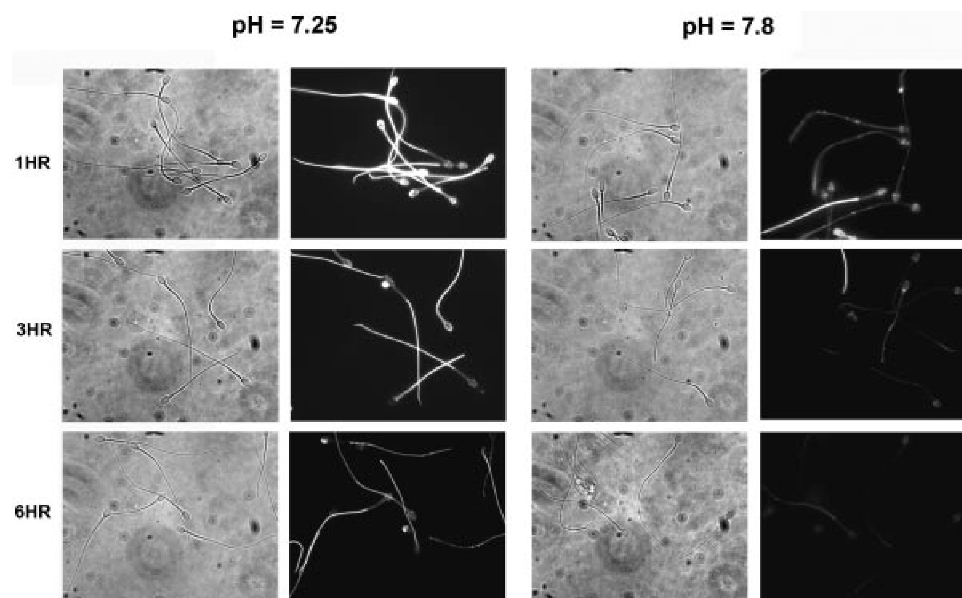

Figure 6. Micrographs of immunofluorescent labeling of DEFB126 on sperm. Pictures are of sperm incubated in either BWW medium buffered with HEPES and adjusted to either pH 7.2 or 7.8. Both media contained 5.5 mM glucose/21 mM lactate. Sperm were fixed and labeled for DEFB126 after 1, 3, and 6 hr of incubation.

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(Harrison and Gadella, 2005, for review). Galantino-Homer et al. (2004) demonstrated that a sustained increase in extracellular pH (pH_o) by decreasing %CO_2 leads to an increase in pH_i in the absence of heparin. As CO_2 exits the sperm, the buffer equilibrium shifts to the left, reducing $[H^+]_i$ again in accordance with Henderson–Hasselbalch equation. This in turn promotes intracellular Cl^- exchange for HCO_3^- via the Cl^-/HCO_3^--exchanger system present in mammalian sperm (Uguz et al., 1994; Zeng et al., 1996) resulting in an elevation of $[HCO_3^-]_i$. Under these conditions sperm appear to be fully capacitated as assessed by protein tyrosine phosphorylation and the LC-induced acrosome reaction (Galantino-Homer et al., 2004). We speculate that under conditions of high levels of glucose, acidification of the sperm cytosol potentially reduces $[HCO_3^-]_i$ by both shifting the buffer equilibrium to the left and by inhibiting Cl^-/HCO_3^--exchanger. Without an elevation of in $[HCO_3^-]_i$, the down stream events resulting in capacitation would not be enabled.

Conditions of *series 2* experiments, either extreme extracellular alkalinity or the elimination of energy substrates, result in greater synchronization of capacitation potentially by increasing $[HCO_3^-]_i$. Driving the pH_o of medium D above 8.0 resulted in over double the acrosome reaction rates observed for same medium at $pH = 7.8$ (78.6% vs. 31.3%, respectively). Assuming that pH_i also increased 0.2 units, this would be roughly the equivalent of increasing the availability of $HCO_3^-{}_i$ by another 52.2 mM (according to Henderson–Hasselbalch). Similarly, incubation in MES medium resulted in high acrosome reaction rates (46.8%; Fig. 4). A 20-fold reduction in glucose (~0.25 mM) would result in less lactic acid production than in medium D, and therefore we would expect a higher pH_i and greater $[HCO_3^-]_i$.

