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Name	ETH-Bibliothek, ILV Spezial
Kunden Adresse	
	ILV SPEC, HG H 31.5, Rämistrasse 101
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Bestelldatum	2015-07-10 14:53:02
Lieferprotokoll	WEB

Name	**ETH-Bibliothek, ILV Spezial**
Benutzer_Nr.	
Zeitschrift	**Fertility and Sterility**
ISSN	
Jahr	1986
Band/Heft	Band 46 Heft 5
Seiten	938-944
Aufsatz-Autor	Fakih
Aufsatz-Titel	Enhancement of human sperm motility and velocity in vitro: effects of calcium
Notiz	BeZ PAB 674 : 46:1 / Speicher L6
Bemerkung	Nur in der Schweiz bestellen
Weiterleitungs-Liste	
Kundenspezifische Information	Alumni 17279/Fertility /mc
Mediennummer	

vol. 46, 1986 II

FERTILITY AND STERILITY

Official Journal of The American Fertility Society,
Society of Reproductive Endocrinologists, Society of Reproductive Surgeons,
Pacific Coast Fertility Society, and The Canadian Fertility and Andrology Society

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Fertility and Sterility (ISSN 0015-0282), published monthly by The American Fertility Society, 2131 Magnolia Avenue, Suite 201, Birmingham, Alabama 35282-9990. *International Edition* (containing two issues) sent bimonthly to foreign subscribers. Membership dues to The American Fertility Society include $45 for *Fertility and Sterility*. Individual non-member subscription rate $90 per year (outside the United States add $17 for surface postage). Second-class postage paid at Birmingham, Alabama, and additional mailing offices at Rochester, Minnesota.

Postmaster: Send address change to The American Fertility Society, 2131 Magnolia Avenue, Suite 201, Birmingham, Alabama 35282-9990.

FERTILITY AND STERILITY
Vol. 46, No. 5, November 1986
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Printed in U.S.A.

Enhancement of human sperm motility and velocity in vitro: effects of calcium and creatine phosphate*

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Because of their roles in motility regulation and energy transport, calcium and creatine phosphate were examined for their effects on sperm motility and velocity in specimens of normal donors. Semen or migrated sperm fractions were incubated with of 1 mmol of calcium, 5 mmol magnesium, and 10 mmol of creatine phosphate (n = 28) or in the presence of 4 μmol of Verapamil, calcium, and creatine phosphate (n = 10). The samples were subjected to multiple exposure photography (four picture frames of two different drops) at 0, 1, 4, or 5 and at 10 hours and sperm motility and velocity were analyzed. In both calcium and calcium-creatine phosphate conditions, sperm motility and velocity were significantly increased, compared with control values (P = between < 0.001 and 0.05). Sperm motility declined following Verapamil exposure, but the motility values remained at the level of the control in the presence of additional calcium or creatine phosphate. The effects of calcium and creatine phosphate take place rapidly; within 1 minute all improvements in sperm velocity and motility are fully achieved. There is no loading effect of calcium, and when the sperm is transferred into media without the additional calcium, the velocity decreased to that of the initial control value. Magnesium alone had no effect on motility or velocity. These experiments indicate that calcium or creatine phosphate can support sperm motility and velocity at a significantly increased level. Thus the addition of calcium or creatine phosphate to the insemination media may enhance the fertilizing capacity of sperm during in vitro fertilization or gamete intrafallopian transfer procedures. Fertil Steril 46:938, 1986

Received April 24, 1986; revised and accepted July 21, 1986.
*Presented in part at the Forty-First Annual Meeting of The American Fertility Society, Chicago, Illinois, September 27 to October 2, 1985.
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The role of calcium and other cations in the regulation of sperm motility has been recognized.[1-4] However, most reports present conflicting results on the effects of calcium (e.g., activation, inhibition, or no effect), due in part to species differences in sperm response and to the lack of objective methods in the measurement of sperm motility and velocity. Another contributing factor is the lack of proper discrimination between sperm motility (the percent of motile and immotile sperm) and sperm velocity (the speed of forward sperm motion), which often are used interchangeably. We addressed the latter problems in this study by measuring both sperm motility and velocity in the various specimens with the use of multiple-exposure photography (MEP) and subsequent computer-assisted analysis.[5, 6] MEP not only allows the measurement of sperm motility and average velocity, it also provides a permanent photographic record for reinvestigation or comparison of the experimental data.

