OPTIMAL DOSE AND DURATION OF EXPOSURE TO ARTIFICIAL STIMULANTS IN CRYOPRESERVED HUMAN SPERMATOZOA

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ABSTRACT

Purpose: Poor sperm motility after cryopreservation is associated with infertility. For any pharmacological stimulation to be of clinical value, its effect in enhancing motility and other motion characteristics should be maintained for at least 1 hour.

Materials and Methods: Three motility stimulants (pentoxifylline, caffeine and 2-deoxyadenosine) were incubated with post-thaw semen samples from 11 healthy donors for 0, 30, 60, 120 and 180 minutes. The final concentrations used were 2.5 mM., 5 mM. and 10 mM. for pentoxifylline; 1 mM., 2 mM. and 5 mM. for caffeine, and 0.5 mM., 1 mM. and 2.5 mM. for 2-deoxyadenosine. Percent motility and changes in motion characteristics were measured on a computer assisted semen analyzer.

Results: Compared to controls (0 minutes, no stimulant), an immediate increase in motility and other motion parameters was noted with all 3 stimulants. All stimulants caused a significant increase in percentage motility at all periods studied (p < 0.01). Similarly, pentoxifylline increased other motion parameters at the 2.5 mM. concentration (p < 0.01), caffeine was effective in increasing curvilinear velocity, average path velocity and amplitude of lateral head displacement (p < 0.01) at 5 mM., and 1 and 2.5 mM. of 2-deoxyadenosine increased the curvilinear velocity, straight line velocity, average path velocity and amplitude of lateral head displacement (p < 0.01). Among all stimulants only 2-deoxyadenosine increased linearity only at the 1 mM. concentration.

Conclusions: Our study suggests that these stimulants, when used at optimum concentrations, can maintain the improved sperm quality for durations longer than the minimum needed for fertilization. This finding may be significant in improving the poor semen quality observed after cryopreservation in oligospermic samples and in semen specimens from cancer patients.

KEY WORDS: spermatozoa; infertility, male; caffeine; pentoxifylline; deoxyadenosines

The prognosis for infertile couples has become more favorable in recent years with the improved efficacy of assisted reproductive techniques. However, in cases of male factor infertility, effective treatment is still lacking. Poor sperm motility rather than a male infertility and the complicating factor most frequently encountered in assisted reproduction. Sperm must be vigorously motile to penetrate the cervical mucus, migrate to the fertilizing site and penetrate the zona pellucida. Motility correlates directly with the fertilization rates of human oocytes in vitro and with pregnancy rates after artificial insemination, adding support to its importance in male fertility.

Sperm cryopreservation continues to gain importance in assisted reproduction, especially due to the acquired immune deficiency crisis, which has necessitated quarantining semen samples for 6 months before use. A 25 to 75% decrease in sperm motility is noted after semen is cryopreserved and poor recovery of motile sperm correlates with a decreased pregnancy rate. Of semen cryopreserved from healthy subjects, only 65 to 70% retain sufficient sperm motility to be useful for artificial insemination. The ability to stimulate motility among thawed sperm and lessen damage during cryopreservation would improve the fertilizing capability of sperm. Therefore, a stimulant that enhances sperm motility in vitro may be of clinical value in treating male infertility. Improved motility would need to be maintained for a period sufficient to complete artificial insemination and other assisted reproductive procedures.

Methylxanthine derivatives have been used to increase the motility of freshly ejaculated sperm. They act by inhibiting 3’5’-monophosphate (cyclic adenosine monophosphate) phosphodiesterase and, thus, increase intracellular cyclic adenosine monophosphate concentration. The effect of phosphodiesterase inhibitors is more pronounced on sperm manifesting less than optimal motility.

Even though several investigators have described the stimulatory effects of methylxanthines on human spermatozoa, the literature is inconsistent about the precise effects on motility or the drug concentrations needed to achieve these effects, especially when cryopreserved human spermatozoa are used. We determine the effects of varying concentrations of 2 methylxanthine derivatives (pentoxifylline and caffeine) and an adenosine analogue (2-deoxyadenosine) on sperm motion for a range of exposure times and establish the optimum concentration for each stimulant.

