

however, more randomized, double-blind, controlled, and well-designed studies are needed to confirm the pregnancy rates after using these stimulants in cryopreserved samples.

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TABLE I. Effect of pentoxifylline, caffeine, and 2-deoxyadenosine on viability, membrane integrity, and penetration scores

Stimulant or Control	Viability (%) (Mean ± SD*)	HOS (%) (Mean ± SD*)	BCMP (mm) (Mean ± SD*)
Control	42.9 ± 9.3	48.9 ± 8.8	48.8 ± 12.2
Pentoxifylline (2.5 mmol)	35.3 ± 10.9 [†] (0.07)	44.1 ± 16.5 [†] (0.15)	53.8 ± 11.0 [†] (0.15)
Caffeine (5.0 mmol)	39.7 ± 12.2 [†] (0.39)	44.6 ± 17.4 [†] (0.24)	50.4 ± 2.3 [†] (0.71)
2-Deoxyadenosine (2.5 mmol)	39.4 ± 13.0 [†] (0.13)	45.3 ± 14.8 [†] (0.21)	51.7 ± 10.5 [†] (0.47)

KEY: HOS = hypo-osmotic swelling test; BCMP = bovine cervical mucus penetration test; SD = standard deviation.

*P value from repeated measures ANOVA, comparison between various stimulants and control.

[†]Not significant.

Also, in our study, the mean average path velocity increased by 55%, and the amplitude of lateral head movement increased by 47% with pentoxifylline treatment. Linearity did not change significantly.

Although motility and motion characteristics improved, other functional characteristics did not significantly change. These observations further strengthen our belief that these stimulants probably increase motility in otherwise quiescent spermatozoa, especially after cryopreservation. The increase in sperm cAMP level is probably the main mechanism underlying the increase in motility observed with pentoxifylline. Pentoxifylline also inhibits reactive oxygen species,¹² causes hyperactivation of sperm,¹³ and enhances the acrosome reaction.¹⁴ Consequently, pentoxifylline is useful for managing patients with oligozoospermia and asthenozoospermia.¹⁵

Some authors believe caffeine increases sperm movement and fertilizing ability,^{16,17} but others question its therapeutic use because of adverse effects.¹⁸ Earlier studies used higher concentrations of caffeine, which could be responsible for the adverse effects observed,¹⁷ and no objective studies of the various motion parameters were conducted. Using a 5 mmol concentration, we found that motility was 55% higher compared with controls. Similarly, curvilinear velocity and straight-line velocity increased by 60% and 34%, respectively, compared with controls. Average path velocity increased by 51%, and the amplitude of lateral head movement exhibited an average increase of 54%. Our results are similar to those of Ruzich *et al.*,¹⁹ who observed that caffeine did not affect linearity, that the effect of caffeine increased as sperm motility decreased, and that caffeine inhibited the rate of decline in sperm motility with time. Moussa²⁰ concluded that 3 mmol and 6 mmol concentrations of caffeine best stimulated motility. Recent work suggests that caffeine has an action similar to pentoxifylline and may stimulate sperm motility by two related pathways that may act synergistically.⁴

2-Deoxyadenosine stimulates cAMP production in sperm and increases intracellular cAMP. In our study, 2.5 mmol 2-deoxyadenosine increased sperm motility by 50% compared with controls. In addition, the curvilinear velocity increased by 47%, the straight-line velocity increased by 52%, the average path velocity increased by 49%, and the amplitude of lateral head movement increased by 42%. Mbizvo *et al.*²¹ reported longer lasting effects with 2-deoxyadenosine than with pentoxifylline or caffeine, whereas Imoedemhe *et al.*²² showed improved fertilization rates in addition to improvement in motility. Our results show that all three stimulants enhance sperm motility in cryopreserved semen samples. Care must be taken to avoid exposure of oocytes to these stimulants.

Practicing urologists and oncologists are frequently asked to provide referrals to a sperm bank for patients diagnosed with infertility or testicular cancer. Thus, a good understanding of cryopreservation techniques as well as artificial stimulation of sperm motility is necessary.

These stimulants have the potential to improve pregnancy rates when using cryopreserved spermatozoa. They have been used successfully in fresh spermatozoa in conjunction with a variety of assisted reproductive techniques.²³⁻²⁶ Many of these procedures improve fertilization and pregnancy rates in patients with previous episodes of fertilization failure.^{9,14,23-28} These studies, therefore, have important clinical applications in male infertility treatment, as well as in in vitro fertilization programs.