Another area of sperm physiology, the mechanism of energy production and use, has advanced recently. Although it is clear that adenosine triphosphate (ATP) is the source of energy for sperm motility, the size and turnover rate of the ATP pool, and the means of energy transport from the mitochondria to the tail are unknown in the human sperm. The concept of the "creatine phosphate shuttle" and the central role of creatine kinase were described in the sea urchin and chicken sperm.[7, 8] This hypothesis suggests that, similarly to muscle tissue, creatine phosphate and creatine kinase are instrumental in the regeneration and transport of energy and in the replenishment of the ATP pool. In support of this idea, the presence of mitochondrial and brain-type (same as muscle-type) creatine kinase isozymes were demonstrated in chicken sperm.[8] We investigated this question further in the human sperm and also found these two creatine kinase isozymes.[9] Further experiments with various inhibitors have demonstrated that, due to its roles in mitochondrial creatine phosphorylation and in the rephosphorylation of adenosine diphosphate (ADP) to ATP in the sperm tail, creatine kinase is instrumental in sperm motility.[7, 8] These data are also supported by the finding that the ATP/ADP ratio declines simultaneously with the rate of motility in human sperm samples.[10]

Toward a better understanding of the regulatory functions in human sperm, we examined the effects of calcium, magnesium, and creatine phosphate on sperm motility and velocity. We found significant enhancement in both parameters in response to administration of calcium or creatine phosphate; magnesium had no effect. The actions of creatine phosphate took place even in the presence of the calcium channel blocker drug Verapamil, which indicate that the enhancement of sperm motility and velocity by creatine phosphate is independent from the influx of calcium.

MATERIALS AND METHODS

Fine chemicals and creatine phosphate were from the Sigma Company, St. Louis, MO, and Verapamil (Knoll Co., Whippany, NJ) and Ethiodol (Sauvage Laboratories, Melville, NY) were purchased. Disposable test tubes, pipettes, and other laboratory ware were from American Scientific Products, Edison, NJ. Fresh semen samples of normospermic donors ($> 20 \times 10^6$ sperm/ml, > 40% motility, > 40% normal sperm forms) were collected by masturbation. The experiments were carried out with either semen or a migrated sperm fraction, prepared as described previously.[11, 12] After liquefaction, the semen was diluted 1/5 with Ham's F-10 medium (GIBCO, Grand Island, NY) containing 15% human umbilical cord serum, 280 mg/l of calcium lactate, 1 mg/ml of penicillin, 0.5 mg/ml of streptomycin and 15 mmol of sodium bicarbonate, pH 7.4. The specimen was layered slowly onto 1 ml of ethiodol (oily contrast medium, specific gravity 1.28). After centrifugation at $400 \times g$ for 10 minutes, the sperm and other particulate matter sedimented to the top of the oil as a flat pellet. The supernatant (with the exception of the lower 1.5 ml) was aspirated gently and discarded. During a 30-minute incubation period at 36°C, the motile sperm fraction migrated into the clear fluid. Approximately 1.0 ml of the supernatant, containing 40 to 100×10^6 sperm, was distributed into aliquots for further incubation with the various agents, including 1 mmol of calcium chloride, 5 mmol of magnesium chloride, and 10 mmol of creatine phosphate. Free calcium concentrations in the media were monitored with the use of an Orion-calcium electrode. The actual concentrations were approximately 1.2 mmol after the addition of 1.0 mmol of calcium chloride, with slight variation among preparations. The sperm motility parameters were unchanged within the free calcium concentrations of 0.5 to 4.0 mmol tested. The sperm ve-

Table 1. *Effects of Calcium (C), Magnesium (M), Creatine Phosphate (R), and Verapamil (V) on Sperm*[a]

	Motility (%)			Velocity (μm/second)		
	F	*df*	*P*	*F*	*df*	*P*
No Verapamil						
Treatment (control, CM, RCM)	3.9	2,162	= 0.03	31.9	2,162	< 0.001
Time (0, 1, 5, and 10 hours)	169	3,162	< 0.001	42.9	3,162	< 0.001
Treatment × time	2.4	6,162	= 0.03	7.7	6,162	< 0.001
2 μg/ml Verapamil						
Treatment (control, V, VC, VR)	9.63	3,81	< 0.001	14.0	3,81	< 0.001
Time (0, 1, 4, and 10 hours)	27.72	3,81	< 0.001	2.9	3,81	< 0.001
Treatment × time	1.91	9,81	= 0.062	6.4	9,81	< 0.001

[a]Statistical analysis with the two-way ANOVA test.

locity and motility in the samples were determined according to the MEP method.[5] The various sperm parameters were quantified with computer-assisted analysis of the films.[6] Statistical analysis of the data was carried out with the two-way ANOVA program and with the Duncan ranked analysis test.

