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Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction rate

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Cryopreservation causes extensive damage to spermatozoa, thereby impairing their fertilizing ability. The purpose of this study was to determine if the direct addition of pentoxifylline to the seminal plasma before cryopreservation improved sperm motility and acrosome reaction. Semen specimens from 15 healthy volunteers were divided into two aliquots. One aliquot was treated by adding 5 mM pentoxifylline directly to the seminal plasma (treatment group) and the other aliquot received no treatment (control group). Both aliquots were then cryopreserved by using the liquid nitrogen freezing method. The percentage of motile spermatozoa and various motion characteristics were then evaluated by performing computer-assisted semen analysis. The sperm viability was determined with a supra-vital dye, Hoechst-33258, and the acrosome reaction (spontaneous and calcium ionophore-induced) was monitored using fluorescein isothiocyanate-conjugated peanut lectin (FITC-PNA) binding assays. Pentoxifylline treatment significantly increased the sperm motility, the amplitude of lateral head displacement, the hyperactivation status, and the frequency of spontaneous acrosome reactions before freezing ($P < 0.05$). After post-thaw, no difference in motion characteristics (except percentage motility) between treated and control groups were observed. Acrosome loss due to the freeze–thaw process was less in the pentoxifylline-treated group ($P = 0.0003$). In addition, the percentage of cryopreserved acrosome-intact spermatozoa that underwent further acrosome reactions in response to calcium-ionophore challenge was significantly higher in the treated group ($P = 0.03$). Pentoxifylline treatment before freezing improved the acrosome reaction to ionophore challenge in cryopreserved spermatozoa. Treatment with pentoxifylline appears to minimize sperm damage during the freeze-thaw process and may improve fertilization rates with assisted reproductive procedures such as intrauterine insemination or in-vitro fertilization.

Key words: acrosome/cryopreservation/ionophore/pentoxifylline/sperm motility

Introduction

Sperm cryopreservation results in adverse changes in sperm motility (Critser *et al.*, 1987), viability (Alvarez and Storey, 1993), the ability of spermatozoa to penetrate into cervical mucus, zona-free hamster eggs (Critser *et al.*, 1987), and the acrosome reaction (McLaughlin *et al.*, 1992). The fertilizing ability of human spermatozoa decreases after cryopreservation and can be partially explained by the reduced percentage of spermatozoa with normal, intact acrosomes and diminished acrosin activity (Mack and Zaneveld, 1987). Therefore, efforts to improve the fertilizing ability of cryopreserved spermatozoa are clinically useful.

Phosphodiesterase inhibitors such as pentoxifylline have been used to stimulate sperm motility (Sikka and Hellstrom, 1991; Tesarik *et al.*, 1992a; Sharma *et al.*, 1996). The treatment of highly selected motile sperm populations by incubation with pentoxifylline stimulates in-vitro fertilization (IVF) rates (Yovich *et al.*, 1993). Pentoxifylline exerts its effects on sperm motility probably by inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterase, thus increasing the intracellular cAMP concentration (Garbers *et al.*, 1971). Cyclic AMP is also involved in the acrosome reaction which is mediated by a second-messenger system (De Jonge *et al.*, 1991). Treatments that increase intracellular cAMP concentrations often cause an increase in the agonist-induced acrosome reaction (Tesarik *et al.*, 1992a,b) and fertilization rate (Yovich *et al.*, 1990). However, others have reported that the beneficial effects of pentoxifylline on human spermatozoa may be mediated through its powerful scavenging capacity for reactive oxygen radicals (Yovich *et al.*, 1993; Mckinney *et al.*, 1996) rather than by phosphodiesterase inhibition alone.

Studies analysing the effects of pentoxifylline on cryopreserved spermatozoa have focused mainly on post-thaw sperm motility (Hammitt *et al.*, 1989; Wang *et al.*, 1993; Brennan and Holden, 1995) and motion characteristics (Mbizvo *et al.*, 1993; Sharma *et al.*, 1996) rather than on the functional aspects of the spermatozoa, namely the acrosome status. In this study we attempted to evaluate whether the addition of pentoxifylline directly to the liquefied semen before cryopreservation improved post-thaw sperm function.