In conclusion, these stimulants increase sperm motility and other motion parameters without altering sperm viability and membrane integrity. Spermatozoa may save the extra energy generated by these stimulants for subsequent steps of fertilization.¹⁵ The net effect of these stimulants may not be limited to increased motility alone; other sperm functions required for successful fertilization, such as egg penetration, may also be modulated by cAMP;

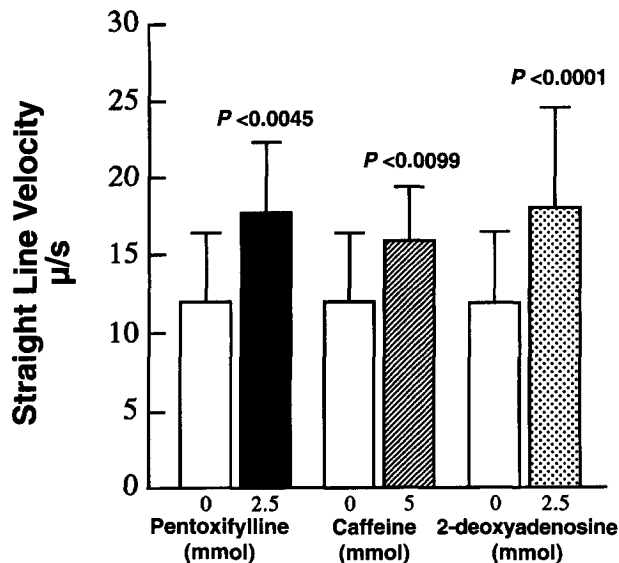


FIGURE 3. Effect of pentoxifylline, caffeine, and 2-deoxyadenosine on straight line velocity. Values represent mean \pm standard deviation ($n = 10$). $P < 0.01$ significantly different from controls.

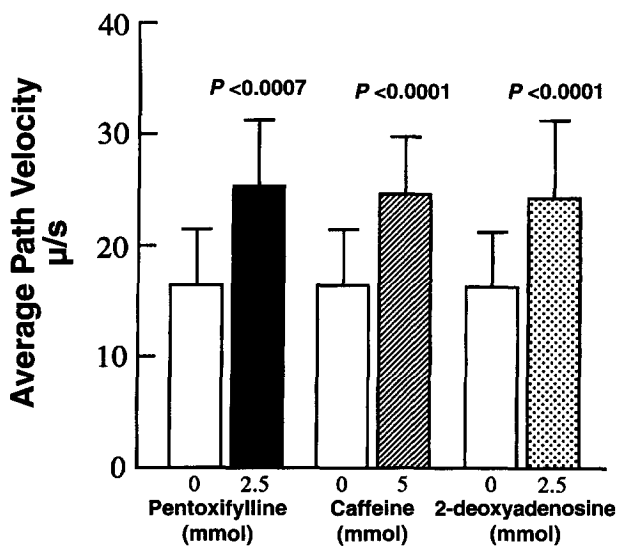


FIGURE 4. Effect of pentoxifylline, caffeine, and 2-deoxyadenosine on average path velocity. Values represent mean \pm standard deviation ($n = 10$). $P < 0.01$ significantly different from controls.

with pentoxifylline; $24.6 \pm 5.0 \mu/s$ ($P < 0.0001$) with caffeine; and to $24.3 \pm 6.9 \mu/s$ ($P < 0.0001$) with 2-deoxyadenosine (Fig. 4).

Amplitude of lateral head displacement increased from $1.9 \pm 0.4 \mu$ in control samples to $2.8 \pm 0.5 \mu$ ($P < 0.0002$) with pentoxifylline; $3.0 \pm 0.5 \mu$ ($P < 0.0001$) with caffeine; and $2.7 \pm 0.4 \mu$ ($P < 0.0002$) with 2-deoxyadenosine (Fig. 5).

Viability, hypo-osmotic swelling, and cervical mucus penetration scores did not significantly increase with treatment and were comparable to control results (Table I).