RESULTS

In the first phase of study, semen or migrated sperm were exposed to calcium, magnesium, and creatine phosphate. Three conditions (control, calcium-magnesium, and calcium-magnesium-creatine phosphate) were tested in 28 determinations (four picture frames each) at 0, 1, 4, and 10 hours. In the second phase of the experiments, the effects of Verapamil were examined in the presence of calcium or creatine phosphate on migrated sperm fractions. The concentrations of Verapamil (4 μmol) were chosen so that they attenuated sperm motility or velocity to approximately 75% of that of the control values. Higher Verapamil concentrations (e.g., 20 to 40 μmol) immobilized the sperm irreversibly. Four conditions were studied: control (CON), Verapamil (V), Verapamil-calcium (VC), and Verapamil-creatine phosphate (VR). Ten determinations were done (four picture frames each) at each time period and condition.

In both experiments, there was a significant decline in both motility and velocity during the incubation time (Table 1). There were highly significant ($P < 0.001$) treatment effects of creatine phosphate, calcium,and magnesium on sperm velocity and of Verapamil on both motility and velocity. A lower significance was observed for the effects of the divalent cations and creatine phosphate on motility. Highly significant time and treatment effects were observed in all velocity measurements ($P < 0.001$). In the motility results, a borderline significant interaction effect was seen only in the absence of Verapamil. Thus the overall analysis of the data indicated that there is a highly significant relationship among the treatment and time factors. Magnesium alone did not affect sperm motility or velocity (data not shown).

SPERM MOTILITY

Sperm motility declined during the 10-hour observation period regardless of the experimental conditions. In the experiments without Verapamil (Fig. 1A), significant improvement ($P = 0.05$) in motility could be observed at 10 hours, whereas the calcium-magnesium or calcium-magnesium-creatine phosphate groups maintained twice the motility (19% and 16%) of the control sperm (8%).

With 4 μmol of Verapamil in the assay (Fig. 2A), in addition to the decline in the overall motility there was a relative inhibition of motility at all time points (CON versus V conditions). The Verapamil apparently has a biphasic effect on sperm motility. It inhibits motility instantaneously (e.g., 82% versus 64% in the CON versus V groups at 0 time; $P = 0.05$), but the presence of calcium or creatine phosphate protects the sperm from this inhibition ($P = 0.05$ for V versus VR or VC at 0 time). Also, the sperm recover partially from the Verapamil inhibition within 1 hour (VO versus V, 1 hour). However, the motility in the Verapamil-exposed sample at 10 hours is only one-half as active as in the control sperm or in the samples with added calcium or creatine phosphate (V versus CON, and V versus VC and VR at 10 hours; $P = 0.01$). Thus Verapamil has a short-term and a long-term detrimental effect on sperm

