Relationship Between Fertilizing Ability and cAMP in Human Spermatozoa

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The goal of this study was to investigate the relationship between cAMP and the fertilizing ability of human spermatozoa. Levels of cAMP were measured in human spermatozoa during 6-hour *in vitro* incubation in capacitation medium, in both the presence and absence of phosphodiesterase (PDE) inhibitor. The fertilizing ability of the same samples was assayed with zona-free hamster eggs. In the absence of PDE inhibitor, no relationship was apparent between mean cAMP levels, which remained at 3–4 pmol cAMP/10^7 spermatozoa, and mean fertilizing ability, which increased from 4% at the start of the incubation to 33% at 6 hours. In the presence of PDE inhibitor (7 mM caffeine or 10 mM theophylline), cAMP concentrations increased within minutes to 3–4 times control levels. Despite this increase in cAMP, there was no immediate change in fertilizing ability. This was true whether PDE inhibitor was present from the start or added to control sperm after 6 or 22 hours of incubation. However, once the sperm were exposed to PDE inhibitor for 4–6 hours, they fertilized a significantly greater proportion of eggs than did control samples. These results suggest that PDE inhibitors, or elevated cAMP levels, do not immediately induce the acrosome reaction, but rather appear to reduce the amount of time required for capacitation to occur *in vitro*.

Key words: capacitation of human spermatozoa, cAMP in human spermatozoa, fertilization of zona-free hamster eggs.

Mammalian spermatozoa acquire the ability to fuse with an egg only after undergoing the acrosome reaction (Yanagimachi and Noda, 1970)—a morphologic change preceded by certain physiologic processes, collectively termed capacitation (Austin, 1951; Chang, 1951). Our understanding of the biochemical bases of capacitation and acrosome reaction is incomplete (Meizel, 1978; Yanagimachi, 1981). In some studies, cAMP has been implicated as playing a regulatory role in these processes (Hoskins and Casillas, 1975; Garbers and Kopf, 1980). For example, stimulatory effects on *in vitro* acrosome reaction and/or fertilization have been produced by the addition of cAMP analogs or phosphodiesterase (PDE) inhibitors (which elevate intracellular cAMP) to the sperm of the rabbit (Rosado et al, 1974; Reyes et al, 1978), rat (Toyoda and Chang, 1974), mouse (Fraser, 1979), guinea pig (Hyne and Garbers, 1979), and hamster (Mrsny and Meizel, 1980). However, inhibitory effects of these treatments have also been observed in hamster and guinea pig spermatozoa (Rogers and Garcia, 1979). In addition to effects on the acrosome reaction and fertilization, a stimulation of metabolism and motility has been induced in the sperm of many species, including the human, by treatments which elevate cAMP (Garbers and Kopf, 1980).

The goal of the present study was to assess the possible relationship between cAMP content and the fertilizing ability of human spermatozoa. Little is known about the capacitation process in human spermatozoa, since the culmination of that process—the acrosome reaction—is difficult to monitor by light microscopy, due to the small size of the acrosome. However, the fertilizing ability of human spermatozoa, incubated with zona-free hamster eggs, can be used as an indirect assay for capacitation and acrosome reaction, since only acrosome-reacted spermatozoa will fertilize the eggs (Yanagimachi et al, 1976). Using this fertilization assay to monitor capacitation, we measured the cAMP content of human spermatozoa during a 6-hour capacitation period to determine whether

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cAMP levels change during capacitation. Samples of the same spermatozoa were also incubated with PDE inhibitors to acertain the degree to which the inhibitors raise cAMP levels in human spermatozoa and to determine whether PDE inhibitor treatment alters the fertilizing ability of the spermatozoa. Results indicate that while the absolute levels of cAMP in human spermatozoa do not fluctuate significantly during capacitation, PDE inhibitors, which rapidly elevate cAMP levels, stimulate the fertilizing ability of the spermatozoa following 4–6 hours of incubation with the drugs.

Materials and Methods

Semen Samples

Semen samples for this study were donated by a total of ten healthy volunteers. Samples were collected following at least 48 hours of sexual abstinence, allowed to liquify (30–45 minutes) at room temperature, and were examined by routine semen analysis (Rogers et al, 1979). Only samples which met the following criteria were used in this study: concentration, >80 million spermatozoa/ml; volume, >1 ml; motile spermatozoa, >50%; quality of motility, 3–4 on a subjective scale of 1 (weakly motile) to 4 (vigorously motile); progressive motility, 3–4 on a subjective scale of 1 (motile but not progressing) to 4 (majority of spermatozoa showing rapid progression in a straight line); and oval forms (normal morphology), >60%.