MATERIALS AND METHODS

Semen collection. Semen samples were evaluated according to World Health Organization criteria. All subjects were asked to abstain from ejaculation for 48 hours. The 11 samples were produced by masturbation into sterile specimen
The ejaculate was allowed to liquefy at 37°C before the sperm count and other semen characteristics were evaluated. The semen samples were diluted with an equal volume of TEST yolk-buffer freezing medium, placed in sterile vials and cryopreserved by slow freezing at -20°C for 8 minutes, followed by immersion in liquid nitrogen vapor (-79°C for 2 hours) and submersion in liquid nitrogen at -196°C.

Cryopreserved samples were thawed at 37°C, washed with human tubal fluid containing 5% human serum albumin, centrifuged and resuspended at a concentration of 25 to 30 x 10⁶ sperm per ml. Each stimulant used was dissolved in human tubal fluid medium at a volume ratio of 1:1. Control aliquots contained human tubal fluid medium alone. All samples were then incubated for 30, 60, 120 and 180 minutes. The final concentrations of the stimulants used were 2.5 mM., 5 mM. and 10 mM. pentoxifylline; 1 mM., 2.5 mM. and 5 mM. caffeine, and 0.5 mM., 1 mM. and 2.5 mM. 2-deoxyadenosine.

Semen analysis. Sperm samples were analyzed on a computer-assisted motion analyzer before freezing and after thawing. For each measurement 5 μl aliquots from the control and treated samples were loaded on a cell slide and analyzed for percent motility, straight-line velocity, curvilinear velocity, average path velocity, amplitude of lateral head displacement and linearity.

Statistical analysis. Data were analyzed for statistical differences in sperm motion parameters between the treated samples and controls by repeated measures of analysis of variance using statistical software. A value of p = 0.01 was considered significant. Semen samples were studied individually to avoid any possible interaction from pooling of the specimens.

RESULTS

Compared to control samples (no stimulation at 0 minute), an immediate increase in motility and all motion parameters except linearity was observed with all 3 stimulants (p <0.01). Similarly, all concentrations of pentoxifylline, caffeine and 2-deoxyadenosine increased motility following 30 minutes of incubation. This increase was maintained even after 180 minutes at 2.5 mM. concentration of pentoxifylline, 5 mM. caffeine and all concentrations of 2-deoxyadenosine (fig. 1). Improvement in curvilinear velocity was maintained for up to 180 minutes with 2.5 mM. pentoxifylline, and 5 mM. and 2-deoxyadenosine (fig. 2).

Straight line velocity was maintained for up to 180 minutes with 1 mM. 2-deoxyadenosine (p <0.01) and for shorter periods with pentoxifylline (30 minutes, p <0.01) and caffeine (120 minutes, p <0.01, fig. 3). Similarly, pentoxifylline and 2-deoxyadenosine maintained an average path velocity for up to 180 minutes (p <0.01) and for up to 120 minutes with caffeine (fig. 4). Increase in amplitude of lateral head displacement was significantly maintained with 2-deoxyadenosine (120 minutes) and caffeine (180 minutes), and for a shorter period (30 minutes) with pentoxifylline (fig. 5).

Compared to controls, no significant change in linearity was noted with pentoxifylline and caffeine at any of the concentrations and periods studied. 2-Deoxyadenosine at a 1 mM. concentration increased linearity at 60 and 180 minutes.

DISCUSSION

Different methylxanthines produce subtly different effects on sperm motility in fresh and cryopreserved spermatozoa, and individuals showed a range of response. Our findings suggest that 2.5 mM. and 5 mM. concentrations of pentoxifylline improve sperm motility, although the lower concentration is preferred to avoid unnecessary drug toxicity. Most other studies with fresh spermatozoa have used 3.6 mM. pentoxifylline, which increases the curvilinear velocity, straight line velocity, average path velocity and amplitude of lateral head displacement of spermatozoa in suspension, although other concentrations have also been effective. Paul et al applied pentoxifylline at concentrations of 1 to 12 mM. directly to semen samples and concluded that 6 mM. was best. Our study strengthens the observation that pentoxifylline stimulates sperm motility optimally at 2 mM. in

![Graph of motility percentage over incubation time](image-url)
spermatozoa from normospermic individuals, and at 3 mM and 3.6 mM in cryopreserved donor spermatozoa.\textsuperscript{19} These results suggest that pentoxifylline may exert its influence over a narrow therapeutic range of concentration.