Materials and methods

Semen collection and sperm preparation

Sperm specimens from 15 healthy volunteers of proven fertility were obtained by masturbation into sterile specimen cups. All subjects were asked to observe 2–3 days of sexual abstinence before donating samples. The ejaculates were then allowed to liquefy for 30 min at 37°C after which a small aliquot was removed from each specimen

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and analysed on a computer-assisted semen analyser equipped with a hyperactivation module (CASA; Motion Analysis Corporation, Cell-Trak, model VP 110, Santa Rosa, CA, USA). All analyses were done within 1 h of sample collection. The following sperm characteristics were assessed: concentration, percentage motility, and motion characteristics including curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH). All ejaculates were of normal semen volume and sperm count according to the World Health Organization (1992) criteria.

Cryopreservation

After initial semen analyses, each specimen was divided into two equal aliquots. The first aliquot was treated by adding 5 mM pentoxifylline (Sigma Chemical Company, St Louis, MO, USA) directly to the sample. The second aliquot received no treatment (control). Both aliquots were then incubated for 30 min at 37°C. After the incubation period both the control and treated specimens were evaluated by using the CASA. A small aliquot (0.1 ml) was also removed from each raw specimen to assess the acrosome status.

The control and treated samples were cryopreserved by using the liquid nitrogen vapour freezing method (Sharma *et al*, 1996), with TEST–yolk buffer containing glycerol (Irvine Scientific, Santa Ana, CA, USA) as the medium for cryopreservation. Briefly, a 5 ml vial of the frozen medium was thawed by incubation at 37°C and an aliquot equal to 25% of the original specimen volume was added to the specimen. The specimen was then gently mixed for 5 min in a Hema-Tek aliquot mixer (Miles, Elkhart, IN, USA). This process was repeated four times to give a final 1:1 volume ratio of freezing medium to ejaculate. These aliquots were then placed in cryovials at –20°C for 8 min. Subsequently, the vials were submerged in liquid nitrogen vapour at –79°C for 2 h before finally being immersed in liquid nitrogen at –196°C for long-term storage. The post-thaw analyses were done after storage for 48 h in liquid nitrogen when a small aliquot (0.1 ml) was removed from each cryovial for semen analysis and monitoring of the spontaneous acrosome reaction.

Calibration set-up of the motion analyser

To assess the sperm motion characteristics, the CASA calibration set-up was done as follows: 2-well, 20 µm; duration of data capture (frames): 15 (raw) and 30 (washed); minimum motile speed (µm/s): 600 (raw) and 800 (washed); distance scale factor (µm/pixel): 0.9457; centroid cell size minimum (pixels): 2; centroid cell size maximum (pixels): 8; number of cells to find per well: 200; minimum number of fields per sample: 3. A high degree of correlation was seen between the CASA and both the manual sperm counts (r^2 =1, slope 1) and motility (r^2 = 0.97, slope 0.97), thereby establishing the accuracy of our CASA measurements. The reproducibility of the analyser results was determined by using a calibration video tape recording. The baseline values for the raw specimens were: sperm count 38.3 to 42.5×10^6/ml (30 frame/s) and motility 61.6 to 75.0 % (30 frame/s); the washed specimens had baseline values of 33.1 to 34.7×10^6/ml (60 frame/s) for sperm count and 78.1 to 81.5 % (60 frame/s) for motility. For these computations results greater than two standard deviations were rejected.

Sperm capacitation and ionophore challenge

A highly motile sperm population from each specimen was isolated by using the swim-up method after thawing (Sharma *et al*., 1996). Capacitation of these motile spermatozoa was achieved by incubating the specimen in a Biggers–Whitten–Whittingham (BWW) medium containing 3% human serum albumin (HSA; Irvine Scientific) at 37°C, under 5% CO_2 in air. Each capacitated sample was divided into two equal aliquots containing 1.0×10^6 spermatozoa: one was challenged with 2.5 mol/l calcium ionophore A23187 (Sigma), and the other was treated with dimethylsulphoxide (10% v/v) solution to serve as a control. Both aliquots were then incubated at 37°C for 1 h under identical incubation conditions.