In vitro *capacitation and fertilization assay*

Human spermatozoa were capacitated *in vitro* as described previously (Rogers et al., 1979), except that incubation times were varied as described below. Briefly, spermatozoa were washed three times by centrifugation (600 × g, 5 minutes each) to remove seminal fluids and were resuspended in BWW culture medium (Biggers et al., 1971) with or without the phosphodiesterase (PDE) inhibitors, caffeine (7 mM), or theophylline (10 mM) (Sigma Chemical Co., St. Louis, MO). The concentrations of PDE inhibitor tested are representative of those reported to stimulate the motility and metabolism of human spermatozoa. (Garbers and Kopf, 1980; Cheng and Boettcher, 1981).

Spermatozoa were incubated (37 C in air) in capped polypropylene culture tubes (#2063, Falcon Plastics, Oxnard, CA, or Provials, Cooke Laboratory Products, Alexandria, VA) in volumes of 0.5 ml, at a concentration of 1×10^7 spermatozoa/ml. Incubations were carried out for 6 hours, a sufficient period of time to allow capacitation and acrosome reaction of human spermatozoa, as was evidenced by their ability to fertilize zona-free hamster eggs (Yanagimachi et al, 1976). At four times (0, 2, 4, and 6 hours) during this incubation, tubes were removed from the incubator, and the spermatozoa were assayed for both fertilizing ability and cAMP content. At each time, a small aliquot (0.02 ml) was also examined for percent motile spermatozoa, for quality of motility, and for progressive motility as described above.

To carry out the fertilization assay, an aliquot (0.1 ml) of sperm was transferred to a plastic Petri dish (#1008, Falcon Plastics) and covered with mineral oil. Approximately 15–25 zona-free hamster eggs (obtained by superovulation and processed as described in Rogers et al, 1979) were added to the spermatozoa, and the eggs and sperm were incubated together (37 C in air) for 2 hours to allow fertilization to occur. Fertilization was assessed by examining the eggs with phase contrast microscopy for the presence of decondensed sperm heads or male pronuclei within the egg cytoplasm (Yanagimachi et al, 1976). The percentage of eggs fertilized and the average number of fertilizing spermatozoa per egg tested (designated the "Fertilization Index") were recorded.

Assay of cAMP Content of Human Spermatozoa

Tubes of spermatozoa were frozen in liquid nitrogen after 0, 2, 4, and 6 hours of incubation in the presence and absence of PDE inhibitor, and were stored frozen until assayed for cAMP. Cyclic AMP was extracted from the sperm by submerging the tubes in boiling water for 15 minutes (Erickson et al, 1979; Homonnai et al, 1975). Disrupted sperm and precipitated protein were pelleted by centrifugation (2000 × g, 20 minutes at 4 C) and the supernatant fluid was assayed for cAMP content by radioimmunoassay (Steiner et al, 1969), using a kit from New England Nuclear (Cambridge, MA). The sensitivity of this assay was increased to the femtomole range by acetylation (Harper and Brooker, 1975).

Statistical Analysis

Differences between the percent fertilization or the fertilization index observed for control spermatozoa and for spermatozoa incubated with PDE inhibitor were analysed with a nonparametric test—the Wilcoxon signed-rank test for paired data (Dixon and Massey, 1969). This test was selected because the fertilization statistics (proportion of eggs fertilized or fertilization index) are nonparametric statistics, and because we could not assume normal distribution of the data. Mean concentrations of cAMP in spermatozoa incubated with or without PDE inhibitor were compared using Student's t test. In each case, differences were considered statistically significant at the level of $P < 0.05$.

Results

Levels of cAMP in Human Spermatozoa during Capacitation in the Presence and Absence of PDE Inhibitor

The levels of cAMP in human spermatozoa incubated for 0–6 hours in capacitation medium in the presence or absence of caffeine or theophylline are shown in Figure 1. In the absence of PDE inhibitor, the mean cAMP content remained at 3–4 pmol cAMP/10^7 spermatozoa during the 6-hour incubation. In the presence of caffeine or theophylline, cAMP levels, at each time tested, were significantly elevated to 3–4 times the levels observed in the untreated controls (Fig. 1). There