The rate of capacitation is considerably faster, however, for sperm incubated in MES than for sperm incubated medium D. A high percentage of sperm incubated in MES medium are capacitated after only 2 hr following a 30 min pre-incubation period while sperm require an overnight pre-incubation before they can undergo capacitation in medium D at pH 8.0 The difference in the kinetics of sperm response in these incubation conditions is not adequately explained solely by changes in sperm pH_i. While there is an incremental decrease in lactic acid produced in sperm when glucose is dropped below 0.5 mM (Voglmayr and Amann, 1973), there was no significant increase in bull sperm capacitation rates (Parrish et al., 1989). Significant reduction of all energy substrates likely affects other regulatory aspects of capacitation. Reducing glucose along with other energy substrates also promotes capacitation in dog and human sperm (DasGupta et al., 1994; Albarracin et al., 2004). Researchers proposed in these reports that a lack of rapidly glycolizable substrates results in a reduction in ATP levels required for maintaining baseline $Ca^{++}{}_i$ via the action of Ca^{++}-ATPase pumps (DasGupta et al., 1994; Albarracin et al., 2004). These pumps have been implicated in the displacement of Ca^{++} from the cytosol of sperm into either the extracellular space or into intracellular storage sites (Roldan and Fleming, 1989; DasGupta et al., 1994; Baker et al., 2004). As a result of reduced ATP, $Ca^{++}{}_i$ rises (DasGupta et al., 1994; Baker et al., 2004). An increase in free cytosolic Ca^{++} in sperm regulates various aspects of capacitation including hyperactivated motility, adenylate cyclase activity, and chemotaxis (Jaiswal and Eisenbach, 2002; Publicover et al., 2007, for review). As sperm can utilize both lactate and pyruvate to generate ATP through oxidative mechanisms (Jones and Bubb, 2000; Medrano et al., 2006), we speculate that the significant reduction of all energy substrates results in markedly reduced sperm ATP levels. Subsequently, the capacitation-associated rise in $Ca^{++}{}_i$ may be accelerated.

Macaques are reproductively similar to humans and are an effective model system for understanding many aspects of fertilization. Similarities in embryogenesis, gametogenesis, sperm transport, and reproductive immunology between humans and these nonhuman primate counterparts have been well documented (Overstreet and VandeVoort, 1990; Kennedy et al., 1997; Buse et al., 2003; Inder et al., 2004; Luetjens et al., 2005). Furthermore, studies in macaques have been crucial for the refinement of assisted reproductive technologies as IVF and intra-cytoplasmic sperm injection (Wolf et al., 1990; Hewitson, 2004). Yet, differences in the details of gamete interaction do exist. Unlike human sperm that capacitate spontaneously in vitro, macaque sperm require activation with exogenous compounds, typically a combination of exogenous cyclic adenosine monophosphate (cAMP) and caffeine (Boatman and Bavister, 1984). As a result, activator compounds are essential for IVF in the macaque (VandeVoort, 2004). Pretreatment of macaque sperm with activators usually results in fertilization rates similar to those observed in human IVF utilizing normal sperm (VandeVoort et al., 2003; Liu et al., 2004). On the other hand, blastocyst development in vitro in the macaque (Wolf et al., 1990; Schramm and Bavister, 1996) lags behind that in the human (Menezo et al., 1998; Sandalinas et al., 2001) using similar embryo culture conditions.

Some reports suggest that low concentrations of the activator compounds residual in insemination drops could have deleterious effects on early embryo development. Treatment of sperm with caffeine is common in some hooved species, and while treatment improves IVF rates it can also decrease the rate of blastocyst formation (Tatham et al., 2003; Mao et al., 2005). Like wise, treatment of human sperm with caffeine to improve motility for IVF resulted in delayed embryo development compared to IVF with untreated sperm (Imoedemhe et al., 1992). Similarly, pronuclear mouse embryos incubated with low levels of caffeine (0.16 mM) frequently stopped developing after one to three cell cycles (Scott and Smith, 1995). We speculate that exposure of macaque pronuclear embryos to caffeine during extended coincubations with sperm could explain the differences in embryo development rates observed between macaques and humans following IVF.

