Influence of caffeine on movement characteristics, fertilizing capacity and ability to penetrate cervical mucus of human spermatozoa

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Summary. When added to frozen–thawed human semen, the 3 doses of caffeine tested (2, 5 and 10 mM) induced a significant increase in the percentage of motile spermatozoa but did not influence the quality of movement. Considerable variability was noted between samples in their responsiveness to caffeine which, at the 5 and 10 mM doses, was significantly correlated with the degree of motility lost during cryostorage. Caffeine treatment of frozen–thawed human spermatozoa also increased the number of spermatozoa penetrating cervical mucus in unit time, by increasing the frequency rather than the success of collisions between spermatozoa and the cervical mucus interface. When caffeine-stimulated spermatozoa were washed free of seminal plasma containing this compound they were no longer at an advantage with respect to their motility or fertilizing ability. When 2 mM-caffeine was added to washed suspensions of capacitated spermatozoa it failed to stimulate motility but did significantly enhance the fertilizing ability of the spermatozoa, indicating a possible clinical role for this compound in in-vitro fertilization therapy.

Introduction

The loss of motility exhibited by human spermatozoa after cryostorage and the concomitant loss of fertilizing potential is a major problem for artificial insemination programmes (Sherman 1964; Serres, Jouannet, Czyglik & David, 1980; Steinberger, Rodriguez-Rigau & Smith, 1980). Of the many drugs that have been assessed for their effect on sperm motility (Dott, 1979) the phosphodiesterase inhibitors and, in particular, caffeine have been repeatedly shown to be amongst the most potent (Casillas & Hoskins, 1970; Garbers, Lust, First & Lardy, 1971; Burge, 1973; Schoenfeld, Amelar & Dubin, 1975; Homonnai, Paz, Sofer, Kraicer & Harell, 1976; Makler, Makler, Itzkovitz & Brandes, 1980).

The possible use of caffeine to stimulate the motility of cryostored human spermatozoa has been suggested by the positive results obtained by Barkay, Zuckerman, Sklan & Gordon (1977) and Schill, Pritsch & Preissler (1979), although caffeine was found to be ineffective in this respect by Read & Schnieden (1978). Whether caffeine could also affect the ability of cryostored spermatozoa to penetrate cervical mucus and fertilize ova has not been fully explored.

Caffeine might also be used to stimulate the fertilizing ability of washed suspensions of human spermatozoa, to facilitate the use of the in-vitro fertilization/embryo transfer procedure in the treatment of infertility due to poor semen quality. Although appropriate studies have been carried out on the influence of caffeine on the fertilizing ability of guinea-pig (Rogers & Garcia, 1979) and

mouse (Fraser, 1979) spermatozoa, no equivalent investigations have been conducted for human gametes.

The purpose of this study was therefore to obtain information relevant to these two potential clinical applications of caffeine.

Materials and Methods

Donors. The semen samples, obtained from normal healthy donors, contained $>20 \times 10^6$ spermatozoa/ml, had $>40\%$ motility, $>40\%$ with normal morphology, and a hamster egg penetration result of $>10\%$ (Aitken, Best, Richardson, Djahanbakhch & Lees, 1982).

Cryostorage of spermatozoa. The semen samples were frozen and stored by the techniques described by Jackson & Richardson (1977). The cryoprotective medium was composed of glycerol (16% v/v), egg yolk (28% v/v), fructose (1.24% w/v) and 56% (v/v) of a 3.2% (w/v) stock solution of sodium citrate in distilled water, and was gradually added to the semen at room temperature in 0.5 ml aliquants until a 1:1 dilution was attained. The samples were frozen at an average rate of $7.8^\circ\text{C}/\text{min}$ in the vapour phase of a Dewar flask containing liquid nitrogen. After cooling to at least -120°C the samples were rapidly transferred to numbered and colour-coded locations in a sperm storage tank and immersed in liquid nitrogen.

Assessment of movement characteristics. The straws of frozen spermatozoa were thawed out for 5 min at room temperature, followed by 20 min at 37°C . The motility of the sample was objectively assessed using an eye-piece graticule and hand-counter. The movement characteristics of the spermatozoa were then measured at 37°C under dark-field illumination using time-exposure photomicrography (Overstreet, Katz, Hanson & Fonseca, 1979; Katz, Overstreet & Hanson, 1981; Aitken *et al.*, 1982). Four photographs of each sample were taken in rapid succession and the mean velocity of 20 randomly selected tracks was calculated by direct measurement from the photographic prints. These tracks were also analysed to determine the percentage of straight swimming spermatozoa, defined as those spermatozoa exhibiting a progressiveness ratio of 1 (ratio of straight line distance travelled : total distance travelled = 1). The percentage of spermatozoa exhibiting an amplitude of lateral head displacement of $<10\text{ }\mu\text{m}$ was also calculated because this characteristic has been shown to exhibit a good correlation with the fertilizing ability of human spermatozoa (Aitken *et al.*, 1982).