were no significant differences between cAMP levels in the presence of caffeine as compared with that of theophylline at any time period (Fig. 1). However, mean levels of cAMP in the presence of caffeine or theophylline were significantly higher ($P < 0.05$, sign considered) at 6 hours than they had been at 0 hours, an observation which could indicate that cAMP accumulated with time. To examine further the kinetics of the PDE inhibitor-induced elevation of cAMP, caffeine and theophylline were added to tubes of sperm which had been incubated in capacitation medium for 0, 2, 4, or 6 hours (final concentrations: 7 mM caffeine, 10 mM theophylline). These tubes were frozen 15 minutes later and assayed for cAMP content. The levels of cAMP in these samples were not significantly different from those observed when the PDE inhibitors were present from the start of the incubation. Just how rapidly the cAMP levels increased in the presence of PDE inhibitor was demonstrated by freezing samples of freshly prepared control spermatozoa at intervals from 10 seconds to 5 minutes after the addition of caffeine (final concentration, 7 mM). The concentration of cAMP in these tubes increased rapidly with time, plateauing within only 1 minute of exposure to caffeine.

Effect of PDE Inhibitors on the Fertilizing Ability of Human Spermatozoa during Capacitation In Vitro

The fertilizing ability of human spermatozoa, incubated in capacitation medium with and without caffeine (7 mM) or theophylline (10 mM), was assayed at 0, 2, 4, and 6 hours of incubation. Neither caffeine nor theophylline had any observable effect on the fertilizing ability of human spermatozoa at the start of the incubation. At this time, the mean percent fertilization was low in all groups (Table 1), and many of the samples did not fertilize any eggs. After 2 hours of incubation, most of the control samples fertilized a small proportion of eggs (mean = 15%). Some of the caffeine- or theophylline-treated samples fertilized a higher proportion of eggs at this time, but the effect was not consistent among different donors and, as a result, the difference was not statistically significant (Table 1). However, in the 4-hour assay, the caffeine-treated samples showed a significant ($P < 0.05$) increase in fertilizing ability, and in the 6-hour assay, both the caffeine- and theophylline-treated samples fertilized a significantly higher ($P < 0.01$) proportion of eggs when compared with controls (Table 1). The fertilization index (mean number of decondensed sperm per total number of eggs) was also significantly higher in the PDE inhibitor-treated samples than in the controls in the 6-hour assay (Table 1).

The stimulation of fertilization observed at 4–6 hours of incubation did not appear to be due to PDE inhibitor-induced improvements in sperm motility. The percentage of motile sperm and the quality of motility were similar in the treated and untreated samples at the times (4–6 hours) when the fertilizing ability of the treated samples was elevated (Table 2). The only effect of PDE inhibitors on sperm motility which was observed in this study was that the inhibitor-treated samples showed less forward progression, especially at the earlier observation times (0–2 hours), when com-

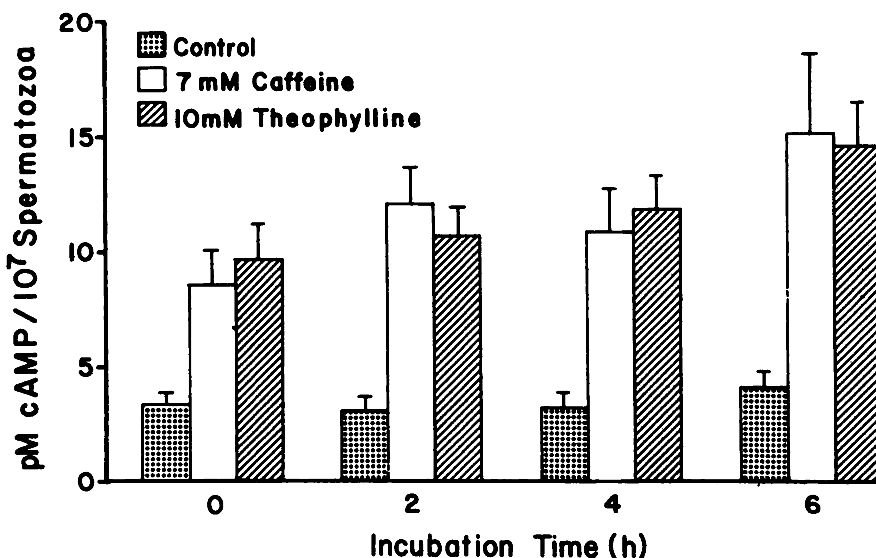

Fig. 1. Concentration of cAMP in human spermatozoa during *in vitro* capacitation in medium with or without PDE inhibitor. Each bar is the mean concentration of cAMP (± SEM) in 9–11 separate experiments. Cyclic-AMP was significantly ($P < 0.02$) elevated in treated as compared with control samples at each time tested.