Assessment of sperm viability

Sperm viability was assessed by centrifugation of sperm specimens and suspension of pellet in 100 µl of 2 µg/ml Hoechst 33258 solution (Sigma) and incubated for 10 min at 37°C in the dark (Esteves *et al*., 1996). The spermatozoa were then washed in phosphate-buffered saline (PBS) solution and centrifuged at 250 *g* for 5 min to remove excess stain. The resulting pellet was resuspended in 100 µl of BWW buffer. Twenty microlitres of this suspension were subsequently smeared on a microscope slide and allowed to dry. At least three slides of each sample were prepared. Viability was classified as follows: viable spermatozoa showed pale-blue fluorescence in the head region, whereas dead spermatozoa fluoresced bright bluish-white.

Assessment of spontaneous and ionophore-induced acrosome reaction

The baseline frequency of acrosome-reacted spermatozoa (spontaneous acrosome reaction) was measured before freezing and after thawing while the calcium ionophore-induced acrosome reaction was measured only after thawing. Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; Sigma) was used to assess the acrosome reaction (Cross *et al*., 1986; Mortimer *et al*., 1990; Aitken *et al*., 1993). The slides were immersed in ice-cold methanol for 30 s to permeabilize the sperm membranes and air-dried at room temperature. The air-dried smears were immersed in a 40 µg/ml FITC-PNA solution, incubated at room temperature for 20 min in foil-covered Coplin jars, and washed gently in PBS to remove the excess label. Scoring was completed within 48 h of staining.

A Leitz Orthoplan fluorescence microscope (Leitz, Germany) equipped with a Ploemopak epi-illumination module and a mercury UV source was used to examine the slides. An anti-quenching immersion oil (Cargille, type DF; Fisher Scientific, Pittsburgh, PA, USA) was used to minimize the loss of fluorescence. A dual filter was used to score the slides, filter cube I.2 was used for FITC-PNA, and filter cube A.2 for Hoechst 33258.

Categorization of staining patterns

Acrosome staining upon FITC-PNA labelling was classified as follows: spermatozoa with intact acrosomes showed uniform apple-green fluorescence in the acrosomal region of the sperm head, acrosome-reacted spermatozoa stained only in the equatorial segment of the acrosome. A total of 200 spermatozoa per sample were scored. The acrosome reaction rate was calculated only in the viable spermatozoa.

Reproducibility of the acrosome scores

The acrosome score was evaluated by two observers scoring the same slide from each donor (n = 15) in a blinded fashion. Similarly, the same slide was scored by a single observer to determine intra-observer reproducibility.

Statistical analyses

Due to the paired nature of the study design, each donor served as his own control. Data are expressed as median and interquartile range. Using the SAS software package (Cary, NC, USA). Wilcoxon signed-rank test was used to assess the differences in the motility and sperm motion characteristics between control and treated specimens before and after cryopreservation. Differences in the frequency of spontan-

Table I. Effects of pre-freeze sperm treatment with pentoxifylline on percentage motility and motion parameters in 15 normozoospermic men before and after cryopreservation

Sperm parameters	Control Median (IQ range)	Pentoxifylline Median (IQ range)	P^{a}
Motility (%)			
Pre-freeze	58.7 (49.1, 62.6)	70.0 (64.7, 73.0)	0.0001
Post-thaw	27.9 (22.1, 32.6)	22.0 (12.4, 26.0)	0.0003
Curvilinear velocity (μm/s)			
Pre-freeze	46.9 (38.3, 52.5)	48.0 (38.9, 58.8)	0.06
Post-thaw	22.4 (17.1, 27.5)	21.6 (18.1, 27.3)	0.49
Straight-line velocity (μm/s)			
Pre-freeze	19.7 (16.3, 25.8)	15.7 (14.4, 20.1)	0.01
Post-thaw	6.2 (4.9, 9.3)	5.4 (4.2, 8.7)	0.71
Average path velocity (μm/s)			
Pre-freeze	29.2 (24.2, 34.3)	25.8 (24.6, 35.4)	0.98
Post-thaw	12.0 (8.4, 13.6)	9.0 (7.2, 14.6)	0.39
Linearity (%)			
Pre-freeze	44.1 (42.5, 46.1)	36.9 (32.6, 38.6)	0.0002
Post-thaw	31.0 (26.5, 34.6)	30.4 (27.9, 35.8)	0.99
Amplitude of lateral head displacement (μm)			
Pre-freeze	2.9 (2.3, 3.1)	3.1 (2.5, 3.5)	0.006
Post-thaw	1.1 (0.9, 1.2)	1.1 (0.9, 1.5)	0.90
Hyperactivation (%)			
Pre-freeze	1.1 (0.5, 1.8)	2.8 (0.9, 4.1)	0.03
Post-thaw	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.50

[a]$P < 0.05$ was considered significant.
IQ = interquartile.