Caffeine was dissolved in a balanced salt solution (BWW medium; pH 5.4) based on the description of Biggers, Whitten & Whittingham (1971) and containing (per litre) 5.54 g NaCl, 0.356 g KCl, 0.25 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.162 g KH_2PO_4 , 0.294 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and phenol red; 10 μl of this solution were added to 190 μl of thawed semen. Control incubations contained 190 μl of thawed semen and 10 μl BWW medium without caffeine. The samples were incubated for 20 min in an atmosphere of 5% carbon dioxide:95% air and a temperature of 37°C . The motility of the specimens was then reassessed and sperm movement was re-analysed. The influence of caffeine on the movement characteristics of washed suspensions of capacitated spermatozoa was similarly examined.

Penetration of cervical mucus. Cervical mucus from patients attending an Artificial Insemination by Donor clinic was obtained at mid-cycle by aspiration with a mucus-sampling syringe (Rocket, Watford, England). Samples exhibiting an Insler Score (Insler, Melmed, Eichen Brenner, Serr & Lunenfeld, 1972) of >11 were used within 1.5 h of collection in a cervical mucus penetration test. The mucus was drawn up into capillary tubes, of rectangular cross-section, exhibiting a depth of $300\text{ }\mu\text{m}$ and a volume of 25 μl (Camlab, Cambridge, England). One end of the capillary tubes was sealed with 'Parafilm' and the tubes were equilibrated in a humidified chamber for 20 min at 37°C in a gas phase of 5% carbon dioxide:95% air. The open end of each capillary tube was then introduced into a 200 μl sample of semen which had previously been examined by time-exposure photomicrography to determine the percentage of motile spermatozoa and their total mean

velocity. After an incubation period of 30 min the capillary tubes were wiped clean and examined at $\times 100$ under dark-field illumination on the stage of a Leitz Ortholux II microscope to determine the distance of sperm migration. The mucus was then extruded into 475 μ l Dulbecco's phosphate-buffered saline (Flow Laboratories, Irvine, U.K.) containing 0.1% 2-mercaptoethanol and 10% formalin. The capillary tube was rinsed repeatedly in this solution and after soaking for 30 min the concentration of spermatozoa in the cervical mucus was determined from counts obtained with an improved Neubauer haemocytometer.

From a knowledge of the dimensions of the capillary tubes, the duration of incubation, the concentration of spermatozoa in cervical mucus, the concentration of motile spermatozoa in semen and the mean velocity of the motile spermatozoa, a ratio can be calculated which compares the number of successful sperm entries into the mucus with the number of original collisions between the spermatozoa and the cervical mucus/semen interface. This figure is termed the PSC value (Percentage Successful Collisions) and is derived according to the calculations described by Katz *et al.* (1980).

Hamster egg penetration test. A measure of the fertilizing capacity of human spermatozoa exposed to caffeine was obtained using the zona-free hamster egg penetration test described originally by Yanagimachi, Yanagimachi & Rogers (1976), as modified by Dor, Rudak & Aitken (1981), Aitken (1982) and Aitken *et al.* (1982).

All statistical comparisons were carried out using the paired *t* test or χ^2 analysis.

Results

Sperm movement characteristics

The 3 doses of caffeine tested all exerted a significant stimulatory effect on motility (Table 1). Although the degree of stimulation was greatest for the 5 mM dose ($88 \pm 21\%$), considerable variation in responsiveness (0–261% increase in motility over the respective control values) was observed between individual samples at all dose levels. At the 5 ($r = 0.536$) and 10 ($r = 0.80$) mM doses, this variability was significantly correlated ($P < 0.001$) with the amount of motility lost as a result of freezing. However, the motility of a sample was not stimulated beyond the value observed before freezing.