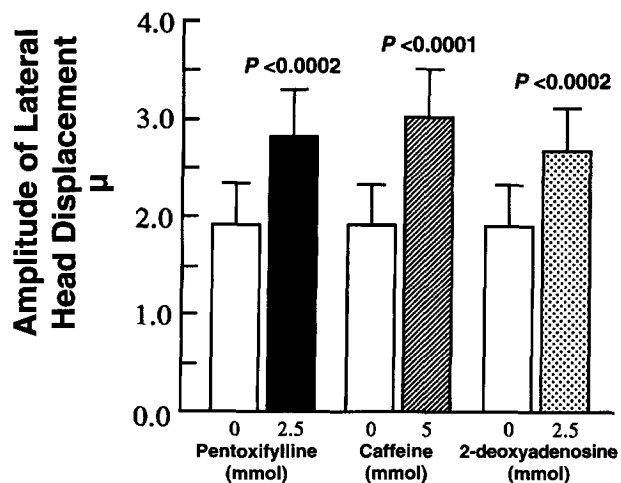


FIGURE 5. Effect of pentoxifylline, caffeine, and 2-deoxyadenosine on amplitude of lateral head displacement. Values represent mean \pm standard deviation ($n = 10$). $P < 0.01$ significantly different from controls.

COMMENT

Motility is crucial for zona pellucida penetration, and motility characteristics are highly correlated with fertilization outcome.² Sperm motility decreases 25% to 75% after cryopreservation.¹ Previously, we established the optimum concentrations and incubation time for each stimulant to maintain improved motion.⁸ This period, 60 minutes, is sufficient for use in assisted reproductive techniques. In the present study, all stimulants enhanced sperm motility in cryopreserved semen samples and significantly improved sample quality.

At 2.5 mmol, pentoxifylline produced a mean increase of 57% in percentage motility compared with control values. In addition, the 60-minute incubation time maintained maximal sperm motion. In another study,⁹ treatment with pentoxifylline greatly increased movement in poor-quality cryopreserved sperm and sperm obtained by electroejaculation. In contrast, Tesarik *et al.*¹⁰ observed no increase, but the drug exposure time was much shorter than ours. This increase in motility suggests that pentoxifylline may have acted on a subpopulation of cryopreserved sperm that was poorly motile or metabolically quiescent sperm that became active in response to an increase in cAMP levels induced by pentoxifylline. Marked changes in sperm quality were observed in the presence of pentoxifylline. In particular, mean curvilinear velocity increased by 59%. The straight-line velocity also increased significantly, by 49%. Other workers have observed increased straight-line velocity in asthenospermic samples after a short exposure to pentoxifylline¹⁰ and after prolonged exposures in samples obtained by electroejaculation.⁹ The significance of enhanced straight-line velocity remains controversial.^{2,11}

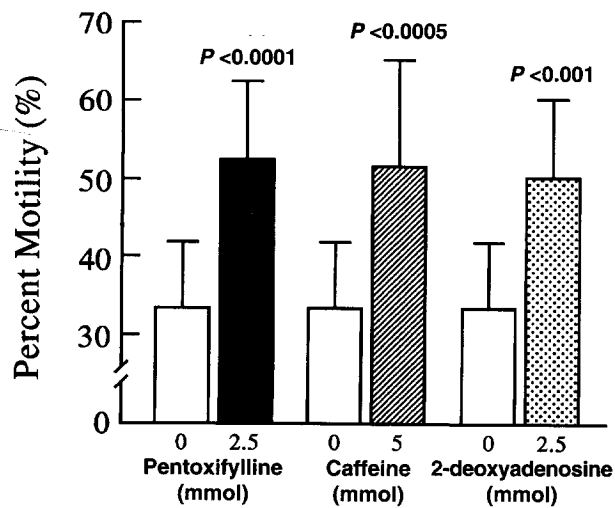


FIGURE 1. Effect of pentoxifylline, caffeine, and 2-deoxyadenosine on percentage motility. Values represent mean \pm standard deviation ($n = 10$). $P < 0.0001$ significantly different from controls.

then resuspended in the same medium to a concentration of 25 to 30×10^6 sperm/mL. The suspensions were divided in four aliquots, one for each stimulant and one for a control. The stimulants were dissolved in HTF in a 1:1 ratio to give the following final concentrations: pentoxifylline 2.5 mmol, caffeine 5.0 mmol, and 2-deoxyadenosine 2.5 mmol. The control aliquot was mixed with HTF alone. The samples were then incubated with the stimulants for 60 minutes at 37°C .