Table II. Effects of pre-freeze sperm treatment with pentoxifylline on spontaneous acrosome reaction in 15 normozoospermic men before and after cryopreservation

Frequency of spontaneous acrosome reaction (%)	Control Median (IQ range)	Pentoxifylline Median (IQ range)	P^{a}
Pre-freeze	10.3 (4.5, 15.5)	17.8 (14.7, 25.5)	0.0001
Post-thaw	28.2 (26.6, 41.7)	32.3 (27.5, 53.6)	0.04
Percentage change	219.5 (163.9, 495.2)[b]	127.2 (23.8, 212.2)[b]	0.0003

[a]$P < 0.05$ was considered significant.
[b]Significant increase after cryopreservation ($P < 0.001$).
IQ = interquartile.

Table III. Effects of pre-freeze sperm treatment with pentoxifylline on post-thaw calcium-ionophore induced acrosome reaction in 15 normozoospermic men after cryopreservation

Frequency of spontaneous acrosome reaction (%)	Control Median (IQ range)	Pentoxifylline Median (IQ range)	P^{a}
Before ionophore challenge	28.2 (26.6, 41.7)	32.3 (27.5, 53.6)	0.04
After ionophore challenge	47.9 (34.9, 52.5)	58.2 (48.6, 64.8)	0.0001
Percentage change	34.8 (10.3, 57.8)[b]	53.3 (22.6, 101.7)[b]	0.03

[a]$P < 0.05$ was considered significant.
[b]Significant increase after cryopreservation ($P < 0.001$).
IQ = interquartile.

eous and calcium ionophore-induced acrosome reaction, and between control and pentoxifylline-treated specimens either before or after cryopreservation were also evaluated by applying the Wilcoxon signed-rank test. $P < 0.05$ was accepted as statistically significant.

Results

The pre-freeze and post-thaw changes in the motility and motion characteristics in both pentoxifylline and control groups are shown in Table I. Pentoxifylline treatment before freezing significantly increased the percentage of motile spermatozoa ($P < 0.001$), ALH ($P < 0.01$), and the hyperactivation status of the spermatozoa ($P < 0.05$). No improvement was seen in VCL or VAP, but the VSL ($P = 0.01$) and LIN ($P < 0.001$) significantly decreased after pentoxifylline treatment. Cryopreservation impaired the motility and other motion parameters in both groups ($P < 0.001$); however, except for the percentage motility, these effects were similar in both control and pentoxifylline-treated groups.

The frequency of spermatozoa undergoing spontaneous acrosome reactions in viable sperm samples before and after cryopreservation is shown in Table II. Before freezing, pentoxifylline-treated specimens exhibited a higher frequency of acrosome-reacted spermatozoa ($P = 0.001$). Similarly, the percentage of spermatozoa exhibiting acrosome reactions was higher in the pentoxifylline-treated group after thawing ($P = 0.04$). The acrosomal loss, assessed by calculating the change in the frequency of viable acrosome-reacted spermatozoa during the freeze-thaw process, was significantly lower in the pentoxifylline-treated group relative to the control-treated group ($P = 0.001$).

The frequency of acrosome reaction occurrences induced by calcium ionophore challenge (ARIC) in both the pentoxifylline and control group is shown in Table III. The ARIC scores were calculated by subtracting the frequency of acrosome reaction occurrence following ionophore challenge from the frequency of occurrence without ionophore challenge. The ARIC score was significantly improved in both groups ($P < 0.001$), but the value was higher in the pentoxifylline-treated spermatozoa ($P = 0.03$). ARIC scores were >10% in 80% (12 out of 15) of patient specimens compared to 60% (9 out of 15) of control specimens. When an individual specimen was compared to its own control, pre-freeze treatment with pentoxifylline was found to increase the ARIC scores above 10% in all control specimens. Even in pentoxifylline-treated specimens with ARIC scores of 5–10% ($n = 3$), the ARIC scores were higher when compared to their individual controls.