Caffeine had a negligible effect on washed suspensions of capacitated spermatozoa in an artificial medium (Table 1). At the 2 mM dose only 14 out of 26 samples showed any change in motility and in 10 of the 14 stimulated samples the increase in motility over the control value was

Table 1. Influence of caffeine on the motility of human spermatozoa

Caffeine (mM)	No. of samples	% Motility	
		Caffeine-free control	Caffeine
<i>Frozen-thawed spermatozoa</i>			
2	13	28.3 ± 3.5	44.5 ± 4.1***
5	15	27.4 ± 3.9	43.0 ± 3.6***
10	12	30.7 ± 3.5	47.3 ± 2.9***
<i>Washed, capacitated spermatozoa</i>			
2	26	40.3 ± 4.5	41.3 ± 4.4
5	23	41.0 ± 4.2	41.2 ± 4.2
10	24	42.2 ± 4.3	43.9 ± 4.2

Values are mean $\pm$ s.e.m.

*** $P < 0.001$.

Table 2. Influence of caffeine on the motility of frozen-thawed human spermatozoa resuspended in seminal plasma or BWB medium

Caffeine (mM)	No. of samples	% Motility	
		BWB medium	Seminal plasma
2	3	26.5 ± 4.7	44.5 ± 3.3*
5	6	25.7 ± 2.5	45.7 ± 3.1***
10	6	23.3 ± 1.7	45.0 ± 3.9***

Values are mean ± s.e.m.

* $P < 0.01$, *** $P < 0.001$.**Table 3.** Influence of caffeine on the velocity of human spermatozoa

Caffeine (mM)	No. of samples	Velocity (μm/sec)	
		Caffeine-free control	Caffeine
<i>Frozen-thawed spermatozoa</i>			
2	13	19.4 ± 1.0	17.8 ± 1.1
5	15	18.6 ± 1.0	19.1 ± 1.2
10	12	16.5 ± 1.7	16.1 ± 0.8
<i>Washed, capacitated spermatozoa</i>			
2	26	25.1 ± 1.5	25.4 ± 1.3
5	23	29.3 ± 1.3	26.7 ± 1.3
10	24	24.4 ± 1.2	27.1 ± 1.5

Values are mean ± s.e.m.

< 10%. At the 5 mM dose, 11 out of 23 samples responded to caffeine with an increase in motility, but in only 2 samples was this response > 10% over the control value. Similarly at the 10 mM dose only 6 out of 24 samples exhibited a response to caffeine that represented > 10% increase over the control value.

As a result of these findings an experiment was designed to control for any mechanical damage inflicted upon the spermatozoa during the centrifugation process involved in the preparation of the washed sperm suspensions and to determine whether the responsiveness of the spermatozoa was related to their state of capacitation. Samples of frozen-thawed human spermatozoa were divided into two aliquots, and centrifuged at 600 *g* for 5 min. The sperm pellet was resuspended in BWB medium or seminal plasma. Caffeine was then added to aliquots of the resuspended spermatozoa and the change in % motility was examined after a 20 min incubation period. The uncapacitated sperm suspensions subjected to a minimum of centrifugation failed to respond to caffeine in an artificial medium, while spermatozoa subjected to an identical centrifugation regimen but resuspended in seminal plasma exhibited full responsiveness (Table 2).

Although a variable proportion of individual semen samples exhibited a response in velocity to caffeine treatment at each dose level, the increase rarely represented more than a 10% elevation above the control values and the total mean velocities were not significantly changed in the presence of caffeine at any concentration (Table 3). Similarly, no significant changes in the percentage of straight swimming spermatozoa or amplitude of lateral head displacement were detected at any dose of caffeine for frozen-thawed spermatozoa in the cryoprotective medium or washed suspensions of capacitated spermatozoa (data not presented).

Penetration of cervical mucus

In 14 out of the 15 samples examined, the mean density of spermatozoa in cervical mucus after the 30 min incubation period was greater in the caffeine-treated samples than in their respective controls (Text-fig. 1a); a result which was significant with respect both to the mean values ($P < 0.01$) and the proportion of samples showing a positive response ($\chi^2 = 5.6$; $P < 0.02$). No significant changes in PSC values were observed (Text-fig. 1b).

Text-fig. 1. Influence of 5 mM-caffeine on (a) the concentration of spermatozoa in cervical mucus and (b) the percentage of successful collisions (PSC value) between spermatozoa and the cervical mucus interface.

Sperm fertilizing ability

Frozen-thawed semen samples were exposed to caffeine for 20 min, resuspended in BWW medium and then capacitated for 7 h before being incubated with zona-free hamster ova. The addition of caffeine resulted in a consistent suppression of the fertilizing ability of the spermatozoa, relative to controls exposed to vehicle alone for 20 min (Table 4). After addition of caffeine to the frozen-thawed semen an increase in motility was observed which was largely lost during the washing procedure and had completely disappeared by the end of the capacitation period when the assessment of fertilizing ability took place (Table 5). Evidently caffeine must remain in contact with the spermatozoa to maintain its influence.