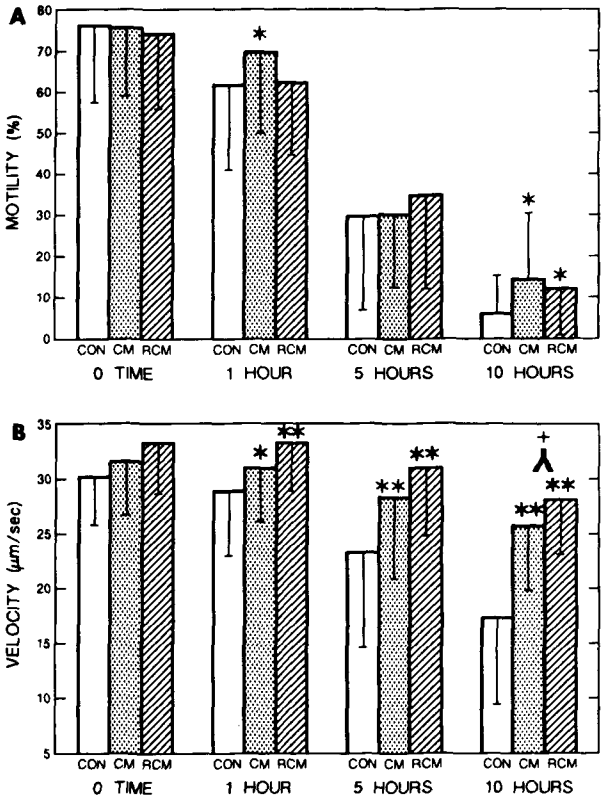

Figure 1
(**A**) Changes in sperm motility in response to calcium (C), magnesium (M), and creatine phosphate (R) at the various time periods after addition of the agents. Significant (*$P \leq 0.05$, **$P < 0.001$), compared with the control. Significant ($\dot{\lambda} P = 0.05$), compared with each other. Data are expressed as mean ± standard deviation (SD), (n = 28). (**B**) Changes in sperm velocity in response to calcium (C), magnesium (M), and creatine phosphate (R) at the various time periods after addition of the agents. Significant (*$P \leq 0.05$, **$P < 0.001$), compared with the control. Significant ($\dot{\lambda} P = 0.05$), compared with each other. Data are expressed as mean ± SD (n = 28).

and calcium or creatine phosphate apparently provide protection against both.

In both experimental groups (in the absence or presence of Verapamil), sperm motility increased after the addition of calcium and creatine phosphate; these agents seem to sustain sperm motility longer than is found in the respective controls ($P = 0.05$ and $P = 0.01$ at 10 hours). In the group treated with Verapamil, the inhibition of motility and velocity was alleviated by the addition of either calcium or creatine phosphate alone. This suggests that creatine phosphate acts by a mechanism unrelated to the influx of calcium. However, the two effects do not appear to be additive. At 22 hours (data not shown), the motility diminished to < 5% in all experiments. This suggests that sperm longevity (viability) is likely to be an inherent property of sperm that cannot be sustained longer, regardless of the experimental conditions.

SPERM VELOCITY

We also measured the velocity (μm/second of forward motion) of the motile sperm population with the MEP method. In the presence of calcium-magnesium or calcium-magnesium-creatine phosphate, velocity is significantly improved at 1 hour, 5 hours, and 10 hours, compared with controls (Fig. 1B). In the CM versus RCM conditions at 10 hours, creatine phosphate provides further improvement ($P = 0.05$) over the effects of cal-

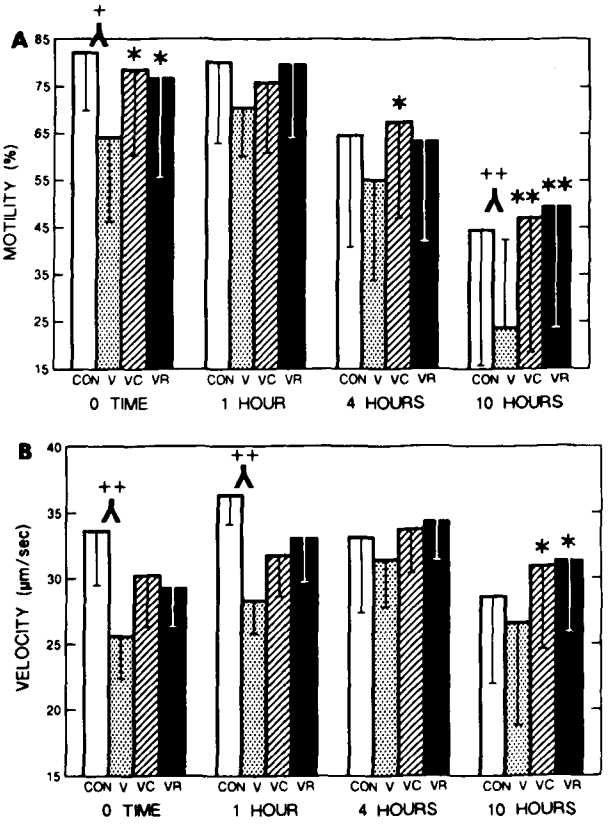

Figure 2
(**A**) Changes in sperm motility in response to Verapamil (V) in the presence or absence of calcium (C) or creatine phosphate (R) at various time periods. Significant (*$P \leq 0.05$, **$P < 0.001$), compared with the respective V. Significant ($\dot{\lambda} P = 0.05$, $\dot{\lambda} P = 0.01$), compared with each other. Data are expressed as mean ± SD (n = 10). (**B**) Changes in sperm velocity in response to Verapamil (V) in the presence or absence of calcium (C) or creatine phosphate (R) at various time periods. Significant (*$P \leq 0.05$, **$P < 0.001$), compared with the respective V. Significant ($\dot{\lambda} P = 0.05$, $\dot{\lambda} P = 0.01$), compared with each other. Data are expressed as mean ± SD (n = 10).