Rapid synchronization of sperm capacitation with MES has usefulness for macaque IVF. To the best of our knowledge, we are the first to achieve fertilization of macaque eggs in vitro without using exogenous compounds to activate sperm. Although the benefits of an "activator-free" system are not clear from this preliminary report, we speculate that long-term studies will show that IVF medium free of

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activator compounds will result in improved rates of early embryo development. Furthermore, elimination of activator compounds should enable researchers in monkey IVF to more conveniently utilize immature oocytes retrieved following superovulation.

In vivo, oocytes acquire meiotic competence at the early antral stage of the follicle but are kept in meiotic arrest at prophase I by high levels of intracellular cAMP (Mehlmann, 2005). Following a surge of luteinizing hormone, or separation from follicular cells in vitro, the level of cAMP in oocytes declines, meiosis resumes, and oocytes advance to metaphase II (MII) and await fertilization (Mehlmann, 2005). Maturation of oocytes removed from their follicles can be inhibited by membrane permeant analogs of cAMP (Sato et al., 1985; Warikoo and Bavister, 1989) or by inhibitors of cAMP-specific phosphodiesterases, such as hypoxanthine (Warikoo and Bavister, 1989; Fagbohun and Downs, 1990) or the methylxanthines caffeine (Jagiello et al., 1972; Prather and Racowsky, 1992; Kren et al., 2004) or 3-isobutyl-1-methylxanthine (Fagbohun and Downs, 1990; Tsafriri and Reich, 1999). Due to the potential inhibitory effects of activator compounds on oocyte maturation, practitioners of monkey IVF select oocytes retrieved from superovulated females that have advanced to metaphase II (Wolf, 2004). Of the remaining oocytes, most are at metaphase I (MI) and will spontaneously progress to MII over the next 6–12 hr. Fertilizing these lagging oocytes is logistically difficult; researchers must repeatedly check for completion of meiosis I (extrusion of first polar body) and arrange for fresh sperm samples. By contrast, use of an activator-free IVF system potentially enables researchers to inseminate MI and MII oocytes at the same time, provided capacitated sperm survive over the maturation interval.

For now, the practicality of MES is perhaps limited, with fertilization about 40% lower than rates reported in studies that use activator compounds (VandeVoort, 2004; for review). Declining sperm vigor may explain the decreased efficiency we experience with MES. After 2 hr in MES, the majority of sperm exhibit hyperactivated motility but by 6 hr few sperm have sustained vigorous progressive motility. Inspection of insemination drops after 12 hr revealed that few sperm bound to eggs and in suspension were still motile (data not shown). This is not surprising in light of considerable evidence that glycolyzable and/or mitochondrial substrates are required for the maintenance of sperm motility (Ford, 2006, for review). At present, we are evaluating the effect of altering the balance of metabolites in fertilization medium with the goal of preserving sperm vigor while inducing capacitation more gradually.

We speculate that alkalinization of the oviduct is the key event that triggers the release of macaque sperm from the oviductal reservoir. Sperm lose DEFB126 from their surface during 1–3 hr of incubation in alkaline conditions similar to what is reported in the macaque oviduct at the time of ovulation (Fig. 3). Absolute pH and not $[HCO_3^-]_o$ appears to mediate this effect as a comparable release of DEFB126 from sperm occurs in HEPES-buffered medium with 5.5 mM glucose (Fig. 6). Since DEFB126 appears to be the primary molecule that tethers sperm to oviductal epithelia (Tollner et al., 2008), the loss of DEFB126 from the sperm surface would enable sperm to detach from the mucosal surface and begin moving towards the ampulla. Alkalinization therefore may represent a signal to coordinate the movement of sperm out of the oviductal reservoir with transport of the egg into and down the oviduct.