TABLE 1. Effect of Caffeine and Theophylline on the Fertilizing Ability of Human Spermatozoa during *In Vitro* Capacitation

Assay Time (Hours)*	Treatment	Mean (± SEM) % Fertilization	Mean (± SEM) Fertilization Index†	Number of Experiments
0	Controls	4 ± 3	0.04 ± 0.05	7
	Caffeine	8 ± 5	0.08 ± 0.04	5
	Theophylline	8 ± 4	0.08 ± 0.04	6
2	Controls	15 ± 5	0.15 ± 0.05	5
	Caffeine	45 ± 18	0.84 ± 0.37	5
	Theophylline	34 ± 12	0.45 ± 0.16	5
4	Controls	27 ± 7	0.41 ± 0.16	10
	Caffeine	57 ± 11‡	0.88 ± 0.20	8
	Theophylline	33 ± 7	0.42 ± 0.13	8
6	Controls	33 ± 11	0.55 ± 0.29	8
	Caffeine	69 ± 8§	1.94 ± 0.74§	8
	Theophylline	65 ± 8§	1.47 ± 0.59§	8

* Amount of time spermatozoa were incubated ± caffeine (7 mM) or theophylline (10 mM) prior to addition of eggs for the fertilization assay.
† Fertilization Index = mean number of decondensed spermatozoa per egg tested.
‡ Significantly different from controls (Wilcoxon signed-rank test for paired data), $P < 0.05$.
§ Significantly different from controls (Wilcoxon signed-rank test for paired data), $P < 0.01$.

pared with controls. Instead of progressing in a straight line, as was typical of control spermatozoa, a proportion (25–50%) of the inhibitor-treated spermatozoa showed an agitated motility, characterized by sudden changes in direction. These samples were given high scores for quality of motility but lower scores for net forward progression (Table 2).

To determine whether a PDE inhibitor would stimulate fertilization with longer exposure times, a second series of experiments was conducted. Spermatozoa were incubated with and without caffeine (7 mM) and tested for fertilizing ability after 10 hours or 18–22 hours (overnight) of exposure. In the 10-hour test, the mean (± SEM) percentage of eggs fertilized by the caffeine-treated samples (61 ± 10%) was not significantly different from that by control samples (53 ± 10%, n = 7). Likewise, in the 18–22-hour test, there was no significant difference between mean proportions of eggs fertilized by caffeine treated samples (50 ± 16%) and controls (61 ± 13%, n = 7). The motility scores of treated and control spermatozoa were also similar at these times.

TABLE 2. Effect of Caffeine and Theophylline on Motility of Human Spermatozoa during Capacitation *In Vitro*

	Incubation Time (Hours)			
Treatment*	0	2	4	6
	% Motile ± SEM			
Control	62 ± 4	72 ± 3	68 ± 4	60 ± 4
Caffeine	74 ± 4	70 ± 3	65 ± 3	57 ± 5
Theophylline	68 ± 6	76 ± 3	68 ± 3	63 ± 4
	Quality of Motility ± SEM (Scale 1–4)			
Control	3.8 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.1
Caffeine	3.8 ± 0.1	3.6 ± 0.1	3.5 ± 0.2	3.3 ± 0.1
Theophylline	3.8 ± 0.0	3.8 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
	Grade of Forward Progression ± SEM (Scale 1–4)			
Control	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.6 ± 0.1
Caffeine	2.4 ± 0.1	2.8 ± 0.1	2.4 ± 0.3	3.2 ± 0.1
Theophylline	2.8 ± 0.1	3.0 ± 0.1	3.5 ± 0.1	3.6 ± 0.1

* Washed spermatozoa were incubated in BWW without PDE inhibitor (controls, n = 11–13) or in the presence of 7 mM caffeine (n = 9–11), or 10 mM theophylline (n = 9–10).

To examine the possibility that caffeine or theophylline might produce immediate effects on fertilizing ability if added to preincubated spermatozoa, these inhibitors were also added to untreated (control) spermatozoa at 6 hours and following an overnight (22 hours) incubation. Zona-free eggs were added 15 minutes later and the incubation continued for 2 hours, in order to assay fertilizing ability. Under these conditions, the PDE inhibitors had no apparent effect on the fertilizing ability of the spermatozoa or on the fertilizability of the eggs (Table 3), despite the fact that cAMP levels were significantly elevated in the treated spermatozoa (as described above).