ASSESSMENT OF SEMEN VARIABLES

Sperm samples were analyzed on a computer-assisted motion analyzer (Cell-Trak, Model VP 110, Santa Rosa, Calif) before freezing and after thawing. For each measurement, a $5\text{-}\mu\text{L}$ sample aliquot from either a control or treated sample was loaded on a MicroCell slide (Conception Technologies, La Jolla, Calif) and analyzed for percentage motility, straight-line velocity (straight-line distance from the beginning of the sperm track divided by time), curvilinear velocity (total distance traveled by a given sperm divided by the total time elapsed), amplitude of lateral head displacement (mean width of sperm head oscillation), average path velocity, and linearity (departure of sperm track from a straight line).

SPERM VIABILITY

The percentage of viable sperm in each aliquot was assessed after incubation. Each sample was mixed with an equal amount of 0.05% eosin-Y-nigrosin to improve contrast. After a 2-minute incubation at 22°C , slides were viewed under light microscopy at $\times 400$. A total of 100 spermatozoa in duplicate from each sample were counted. The dead sperm appear pink; sperm without the dye were counted as viable.

MEMBRANE INTEGRITY

One milliliter of hypo-osmotic solution (150 mOsm/L; 0.025 mmol sodium citrate and 0.075 mmol D[-]fructose) was added to 0.1 mL liquefied semen. After incubation at 37°C for 60 minutes, a minimum of 200 spermatozoa per sample were examined using phase-contrast microscopy (Olympus, Model BH 2, Tokyo, Japan), and the percentage of sperm with intact membranes was calculated.⁷

CERVICAL MUCUS PENETRATION

The bovine cervical mucus penetration test was performed using the Penetrak kit (Seron Diagnostics, Allentown, Pa).

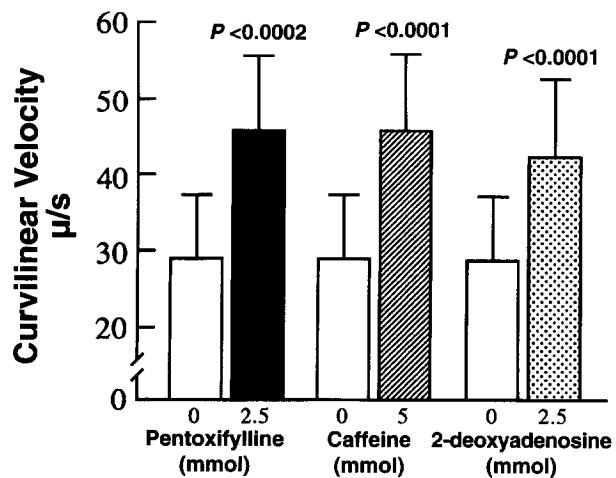


FIGURE 2. Effect of pentoxifylline, caffeine, and 2-deoxyadenosine on curvilinear velocity. Values represent mean \pm standard deviation ($n = 10$). $P < 0.01$ significantly different from controls.

Capillary tubes in duplicate were thawed at room temperature for 30 minutes and snapped at the red score mark above the mucus meniscus. The cut end was placed in a plastic beaker containing $200\ \mu\text{L}$ semen and incubated at room temperature for 90 minutes. The capillary tubes were then placed on a calibrated slide and examined using phase-contrast microscopy. The distance (in millimeters) covered by the vanguard sperm was measured.

STATISTICAL ANALYSIS

Repeated measures analysis of variance (ANOVA) was used to assess the effect of stimulants, using the SAS statistical software package (SAS Institute Inc., 1992). A P value of ≤ 0.01 was considered as statistically significant. All data are shown as mean \pm standard deviation. Semen samples were studied individually to avoid any possible interaction from pooling the samples.

RESULTS

Compared with control samples, specimens treated with pentoxifylline, caffeine, or 2-deoxyadenosine displayed increases in all motion characteristics except linearity.

The percentage motility in controls was 33.3 ± 7.5 . With pentoxifylline, this value increased to 52.3 ± 9.1 ($P < 0.0001$); with caffeine, 51.5 ± 13.5 ($P < 0.0005$); and with 2-deoxyadenosine, 50.1 ± 9.3 ($P < 0.001$) (Fig. 1).

The curvilinear velocity in control samples was $28.7 \pm 8.1\ \mu\text{/s}$; in pentoxifylline-treated samples, it was $45.7 \pm 9.1\ \mu\text{/s}$ ($P < 0.0002$); in caffeine-treated samples, it was $45.8 \pm 9.6\ \mu\text{/s}$, ($P < 0.0001$); and in 2-deoxyadenosine-treated samples, it was $42.3 \pm 10.2\ \mu\text{/s}$ ($P < 0.0001$) (Fig. 2).