The implication of our collective data is that, at the time of ovulation, conditions of pH, HCO_3^-, and glucose in the oviductal lumen are sufficient to trigger the release of sperm from epithelium in a fully capacitated state. It is interesting to note that only a third of sperm incubated in medium D for 6 hr undergo capacitation (Fig. 1). It is clear from *series* 2 experiments that far more sperm can potentially respond if conditions are pushed to either extremes of alkalinization or energy substrate deprivation. Perhaps the means by which the oviduct controls the numbers of capacitated sperm that are ushered to the site of fertilization is by the gradual and simultaneous changes in the luminal fluid HCO_3^-, and glucose concentration. Some sperm will be "early" responders, becoming detached from oviductal epithelia at mildly alkaline conditions and intermediate levels of glucose, other sperm will require more extreme conditions to induce detachment and will therefore leave the reservoir some moments later. The asynchronous response of sperm to capacitation conditions may reflect subtle physiological differences among individual sperm due to differences in the absolute age of sperm from the same ejaculate (Jaiswal and Eisenbach, 2002). The net effect: gradually shifting oviductal fluid conditions ensure that there is a continuous trickle of capacitated sperm leaving the isthmus as the egg is shuttled to the site of fertilization.

MATERIALS AND METHODS

Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) unless stated otherwise.

Antibody Production and Preparation

Antibodies were developed to sperm surface protein DEFB126 as described previously (Yudin et al., 2003). Macaque sperm were washed through 80% Percoll, resuspended in Dulbecco's phosphate buffered saline (DPBS), pelleted by centrifugation (300*g*) for 10 min, and treated with phospho-inositol-phospholipase C (3 units/ 80×10^6 sperm). Sperm samples were incubated at 37°C. The supernatant was passed through a 0.22 μm syringe filter, concentrated and electrophoretically separated on an 8–16% gel. The gels were stained with Gel Code Blue (Pierce, Rockford, IL). For DEFB126 collection, the 53 kDa band was cut from the gel and electro-eluted (Yudin et al., 2003). After complete electro-elution, samples were chemically reduced with 0.1 M dithiothreitol (DTT) and electrophoresed on a 16% gel. The entire 31–35 kDa band (the apparent molecular weight of the reduced protein) was cut from the gel and electro-eluted for immunization. Development of antibodies to DEFB126 in rabbits was thoroughly described previously (Yudin et al., 2003). The resulting serum samples were initially heat inactivated (56°C/30 min) and then precipitated with ammonium sulfate (0.24 g/ml). The ammonium sulfate was added slowly over a 4 hr period at 4°C. The precipitated Ig was pelleted and resuspended in DPBS (Life Technologies, Rockville, MD) and dialyzed overnight. Total protein concentration of precipitated Ig was determined with a BCA protein analysis system (Pierce). The Ig was

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stored at −20°C. Ig only recognized DEFB126 (a single band at 53 kDa) on Western blots of whole sperm (Yudin et al., 2003). Anti-DEFB126 Ig was used for localization of DEFB126 on sperm.

Semen Collection and Sperm Preparation

Six adult male cynomolgus macaques (*Macaca fascicularis*) were housed at the California National Primate Research Center (CNPRC). Animal protocols were reviewed and approved in advance by the Animal Care and Use Committee of the University of California, Davis; all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Semen samples were collected by electro-ejaculation (Sarason et al., 1991). Each ejaculate was collected into a 15 ml centrifuge tube containing 5 ml of Biggers, Whitten and Whittingham medium modified with addition of 21 mM HEPES buffer and 4 mM sodium bicarbonate (here after referred to as mBWW; Irvine Scientific, Santa Ana, CA). After 1 hr, the samples were checked for motility and only those samples having greater than 70% motile sperm were used in the experiments. Following removal of coagulum, sperm were pelleted by centrifugation at 300*g* for further processing. Sperm were then capacitated overnight as described previously (Tollner et al., 2003). Briefly, sperm were washed through a 3.5 ml column of 80% Percoll and suspended in BWW medium containing 30 mg/ml BSA buffered with 35.7 mM sodium bicarbonate (here after referred to as BWW). Sperm were washed two more times at 300*g* and resuspended in BWW medium to a concentration of 10–15 × 10^6/ml. Sperm underwent one of two incubation periods (or pre-incubations) before experimentation. Sperm were either pre-incubated at 39°C and 5% CO_2 for 1 hr (short) or overnight (long) at 28°C and 5% CO_2. Following pre-incubation, sperm were washed into assorted media and placed into a 39°C incubator and 5% CO_2 for an additional 1, 3, or 6 hr depending on the experiment. For some experiments sperm were treated with 2 µg/ml H33348, a fully permeable chromatin dye, for 15 min prior to being washed into final incubation medium to later facilitate scoring numbers of sperm adhered to the zona pellucida.