The inter- and intra-observer reproducibility analysis revealed that the mean difference in the measured frequency of spontaneous acrosome reaction occurrence between observers was 1.31 ± 9.53% (not significant) and the average coefficient of variation (CV) was 6.5%. The intraclass correlation coefficient (ICC) between the observers was 0.81 at a 95% confidence interval (CI) of 0.62–0.91. The mean difference for duplicate evaluation of the same 15 slides by one observer was 0.29 ± 2.41% (not significant) and the CV was 1.6%. The ICC between the two readings by the same observer was 0.98 at a 95% CI of 0.95–0.99.

Discussion

Despite the availability of sophisticated assisted reproductive techniques, the cryosurvival rates and pregnancy rates with

frozen semen are lower than those with fresh semen (Polanski and Lamb, 1988; Topfer-Peterson *et al*., 1988). Although damage to the acrosome after cryopreservation may be secondary to cell death (Cross and Hanks, 1991), an increase in the proportion of viable acrosome-reacted spermatozoa after cryopreservation has been reported (McLaughlin *et al*., 1992). In the present study, we observed a detrimental effect of cryopreservation on overall sperm quality. Irrespective of whether the spermatozoa were treated with pentoxifylline before freezing, significant decreases in the percentage of motile spermatozoa, motion characteristics, and in the percentage of viable spermatozoa with intact acrosomes were seen, corroborating existing reports (Critser *et al*., 1987; Centola *et al*., 1990; McLaughlin *et al*., 1992).

Pentoxifylline, a phosphodiesterase inhibitor in the methylxanthine group, exerts a stimulating influence on sperm motion characteristics by inhibiting cAMP phosphodiesterase (Garbers *et al*., 1971; Sikka *et al*., 1991). Although pentoxifylline does not increase the number of motile spermatozoa, it does improve sperm motion characteristics in pre-selected sub-populations of spermatozoa (Yovich *et al*., 1990; Tesarik *et al*., 1992b). However, these previous studies were performed on selected sperm populations enriched with highly motile cells, and therefore the usefulness of these results in improving IVF protocols is questionable (Tournaye *et al*., 1993a). Nevertheless, patients whose semen parameters are sub-optimal may benefit from the addition of pentoxifylline directly to the semen specimens (Paul *et al*., 1995). Increased recovery and enhanced motility of spermatozoa would be advantageous for oligozoospermic and asthenozoospermic patients undergoing either IVF or IUI.

Whereas most studies have used a concentration of 3.6 mM pentoxyfylline for 3 h (Tesarik *et al*., 1992b; Kay *et al*., 1993; Lewis *et al*., 1993), others have demonstrated similar beneficial effects both with lower and higher concentrations (Sikka and Hellstrom, 1991; Tesarik *et al*., 1992a; Moohan *et al*., 1993; Centola *et al*., 1995; Sharma *et al*, 1996). However, a detrimental effect on membrane integrity was seen at the pentoxifylline concentration of 10 mM (Tournaye *et al*., 1994). Moreover, there is a considerable variation in the response of spermatozoa to pentoxifylline in both normozoospermic (Moohan *et al*., 1993) and oligozoospermic semen samples (Yovich, 1993). In ~10% of patients, spermatozoa do not respond to a pentoxifylline challenge. Therefore, an assessment of the individual responses to pentoxifylline is necessary before routinely employing this agent in assisted reproduction.

We selected a concentration of 5 mM pentoxifylline not only because it stimulates sperm motility when added directly to the seminal plasma, but also because of its oxygen free radical scavenging capacity (Gavella *et al*, 1991; Yovich *et al*., 1993; McKinney *et al*., 1996). The increase in sperm motion characteristics including the motility percentage, the ALH, and the hyperactivation status occured after 30 min exposure to pentoxifylline. Notably, the variations in the response of individual sperm specimens to pentoxifylline treatment seen in our study have been reported by others (Paul *et al*., 1995). Pentoxifylline at a 5 mM concentration decreased the VSL and LIN, but had no effect on VAP and VCL. This is in agreement with previous studies although different concentrations of pentoxifylline were used (Kay *et al*., 1993; Pang *et al*., 1993). It is possible that the freezing of semen specimens with pentoxifylline may afford additional cryoprotection. No beneficial effect was seen on the post-thaw sperm motion characteristics regardless of whether pentoxifylline was present in the freezing medium. In addition, no improvements in post-thaw donor sperm motion characteristics after a 2 h incubation with pentoxifylline were reported, except for an increase in the percentage of hyperactivated spermatozoa (Centola *et al*., 1995). These findings are in contrast to previous studies that demonstrated an enhancement in post-thaw sperm motility when pentoxifylline was added prior to cryopreservation (Hammitt *et al*., 1989). The addition of pentoxifylline to the freezing media may protect the sperm membranes from damage caused by freezing and allow a better recovery of motile spermatozoa (Bell *et al*., 1993). Our findings indicate that at the concentration used, post-thaw spermatozoa may be unable to respond effectively to pentoxifylline treatment, either because of membrane damage caused by cryopreservation, or because they may be sensitized and require a lower concentration.