Discussion

The aim of this study was to investigate the possible relationship between cAMP levels and fertilizing ability of human spermatozoa. Cyclic AMP concentrations in human spermatozoa, incubated with and without the phosphodiesterase (PDE) inhibitors, caffeine (7 mM), or theophylline (10 mM), were monitored over a 6-hour incubation period. During this period, the fertilizing ability of the same sperm samples was monitored using zona-free hamster eggs (Yanagimachi et al, 1976). In light of previous reports of PDE inhibitor-induced changes in sperm motility, this study also included evaluations of the percentage of motile spermatozoa, the quality of sperm motility, and the grade of forward progression of the sperm in the presence and absence of PDE inhibitor.

In the absence of PDE inhibitor, the mean concentration of cAMP in human spermatozoa did not fluctuate significantly over the 6-hour incubation. However, the mean percent fertilization achieved by the same samples increased from 4% at 0 hours to 33% at 6 hours. This lack of correlation between cAMP concentrations and fertilizing ability does not necessarily rule out a possible role for cAMP in capacitation or acrosome reaction. It is important to note that the proportion of human spermatozoa which were capacitated and acrosome-reacted at any given time during the incubation is unknown. The assumption was made here that increases in fertilizing ability (or fertilization index) reflect increases in the number of capacitated acrosome-reacted sperm. Since the fertilizing ability of most samples increased gradually over the incubation time and was maintained over long periods of time (up to 22 hours), it is likely that individual human spermatozoa undergo capacitation at different rates and that, at any given time, only a small percentage of spermatozoa are undergoing the acrosome reaction. If this is the case, then changes in cAMP content of any one small population of sperm cells might not be detectable.

Following the addition of caffeine or theophylline, either at the start of the incubation or at intervals during the incubation, the cAMP content of the spermatozoa increased to levels 3–4 times those found in control samples. The increase occurred rapidly (within minutes), after which time cAMP levels appeared to be maintained in an equilibrium state. The increased cAMP did not immediately stimulate fertilizing ability as might be expected if cAMP triggered the acrosome reaction directly. This was true whether the PDE inhibitor was added at the start of the incubation or was added to preincubated sperm at 6 or 22 hours of incubation. Rather, the fertilizing ability of the treated samples was stimulated after 4–6 hours of exposure to the drugs. This stimulatory effect was no longer apparent at longer (10 hours or 18–22 hours) incubation times. A similar time lag between application of treatments which increase cAMP and stimulatory effects on fertilization or acrosome reaction has been reported in other

TABLE 3. Effect of Caffeine and Theophylline on Fertilizing Ability of Human Spermatozoa When Added to the Spermatozoa after a Preincubation

Assay time (hours)*	Treatment	Mean (± SEM) % Fertilization	Mean (± SEM) Fertilization Index†	Number of Experiments
6	Controls	28 ± 7	0.33 ± 0.09	6
	Caffeine	37 ± 7	0.42 ± 0.07	6
	Theophylline	40 ± 5	0.51 ± 0.12	6
22	Controls	60 ± 9	1.14 ± 0.27	7
	Caffeine	54 ± 12	1.08 ± 0.38	6
	Theophylline	62 ± 18	1.02 ± 0.37	4

* Caffeine (7 mM) or theophylline (10 mM) was added to spermatozoa which had been preincubated in the absence of PDE inhibitor for 6 hours or 22 hours. The eggs were added 15 minutes later to assay for fertilizing ability.

† Fertilization Index = mean number of decondensed spermatozoa per egg tested.

species (Fraser, 1979; Hyne and Garbers, 1979; Mrsny and Meizel, 1979). This time lag is suggestive of an acceleration of capacitation, but not of a direct stimulation of the acrosome reaction.

Since numerous investigators have demonstrated that PDE inhibitors alter sperm motility (Garbers and Kopf, 1980), it was important to consider the possibility that the observed increases in fertilizing ability could have been due to PDE inhibitor-induced improvements in sperm motility. Our evaluations of sperm motility, however, did not provide any evidence that the PDE inhibitor-treated spermatozoa possessed superior motility at the assay times when fertilizing ability was elevated. Changes in progressive motility were observed in treated spermatozoa at the early observation times (0−2 hours): instead of swimming in a straight line as did control sperm, the treated sperm changed direction so often that they appeared to dart about in one location. Whether this form of motility is comparable to the hyperactivated (whiplash) motility characteristic of capacitated spermatozoa of other species (Yanagimachi, 1981) is not known.

In conclusion, the time-dependent stimulation of fertilizing ability of human spermatozoa incubated with caffeine or theophylline suggests that these agents stimulate capacitation. These results contribute evidence to support the theory that cAMP plays an underlying, modulatory role in the capacitation process.

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