Series 1—Experiments With Physiologic Media

Following both short and long pre-incubations, sperm were pelleted by centrifugation (300*g*) over 10 min. Pellets were resuspended into 400–500 µl of BWW. The sperm suspension was divided into four equal parts, each aliquot was washed by centrifugation twice (300*g*, 10 min each) in one of four different media. Sperm were resuspended to 2 × 10^6 sperm/ml into the same medium used for washing and incubated for several hours at 39°C and 5% CO_2. At 1, 3, and 6 hr, sperm were assessed for capacitation, presence of surface coating protein DEFB126, and the ability to fertilize eggs in vitro. All media were based on original BWW formula, containing same composition of salts (except for NaCl which was adjusted to normalize the osmolarity), pyruvate, and antibiotics. All media also contained 3 mg/ml BSA. Media were varied with respect to both energy substrates (glucose and lactate) and HCO_3^- concentration as follows: (A) 5.5 mM glucose + 21 mM lactate/35 mM HCO_3^-, (B) 5.5 mM glucose + 21 mM lactate/90 mM HCO_3^-, (C) 0.5 mM glucose + 10.5 mM lactate/35 mM HCO_3^-, and (D) 0.5 mM glucose + 10.5 mM lactate/90 mM HCO_3^-. Media were incubated at least 1 hr at 39°C and 5% CO_2 before use with sperm. Following incubation, the pH of media containing 35 mM HCO_3^- ranged from 7.2–7.3, and media containing 90 mM HCO_3^- ranged from 7.75–7.85. All media were adjusted to 290 mOsm.

To investigate the role of lactate, experiments were repeated following long pre-incubations using a second set of media conditions. All media were based on BWW as described above and contained 90 mM HCO_3^-, but were varied with respect to glucose and lactate concentration as follows: (a) 5.5 mM glucose + 10 mM lactate, (b) 5.5 mM glucose + 21 mM lactate, (c) 0.5 mM glucose + 10 mM lactate, (d) 0.5 mM glucose + 21 mM lactate.

Series 2—Experiments With Nonphysiologic Media

Following the short incubation, sperm were pelleted by centrifugation (300*g*) over 10 min. Half the pellet (200 µl) was resuspended into 4 ml complete BWW with 3 mg/ml BSA. The remaining half was resuspended into 4 ml of BWW with 3 mg/ml BSA that contained no glucose, lactate or pyruvate, resulting in a 20-fold dilution of the original levels of substrates (minimal energy substrates or MES). Prior to use with sperm, the pH and osmolarity of both media were adjusted to 7.4 and 290 mOsm, respectively. Sperm were incubated for 2 hr at 39°C and 5% CO_2. Also, the experimental setup from *series 1* was repeated using just medium D, but with the pH of the medium adjusted to 8.0–8.1. Following incubations in nonphysiologic media, sperm were assessed for capacitation, presence of surface coating protein DEFB126, and the ability to fertilize in vitro.

Series 3—Conditions That Release DEFB126

Following the long incubation, sperm were pelleted by centrifugation (300*g*) over 10 min. The pellet was divided into two, each half was washed 2× by centrifugation (300*g*, 10 min each) in one of two mBWW media (Irvine Scientific) that differed only in pH (7.2 vs. 7.8). Both media had same levels of salts, energy substrates (same as medium A above), and BSA (3 mg/ml). Sperm were resuspended to 2 × 10^6/ml into the same medium used for washing and incubated for several hours at 39°C, without CO_2. At 1, 3, and 6 hr, sperm were assessed for capacitation and the presence of surface coating protein DEFB126.