When caffeine was added to the spermatozoa at the end of the capacitation period, at the same time as the zona free hamster ova, and left in contact with the gametes during the 3 h insemination period, there was a net dose-dependent inhibition of fertilization (Table 6).

Table 4. Effect of caffeine on the fertilizing ability (% penetration) of human spermatozoa

Sample	Caffeine-free control	Caffeine		
		2 mM	5 mM	10 mM
1	34.7	31.8	5.8	2.7
2	92.0	56.0	53.8	—
3	96.5	—	—	92.0
4	25.0	17.0	20.0	10.0

Table 5. Changes in sperm motility* after the addition of caffeine to frozen-thawed human semen and during the subsequent washing and capacitation episodes

Treatment	% Motility			Penetration (%)
	Semen	After washing	After capacitation	
Control	46	28	33	25
Caffeine 2 mM	53	32	30	17
5 mM	55	32	29	20
10 mM	64	43	27	10

* Values are for 1 of the 3 experiments covered in Table 4.

Table 6. Influence of caffeine on the fertilizing ability of human spermatozoa when added (a) to the insemination medium and (b) during the last 20 min of the capacitation period

Caffeine (mM)	No. of samples	Penetration rates (%)		Net % change in penetration
		Caffeine-free control	Caffeine	
(a) 2	7	52.4 ± 11.0	51.4 ± 11.0	-0.5
5	7	43.7 ± 10.6	31.3 ± 8.5*	-38.7
10	7	59.2 ± 4.9	25.1 ± 4.2***	-59.2
(b) 2	7	37.6 ± 10.7	47.2 ± 12.8*	+20.8
5	5	29.1 ± 13.0	40.1 ± 13.5	-8.8
10	6	61.1 ± 13.1	47.7 ± 11.9**	-19.8

Values are mean ± s.e.m.

Compared with control values: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.001$.

To check whether caffeine might exert a toxic effect on the ova, caffeine was added to the sperm suspensions for the last 20 min of the capacitation period and then removed by centrifugation before introduction of the hamster ova (Table 6). At the 10 mM dose, caffeine inhibited fertilization with every sample tested ($P < 0.02$). At the 5 mM dose, stimulation was observed for 2 of the 5 samples examined although the net change in fertilizing capacity for this group was still negative. At the 2 mM dose, however, every sample examined showed an increase in penetration rate in the presence of caffeine ($P < 0.05$).

Discussion

The present results clearly demonstrate that the motility lost by human spermatozoa during cryo-storage can be regained by treatment with caffeine. Relative to the control incubations, caffeine induced ~80% increase in motility at all dose levels, which is in good accordance with the 81% increase in motility induced by 5 mM-caffeine in the study of Schill *et al.* (1979). Barkay *et al.* (1977) examined the influence of caffeine dose on the increase in motility of cryostored spermatozoa and concluded that 7.2 mM was the optimum concentration for samples stored in excess of 30 days, although the statistical significance of this claim was not calculated. In the present study 5 mM-caffeine gave the highest mean increase in motility (88%) although this was not statistically different from the degree of stimulation observed with the 2 and 10 mM doses. Considerable variation was noted between samples in their responsiveness to caffeine which was, at the 5 and 10

mM doses, significantly correlated with the variable amount of motility lost as a result of cryostorage. The addition of 5 mM-caffeine to semen samples that have lost significant motility during cryostorage might therefore be beneficial.

The stimulation of cryostored spermatozoa with caffeine also increased the number of cells entering cervical mucus in unit time. This effect was achieved by increasing the total number of collisions between spermatozoa and the cervical mucus interface and not by increasing the success of these collisions. Whether an increase in the size of the cervical pool of spermatozoa increases the chances of conception *in vivo* is debatable since this study has also shown that once spermatozoa have lost contact with caffeine they are no longer at an advantage with respect to motility or fertilizing ability. Preliminary experiments in which caffeine was employed as an additive to fresh semen in an Artificial Insemination by Husband clinic tend to support this view (Harrison, 1978) although similar studies with frozen-thawed donor spermatozoa have yet to be carried out.

The influence of caffeine appeared to be confined to an increase in the percentage of motile cells since, within the limits of resolution of time-exposure photomicrography, no significant changes were noted in the mean velocity of the spermatozoa or their patterns of movement. Using similar methods of analysis Haesungcharern & Chulavatnatol (1973) and Makler *et al.* (1980) arrived at the same conclusion. Our method and that used by Makler *et al.* (1980) have been well validated and give highly repeatable objective estimates of the velocity of human spermatozoa. Reports in which an increase in the quality of sperm movement has been observed following caffeine treatment (Schoenfeld *et al.*, 1975; Schill *et al.*, 1979) have used subjective methods for grading the progressiveness of sperm motility and these may be subject to errors of interpretation.