The straight-line velocity increased from $11.8 \pm 4.5\ \mu\text{/s}$ in control samples to $17.6 \pm 4.4\ \mu\text{/s}$ ($P < 0.0045$) with pentoxifylline; $15.8 \pm 3.4\ \mu\text{/s}$ ($P < 0.0099$) with caffeine; and $17.9 \pm 6.6\ \mu\text{/s}$ ($P < 0.0001$) with 2-deoxyadenosine (Fig. 3). The average path velocity also increased, from $16.3 \pm 5.0\ \mu\text{/s}$ in controls to $25.3 \pm 5.9\ \mu\text{/s}$ ($P < 0.0007$)

SPERM KINEMATICS OF CRYOPRESERVED NORMOZOOSPERMIC SPECIMENS AFTER ARTIFICIAL STIMULATION*

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ABSTRACT

Objectives. Cryopreservation of semen is associated with reduced motility after thawing, resulting in a decreased pregnancy rate. Artificial stimulation of motility has been used in fresh semen samples. This study measured the effect of motility stimulants on various motion characteristics and other sperm functions using cryopreserved semen.

Methods. Frozen semen samples from healthy donors were thawed, and motility stimulants were added in vitro and incubated for 60 minutes. The percentages of motile spermatozoa in each specimen and other motion characteristics were measured. In addition, spermatozoon's viability, membrane integrity, and ability to penetrate bovine cervical mucus were studied after addition of stimulants.

Results. Percentage motility and all other motion characteristics improved after stimulation with pentoxifylline, caffeine, or 2-deoxyadenosine. Linearity did not significantly differ in the control samples after adding any of the stimulants. Viability, membrane integrity, and penetration ability did not improve significantly and were comparable with control values.

Conclusions. Pentoxifylline, caffeine, and 2-deoxyadenosine can stimulate sperm motility and other motion characteristics. This may be beneficial in the cryopreservation of sperm from normal donors and oligozoospermic patients for use in assisted reproduction. *UROLOGY*® 47: 77-81, 1996.

Sperm cryopreservation continues to gain importance with newer assisted reproduction techniques. Unfortunately, cryopreserved spermatozoa achieve lower fertilization rates than fresh spermatozoa because recovery of motile sperm is poor.¹ Motility is an important factor in the ability of the sperm to fertilize.² Some cryopreserved donor sperm have intact membranes after thawing, but are immobile owing to a low metabolic state. Artificial stimulation of motility may be beneficial in cryopreserved and oligozoospermic sperm preparations used for assisted reproduction. The ability to stimulate motility and reduce damage during cryopreservation may improve the fertilizing capability of cryopreserved sperm.³

Methylxanthine derivatives, such as pentoxifylline and caffeine, increase the motility of ejac-

ulated sperm by inhibiting cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterase. Sperm motility can also be increased by direct exposure to 2-deoxyadenosine, an analogue of adenosine.^{4,5}

This study determined the effects of pentoxifylline, caffeine, and 2-deoxyadenosine on sperm motion, viability, membrane integrity, and ability to penetrate cervical mucus using cryopreserved sperm specimens from normozoospermic persons.

MATERIAL AND METHODS

CHEMICALS

TEST yolk buffer-freezing medium and modified human tubal fluids with 5% human serum albumin (HTF) were purchased from Irvine Scientific (Santa Ana, Calif). Pentoxifylline, caffeine, and 2-deoxyadenosine were purchased from Sigma Chemical Company (St. Louis, Mo).

SEMEN COLLECTION AND FREEZING

Semen samples from 10 healthy donors were evaluated as per World Health Organization criteria.⁶ All subjects abstained from ejaculation for 48 hours. The samples were obtained by masturbation into a sterile specimen cup. The ejaculate was allowed to liquefy at 37°C, and the semen characteristics were analyzed. The samples were then diluted 1:1 with TEST yolk buffer-freezing medium, placed in sterile vials, and cryopreserved by slow freezing at -20°C for 8 minutes, followed by immersion in liquefied nitrogen vapor at -79°C for 2 hours, and finally submersion in liquid nitrogen at -196°C.

The samples were thawed by incubating at 37°C. They were then washed with HTF, centrifuged at 300g for 7 minutes, and

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