For all experiments, 200 sperm/treatment were scored for percent motility and progressive motility as described previously (Tollner et al., 2004).

Sperm–Zona Pellucida Interaction

Assessment of capacitation was performed using an assay that quantitates the zona-pellucida-induced acrosome reaction in real-time (Tollner et al., 2003). Briefly, ovaries were obtained at necropsy from adult female cynomolgus macaques at the CNPRC. Zona pellucida-intact immature oocytes were collected from the ovaries and were frozen at −80°C in 2 M dimethyl sulfoxide (DMSO) in DPBS according to previously published protocols (VandeVoort et al., 1992). The oocytes were thawed, rinsed, and dried onto glass slides. Four posts of silicon grease containing 50–75 µm silica beads were deposited at four corners around a single zona. The zona was rehydrated with a 5 µl drop of medium and a 22 mm × 22 mm glass coverslip was carefully pressed down onto the posts until the grease was completely flattened. Prior to the addition of sperm, sperm suspensions were treated with fluorochrome-conjugated soybean trypsin inhibitor (ALEXA-SBTI) which labels the anterior head of sperm undergoing the acrosome reaction (Tollner et al., 2000). The slide was warmed for 5 min on a microscope stage warmer set at 37.5°C prior to the addition of sperm. A 40 µl aliquot of sperm was added to the warmed slide at the edge of the coverslip. Sperm were drawn under the coverslip and around the zona by capillary action. Sperm were observed with a Lietz Laborlux S microscope (Wild Leitz, GmbH, Wetzlar, Germany) equipped with 200 W mercury fluorescence vertical illuminator and a 1-Lambda Ploemopac incident light fluorescence illuminator employing an I3 filter cube with a BP 450–490 excitation filter, a RKP 0510 dichromatic mirror, a LP 515 suppression filter, and a 40X fluorescence/phase objective. A timer was started at the moment the first motile sperm attached to the zona. After 3 min, the total number of zona-bound sperm and the number of sperm acrosome labeled with ALEXA-SBTI were counted, starting at the 12 o'clock position of the zona and working clockwise to the starting point. The count required approximately 15 sec for completion. Observations were made in two different chambers, each with one zona.

In the "fixed zona" method, loosely adhered sperm are not rinsed away. As such, this assay is not sensitive in determining

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differences in zona pellucida binding ability of sperm. To assess sperm–zona binding, two zona, thawed and rinsed as described above, were transferred with a fine bore glass pipette into sperm suspensions maintained under equilibrated mineral oil (39°C and 5% CO_2) and allowed to co-incubate for 2 min. After co-incubation, zonae were transferred through three rinse drops of HEPES-buffered BWW (two aspirations per drop) to remove loosely or non-specifically bound sperm (VandeVoort et al. 1992). Zonae were then placed on a glass slide along with 5 μl of medium between four posts of silicon grease containing 100-μm glass beads. A glass coverslip was added and the number of zona-bound sperm was scored by visualizing the Hoechst stain with the Leitz 50× fluorescence objective and an A filter cube with a BP 340–380 excitation filter, a RKP 0400 dichromatic mirror, and a LP 425 suppression filter.