cium-magnesium. As with motility, the velocity of the moving sperm population decreases by time, but both the calcium-magnesium and the calcium-magnesium-creatine phosphate conditions diminished the rate of decline significantly. For example, at 5 and 10 hours, the velocities in the control group declined to 76% and 56% of the speed at 0 time (Fig. 1B). The respective values in the calcium-magnesium group remained 92% and 83% and in the calcium-magnesium-creatine phosphate group were 100% and 91% ($P = 0.01$, with the exception of CON 1 hour versus CM 1 hour, in which $P = 0.05$). The effects of creatine phosphate are additive to that of calcium (CM versus RCM at 10 hours), but creatine phosphate also increases sperm velocity independently from calcium after Verapamil treatment (Table 1), which is likely to be related to the role of creatine kinase as an energy source.

In the samples with Verapamil there was improvement in velocity with the addition of calcium and creatine phosphate (Fig. 2B). In response to the calcium channel blocker, the velocity of the sperm at 0, 1, 4, and 10 hours were reduced to 76% ($P = 0.01$), 78%, 95%, and 93% of that of the respective controls, whereas in the presence of calcium or creatine phosphate the velocity of the motile sperm fraction remained close to the original levels after ejaculation (CON at 0 time). Calcium or creatine phosphate caused an increase to the extent that at 10 hours the velocities were 108% to 117% that of the control sample ($P = 0.05$ for both). Verapamil decreased sperm velocity, but samples that contained calcium or creatine phosphate maintained sperm velocity after 5 hours, even better than the respective untreated controls (CON 10 hours).

To analyze further the mechanism of calcium and creatine phosphate action, we did two more experiments. The first was to address the time scale of the response. We observed sperm at 30 seconds and at 1, 2, 3, 4, and 5 minutes after the addition of calcium and found that the enhancement in velocity occurs within 1 minute. This is also true in the presence of Verapamil: calcium or creatine phosphate provided instantaneous protection (or compensation) to the Verapamil effect on velocity (Fig. 2B). VO versus CON at 0 time was lower by 24% ($P = 0.01$), whereas VC and VR at 0 time decreased by only 10% and 13%, respectively (differences not significant).

The second set of experiments was aimed at the duration of calcium response with respect to a possible long-term "loading" effect of calcium in sperm. We exposed part of the samples to calcium and demonstrated the improved velocity. When these sperm fractions were migrated into the regular Ham's F-10 solution (without the additional 1.0 mmol of calcium), sperm velocity was comparable to that of the untreated fraction of the same specimen. Thus calcium loading did not occur in the sperm. Calcium apparently causes an immediate improvement in sperm velocity, which lasts only as long as the sperm is exposed to this agent.

DISCUSSION

The ability of the mature mammalian sperm to maintain a coordinated and forward motility is dependent upon intracellular free calcium.[1-4] At least two mechanisms regulating the intracellular calcium concentration have been identified (1) collection and release of calcium between the mitochondrial and cytoplasmic compartments of sperm[3, 4, 13]; (2) uptake of calcium via the cell membrane and extrusion of calcium by the calcium-magnesium activated pump.[14, 15] The regulatory role of calcium was demonstrated in various sperm functions, including a calcium-calmodulin-mediated phosphorylation of dynein with the use of a myosin light-chain kinase-type enzyme[16] (as in smooth muscle contraction[17]) which regulates tubulin-dynein interaction, the basis of flagellar beating.

We investigated the effects of calcium and creatine phosphate on sperm motility and velocity. We believe that velocity is a more important sperm function to study, because motility simply describes the percent of motile or nonmotile sperm. The effects of various agents may be assessed better on the motile fraction, which is able to respond with a change in velocity or viability. The MEP technology provided an experimental system previously not available in which statistical analysis of the motility parameters in response to the various agents is possible. Our two major goals were (1) to reach a better understanding of the processes underlying the regulation of motility characteristics and energy synthesis of sperm; and (2) to study in vitro conditions in which the fertilization potential of sperm in subfertile men may be improved.