Pentoxifylline increases the motility speed of spermatozoa by increasing intracellular cAMP concentration (Wang *et al*. 1993); this also improves the rate of the acrosome reaction. Collectively, these processes may be critically involved in enhancing fertilization rate following IUI or IVF (Holt *et al*., 1985), as well as in cases of severe male infertility (Yovich *et al*., 1990). Pentoxifylline treatment increases the capacity of spermatozoa to undergo the acrosome reaction in response to both natural (follicular fluid) and artificial (ionophore A23187) stimuli, and it also improves the fertilizing ability in patients with acrosome reaction insufficiency (Tesarik *et al*., 1992a; Tesarik and Mendoza, 1993). Treatment with pentoxifylline can enhance sperm function in selected patients. If used to enhance the in-vitro sperm function, pentoxifylline should be washed from the preparation used for insemination to minimize toxicity to the embryo (Tournaye *et al*., 1993b).

In the present study, pentoxifylline treatment increased the frequency of the acrosome reaction both before freezing and after thawing, although this response was not seen uniformly in all post-thaw specimens. Notably, the lack of an increase in the frequency of spontaneous acrosome reactions after pentoxifylline treatment as reported by others may be due to the use of lower pentoxifylline concentrations than that employed in our study (Tesarik *et al*., 1992a).

We observed a significant reduction in acrosome loss due to the freeze–thaw process in pentoxifylline-treated specimens. However, the pre-freeze sperm treatment with pentoxifylline improved acrosome reaction to ionophore challenge in cryopreserved spermatozoa. The beneficial effects of pentoxifylline on sperm function may be due to its scavenging property for reactive oxygen species and the increase in intracellular cAMP concentrations. Aitken *et al*. (1996) reported reduction in peroxidative damage by supplementing pentoxifylline with antioxidants. Significant increase in pregnancy rates were observed following IUI when pentoxifylline was added to the semen at the time of sperm preparation (Negri *et al*., 1996). Paul

et al. (1996) demonstrated increased binding of spermatozoa to hemizonae treated with pentoxifylline in the hemizona binding assay. Cryopreserved spermatozoa may have defective sperm function due to elevated levels of reactive oxygen species (Aitken *et al.*, 1989). This may cause peroxidation of unsaturated fatty acids in the acrosomal membranes and decrease the response to calcium influx signals that trigger the acrosome reaction. Thus, the antioxidant properties of pentoxifylline may stabilize the acrosomal membrane and maintain acrosome reaction. Preincubation of spermatozoa with pentoxifylline may therefore increase the fertilizing ability by rendering the spermatozoa more responsive to the acrosome reaction inducers present on the oocyte surface.

The response of the acrosome reaction-to-ionophore challenge test (ARIC) has demonstrated high sensivity and predictive value for IVF (Cummins *et al.*, 1991). Patients showing ARIC scores of >10% have better fertilization rates than those with scores <10% (Yovich *et al.*, 1993). In the present study, only 60% (nine out of 15) of control specimens had ARIC scores >10%, whereas, 80% (12 out of 15) of pentoxifylline-treated specimens exhibited ARIC scores >10%. Furthermore, pentoxifylline improved the ARIC scores in all the treated specimens compared to their controls. Although our study did not examine the relationship of pentoxifylline treatment to subfertility, others have demonstrated beneficial effects of pentoxifylline in improving fertilization rates and pregnancy outcome in couples with male factor infertility and poor fertilization rates following assisted reproduction (Matson *et al.*, 1995; Rizk *et al.*, 1995; Wittemer *et al.*, 1996).