In Vitro Fertilization

Adult female cynomolgus macaques were housed at the CNPRC. Animal protocols were reviewed and approved in advance by the Animal Care and Use Committee of the University of California, Davis; all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. After onset of menstruation, female monkeys were hormonally stimulated by one of two stimulation protocols. Animals were stimulated by twice-daily injections of 37.5 IU (75 IU total/day) recombinant cynomolgus monkey FSH for 7 days and the next morning recombinant hCG (1000 IU Ovidrel; Serono, Rockland, MA) was administered. Oocytes were aspirated from follicles the morning after the last dose of rFSH by an ultrasound-guided procedure (VandeVoort and Tarantal, 1991, 2001). Aspirates containing oocytes were maintained at 35°C within a temperature-controlled isolette at all times during experiments. Aspirates were transferred onto a 24-mm diameter, 70-μm pore-size filter (Netwell Inserts 3479, Corning, Inc., Acton, MA), and blood cells were rinsed away with fresh Tyrode's lactate (TL) medium + HEPES buffer + 0.1 mg/ml polyvinyl alcohol (PVA) medium (TL-HEPES-PVA), and the cumulus–oocyte complexes were recovered from the filter. The medium and the aspiration method itself are described more fully by VandeVoort et al. (2003). All chemicals and solutions were purchased from Sigma Aldrich Chemical Co. or Gibco (Grand Island, NY) unless otherwise specified. Oocytes were recovered and placed in drops of HECM-9 medium (McKiernan and Bavister, 2000) under oil until the time of insemination later on the same day. Oocytes were rinsed and transferred into sperm capacitation medium (either D or MES) at 37°C under oil and inseminated with cynomolgus macaque sperm (pre-incubated in either D or MES) according to standard procedure for IVF of rhesus macaque oocytes (Schramm and Bavister, 1996). Eggs were co-incubated with sperm treated as described in *series 2* experiments for 12 hr and then washed and resuspended in TL-BSA medium (Enders et al., 1989). The next morning, oocytes were transferred into 70 μl drops of HECM-9 medium under oil (37°C) and incubated at 37°C in a humidified atmosphere of 5% CO_2, 10% O_2, and 85% N_2 for 48 hr. After the 48-hr incubation, noncleaved oocytes were fixed for assessment of nuclear status. The remaining embryos were transferred into 70 μl drops of HECM-9 medium with 5% bovine calf serum (Gem Cell, Woodland, CA) under mineral oil and incubated as described above. Embryos were transferred to fresh medium every other day until no further development was observed.

Labeling of DEFB126 on the Sperm Surface

Sperm were labeled immunofluorescently for DEFB126 as described previously (Tollner et al., 2004). Sperm were fixed in 2% paraformaldehyde in DPBS for 20 min. After fixation, sperm were thoroughly washed (2–3×) in blocking solution (1% BSA, 0.1% NaN_3, 1% gelatin/DPBS). Sperm samples were suspended in anti-DEFB126 Ig (10 μg Ig/ml), gently rolled for 1 hr, and then washed 3× in blocking solution and resuspended in a solution of 20 μg/ml goat anti-rabbit IgG Alexa 488 (Molecular Probes, Eugene, OR) in blocking solution. The samples were again rolled for 1 hr and then thoroughly washed and resuspended in a fluorescent stabilization medium (50% glycerol, 0.2% NaN_3, 1% paraformaldehyde/DPBS). Photomicrographs were taken of representative cells using a cooled CCD digital camera (Magnafire, Optronics, Santa Barbara, CA) mounted on a Leitz Laborlux S microscope equipped as described above. Optics included a 3.3X intra-ocular magnifier (Scientific Instruments, Sunnyvale, CA) and a Zeiss 63X oil emersion fluorescence objective (JH Technologies, San Jose, CA). Initial images were captured using Magnafire 2.0 software (Optronics) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA) for production of figures. In some experiments, 200 sperm/treatment were scored for the presence of DEFB126 on their surface. Sperm were considered positive for surface DEFB126 if fluorescence was detectable on either the sperm flagellum or head.

Statistical Analysis

For *series 1* experiments, a two-factor analysis of variance (ANOVA) was used to assess differences in acrosome reaction rates due to changes in HCO_3^- (factor 1) and energy substrates (factor 2) in sperm incubation media. For all other experiments, a single factor (sperm treatment) ANOVA was used. Response means of treatments were compared using Tukey's range test. All experiments (replicates) generated values of either numbers of sperm bound/zona for every treatment or % acrosome reacted sperm of total sperm bound/zona. Data were reported as mean number sperm/zona ± standard error of the mean (SEM) or mean % acrosome reacted sperm/zona ± standard error of the mean. All ANOVAs met assumptions of factor independence, as well as normality of data distribution and variability. Analyses were conducted with SAS statistical program (SAS Institute, Cary, NC) according to the principles described by Steel et al. (1997).

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