We observed a significant improvement in sperm motion using a 5 mM concentration of caffeine for 180 minutes, although the lower concentrations were effective at 60 minutes. Hammitt et al found a concentration of 7.5 mM to be optimum, although they reported decreased in vitro sperm survival and penetrating capacity.\textsuperscript{7} Our findings are similar to those of Ruzich et al, who demonstrated an increase in sperm velocity and percentage of motile sperm in healthy donors.\textsuperscript{20}

In our study the effect of 2-deoxyadenosine on motility and other motion characteristics lasted for up to 60 minutes of incubation at all concentrations. Compared to pentoxifylline and caffeine, 2-deoxyadenosine produced a larger increase in motility, and significantly improved curvilinear velocity,
FIG. 4. Effect of pentoxifylline, caffeine and 2-deoxyadenosine on average path velocity in 11 patients. Values represent mean plus or minus standard deviation. Asterisks indicate values significantly different from controls (*p < 0.01).

FIG. 5. Effect of pentoxifylline, caffeine and 2-deoxyadenosine on amplitude of lateral head displacement in 11 patients. Values represent mean plus or minus standard deviation. Asterisks indicate values significantly different from controls (*p < 0.01).

straight line velocity and average path velocity. For longer periods (120 and 180 minutes) 1 mM. and 2.5 mM. effectively maintained these improved motion parameters. Aitken et al also demonstrated longer lasting effects with 2-deoxyadenosine.10

Different stimulants possess different mechanisms of action. Pentoxifylline inhibits 3'5'-nucleotide phosphodiesterase, catalyzing conversion and buildup of cyclic adenosine monophosphate by stimulating glycolysis.10,11 Caffeine increases motility and respiration by 2 interrelated pathways that could interact synergistically.21-23 At lower concentrations caffeine inhibits calmodulin-dependent phosphodiesterase, thereby increasing the intracellular cyclic adenosine monophosphate levels.7,8 At a higher concentration it modifies calcium translocation, resulting in decreased intracellular calcium levels. 2-Deoxyadenosine increases intracellular cyclic adenosine monophosphate by in-
creasing adenylate cyclase activity. The stimulatory activity is rapid and sustained, and increases the number and quality of motile sperm.

These stimulants not only increase sperm motility but also other sperm functions necessary for penetration and fertilization, such as hyperactivation, capacitation and acrosome reaction. These functions may be modulated through increased influx of calcium and by the amount of cyclic adenosine monophosphate available. Pentoxifylline does not affect the spontaneous acrosome reaction but increases the induced acrosome reaction, which correlates with an improved in vitro fertilization rate, especially in cases of acrosome insufficiency. On the other hand, caffeine increases the spontaneous acrosome reaction without any change in induced acrosome reaction, which may counteract the beneficial effects on sperm motility.

Naturally occurring cyclic adenosine monophosphate phosphodiesterase inhibitors have been implicated in the maintenance of meiotic arrest in mammalian oocytes. Pentoxifylline has a low toxicity, and no effect on embryonic development was observed in a study in which the cumulus-free oocytes were inseminated or when cleavage beyond the 2-cell stage was initiated. This finding suggests that the inhibitory effect is restricted to high concentrations of pentoxifylline and caffeine. Interestingly, the association of caffeine to stimulate human spermatozoa in vitro was dampened by its reported deleterious effects on spermatozoan morphology and fertility. Improvement in sperm motility by caffeine does not translate into enhanced fertilizing ability, and inhibition of human embryo development has been observed in the presence of caffeine. However, Barkay et al demonstrated an improved pregnancy rate in an artificial insemination study when spermatozoa were pretreated with caffeine.

2-Deoxyadenosine has been observed to arrest embryonic development in mice due to inhibition of protein kinase. However, neither pentoxifylline nor 2-deoxyadenosine (2.5 mM) had any detrimental effect on exposed oocytes if they were removed before insemination, and fertilization rates were comparable to those in routine in vitro fertilization. Therefore, exposure of oocytes to these stimulants should be avoided. Fertilization rates have increased after treatment with 2-deoxyadenosine. Use of these stimulants in a fresh sperm sample is helpful in cases of male infertility when donor insemination would otherwise fail, and has resulted in higher fertilization and pregnancy rates. It has also decreased the risk of failed fertilization in certain cases of male factor infertility.

CONCLUSIONS

Our study suggests that concentrations of 2.5 mM pentoxifylline, 5 mM caffeine and 2.5 mM 2-deoxyadenosine are optimum to improve and maintain motility long enough to increase the number of sperm reaching the upper reproductive tract, and increase the fertilizing capacity in assisted reproduction. We believe these stimulants have a role in treating some male infertility problems in which donor insemination would otherwise have been advised. However, removing these chemicals before insemination would be prudent.

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REFERENCES