The effects of calcium on mammalian sperm motility had been investigated, with controversial results in various laboratories. Significant improvements in calcium uptake and sperm mo-

tility in response to extracellular calcium have been reported,[3, 4, 13] but other authors have found that calcium does not enter sperm.[18] The uptake of calcium by sperm and a simultaneous increase in motility and velocity is now well established,[2] but an issue yet to be clarified is the role of calcium in the epididymal versus the seminal fluids with respect to sperm motility.[19, 20] Although there is an apparent inhibitory effect of calcium in the caudal epididymis, other factors (e.g., structural changes in the sperm membrane,[21] binding of proteins to sperm from the seminal fluid,[22] and changes in sperm metabolism due to respiration[22]) may affect motility of the ejaculated sperm. It is also conceivable that the species-to-species differences in calcium response are due to variations in the structure of sperm membrane proteins. This is supported by the gossypol toxicity experiments,[23] in which the calcium-magnesium ATPase activity showed species differences in sperm sensitivity and stability.

Our results indicate that calcium or creatine kinase may cause statistically significant enhancements in sperm velocity and that they reduce the rate of time-related loss of motility. With the maintenance of sperm parameters in the presence of the calcium channel blocker Verapamil, we established that the effects of creatine phosphate occur independently from calcium influx. The effects of creatine kinase were additive to those of calcium, although this additional increase reached statistical significance only in the case of CM versus RCM at 10 hours (Fig. 1B). The findings are consistent with the idea that calcium activates the regulatory mechanism of sperm motility whereas creatine phosphate supports ATP rephosphorylation, thus improving sperm motility parameters by providing additional energy. The improvement in sperm velocity caused by calcium or creatine phosphate was instantaneous, and there was no long-term loading of calcium in the sperm. The enhancement in velocity was present as long as the additional calcium was available. Our finding that magnesium alone had no effect on sperm motility or velocity is in agreement with previous results.[13] We initially included the magnesium because we expected that it would be necessary for the activity of creatine kinase.

The Verapamil concentrations (4 μmol) used in the experiments diminished sperm motility and velocity to about 60% to 70% of that of the original values. Under these conditions, the improvement in motility parameters due to calcium or creatine phosphate was measurable. Higher Verapamil concentrations (20 to 40 μmol) permanently inhibited sperm motility. These data agree with those in the sea urchin sperm, in which Verapamil binding was one-half maximal at 1 μmol.[24] Sperm motility, velocity, and viability (longevity of survival) declined due to Verapamil. Either calcium or creatine phosphate could alleviate the Verapamil effects with respect to motility and velocity. However, the viability of the sperm did not show similar improvements. The short-term recovery of sperm motility and velocity after Verapamil administration and the subsequent long-term effects causing an abbreviated sperm viability are unexplained at this time. The short-term recovery may be due to a change in efficiency of the remaining calcium channels or to transport of calcium from the mitochondria to the sperm cytoplasm. It is known that Verapamil has pharmacologic effects unrelated to blocking of the calcium channels.[24] However, the fact that the inhibitory action of this experiment is overridden by calcium and the lack of evidence that other pharmacologic effects of Verapamil (e.g., modulation of α-receptors) are affected by additional calcium strongly suggest that the attenuation of sperm motility parameters is due to the calcium channel-blocking action of Verapamil.

From the point of view of sperm energetics, these results suggest that creatine phosphate may directly fuel the rephosphorylation of ADP to ATP in the sperm tail by supplementing the creatine phosphate shuttle.[7] Although the generally accepted view suggests that creatine phosphate is not transported into the intracellular space, it is known that creatine phosphate administration improves the metabolism of heart muscle, and uptake of the creatine phosphate into rabbit myocardium has been demonstrated.[25] With the use of radioactive tracer techniques, we will investigate this question in sperm, as well as the relationship between the uptake of calcium and enhancement of motility and velocity.

Although the molecular mechanism of the enhancement of sperm motility and velocity need more investigation, we expect that the presence of creatine phosphate, calcium, or similar agents in the media during in vitro fertilization or the gamete intrafallopian transfer procedures may increase the efficiency of the sperm in subfertile men. This is not likely to be a factor during intrauterine insemination, which requires the move-

ment of sperm to the fallopian tubes or to the pelvic area, because of the lack of calcium loading effects in migrated sperm. At present, we are studying the response of sperm to calcium and creatine phosphate in oligospermic and asthenospermic specimens to determine the limits of sperm concentration and motility that would make a sample appropriate for this type of in vitro potentiation.

Acknowledgments. We wish to thank Andrew Huszar for help in the statistical analysis of the data and Theresa Concelmo for her excellent contributions in typing and editing the manuscript.

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