The mechanism by which caffeine stimulates sperm motility is thought to involve the inhibition of phosphodiesterase activity and the subsequent accumulation of cyclic nucleotides, especially cAMP, within the sperm cells (Garbers *et al.*, 1971; Hicks, Martínez-Manautou, Pedron & Rosado, 1972). The precise way in which elevated intracellular cAMP levels induce an increase in motility is not clearly understood, although the control of calcium ion fluxes across the sperm plasma membrane is probably involved (Peterson, Seyler, Bundman & Freund, 1979). Greatly elevated levels of intracellular calcium certainly appear to have a detrimental effect on sperm motility (Rosado, Hicks, Martínez-Zedillo, Bondani & Martínez-Manautou, 1970; Davis, 1978; Peterson *et al.*, 1979; Shams-Borhan & Harrison, 1981) and cAMP-associated calcium pumps in the plasma membrane appear to be critically important in regulating the content of this ion within the sperm cell (Peterson *et al.*, 1979). Since considerable damage is inflicted on the sperm plasma membrane during cryostorage (Mann & Lutwak-Mann, 1981) it is possible that the loss of motility observed after freezing and thawing spermatozoa is related to the inability of the damaged plasmalemma to control the levels of calcium within the sperm cell. Caffeine may compensate for this damage and the resultant loss of motility by stimulating the activity of an outwardly-directed plasma membrane calcium pump through the mediation of cAMP.

However, in the present study, caffeine could not stimulate the motility of washed suspensions of human spermatozoa before or after capacitation. The most likely explanation for the failure of BWW medium to support caffeine action is the presence of free calcium, since this ion was not present in the Tris-buffered salt solutions employed in previous studies in which caffeine did exert an effect on the motility of washed spermatozoa (e.g. Garbers *et al.*, 1971; Homonnai *et al.*, 1976; Peterson & Freund, 1976). If phosphodiesterase inhibitors stimulate or maintain sperm motility by removing excess calcium from within the cell (Peterson & Freund, 1976; Peterson *et al.*, 1979), this effect would be more readily achieved in artificial media lacking calcium since the efflux of this ion would be facilitated by a steep concentration gradient across the sperm plasma membrane.

If this argument is correct, it is surprising that caffeine can stimulate the motility of spermatozoa in semen which contains some 28.2 ± 3.3 mg calcium/100 ml plasma (Quinn, White & Wirrick, 1965). However a large proportion of the calcium in semen is either complexed with substances such as citric acid or protein bound. In this respect, there is a specific protein component in bovine seminal plasma that interacts with the sperm surface and decreases the

permeability of the plasma membrane to calcium (Babcock, Singh & Lardy, 1979). The anti-fertilization effects of human seminal plasma (Kanwar, Yanagimachi & Lopata, 1979) may well depend upon the presence of an analogous substance. Since the success of in-vitro fertilization depends upon the removal of such seminal components and the concomitant exposure of the spermatozoa to free calcium ions to promote both the acrosome reaction (Yanagimachi & Usui, 1974) and the binding of spermatozoa to the zona pellucida (Saling, Storey & Wolf, 1978), it is unlikely that phosphodiesterase inhibitors such as caffeine (which may depend on calcium efflux from the sperm cell for their motility-stimulating activity) will ever be effective stimulators of motility in situations designed to promote fertilization.

However, phosphodiesterase inhibitors may still be of clinical relevance for in-vitro fertilization therapy because although caffeine may not stimulate sperm motility in the presence of artificial media the results obtained in this study, and the preliminary report of Rogers (1981), indicate that this compound can enhance the fertilizing ability of human sperm cells. In the present study this enhancement was obtained with a low dose (2 mM) of caffeine and a short exposure time (20 min), higher doses proving either occasionally (5 mM) or consistently (10 mM) toxic. Although the toxic effects of caffeine on human spermatozoa have been repeatedly demonstrated (Harrison, Sheppard & Kaliszer, 1980; Traub, Earnshaw, Brannigan & Thompson, 1982), Rogers (1981) reported an improvement in the fertilizing ability of human spermatozoa when using 7 mM caffeine and exposure times as long as 6 h, but some methodological details, particularly the procedures used to solubilize the caffeine, were not included. Further studies are clearly required to determine the optimal conditions for exploiting the stimulatory properties of this compound.

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