In summary, direct addition of pentoxifylline to the seminal plasma before cryopreservation offers significant benefits in improving the acrosome reaction to ionophore challenge and minimizing acrosomal loss during the freeze–thaw process. Pentoxifylline, as proposed, may improve the fertilizing ability of cryopreserved spermatozoa.

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References

Aitken, R.J., Clarkson, J.S., Hargreave, T.B. *et al.* (1989) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J. Androl.*, **10**, 214–220.

Aitken, R.J., Buckingham, D.W. and Fang, H.G. (1993) Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J. Androl.*, **14**, 132–141.

Aitken, R.J., Allan, I.W., Irvine, D.S. and Macnamee, M. (1996) Studies on the development of diluents for the transportation and storage of human semen at ambient temperature. *Hum. Reprod.*, **11**, 2186–2196.

Alvarez, J.G. and Storey, B.T. (1993) Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerols and other polyols as sole cryoprotectants. *J. Androl.*, **14**, 199–208.

Bell, M., Wang, R., Hellstrom, W.J.G. and Sikka, S.C. (1993) Effect of cryoprotective additives and cryopreservation protocol on sperm membrane lipid peroxidation and recovery of motile human sperm. *J. Androl.*, **14**, 472–478.

Brennan, A.P. and Holden, C.A. (1995) Pentoxifylline-supplemented cryoprotectant improves human sperm motility after cryopreservation. *Hum. Reprod.*, **10**, 2308–2312.

Centola, G.M., Mattox, J.H., Burde, S. and Leary, J.F. (1990) Assessment of the viability and acrosome status of fresh and frozen-thawed human spermatozoa using single-wavelength fluorescence microscopy. *Mol. Reprod. Dev.*, **27**, 130–135.

Centola, G.M., Cartie, R.J. and Cox, C. (1995) Differential responses of human sperm to varying concentrations of pentoxifylline with demonstration of toxicity. *J. Androl.*, **16**, 136–142.

Critser, J.K., Arneson, B.W., Asker, D.V. *et al.* (1987) Cryopreservation of human spermatozoa. II. Post-thaw chronology of motility and zona-free hamster ova penetration. *Fertil. Steril.*, **47**, 980–984.

Cross, N.L. and Hanks, S.E. (1991) Effects of cryopreservation on human sperm acrosomes. *Hum. Reprod.*, **6**, 1279–1283.

Cross, N.L., Morales, P., Overstreet, J.W. and Hanson, F.W. (1986) Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res.*, **15**, 213–226.

Cummins, J.M., Pember, S.M., Jequier, A.M. *et al.* (1991) A test of the human sperm acrosome reaction following ionophore challenge. Relationship to fertility and other seminal parameters. *J. Androl.*, **12**, 98–103.

De Jonge, C.D., Han, H.L., Lawrie, H. *et al.* (1991) Modulation of the human sperm acrosome reaction by effectors of the adenylate cyclase, cyclic AMP second-messenger pathway. *J. Exp. Zool.*, **158**, 113–125.

Esteves, S.C., Sharma, R.K., Thomas, A.J., Jr and Agarwal, A. (1996) Suitability of the hypo-osmotic swelling test for assessing the viability of cryopreserved sperm. *Fertil. Steril.*, **66**, 798–804.

Garbers, D.L., Lust, W.D., First, N.L. and Lardy, H.A. (1971) Effect of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. *Biochemistry*, **10**, 1825–1831.

Gavella, M. and Lipovac, V. (1991) Effect of pentoxifylline on experimentally induced lipid peroxidation in human spermatozoa. *Int. J. Androl.*, **17**, 308–313.

Hammitt, D.G., Bedia, E.B., Rogers, P.R. *et al.* (1989) Comparison of motility stimulants for cryopreserved human semen. *Fertil. Steril.*, **52**, 495–502.

Holt, W.V., Moore, H.D.M. and Hillier, S.G. (1985) Computer-assisted measurements of sperm swimming speed in human semen: correlation of results with *in vitro* fertilization assays. *Fertil. Steril.*, **44**, 112–119.

Kay, V.J., Coutts, J.R.T. and Robertson, L. (1993) Pentoxifylline stimulates hyperactivation in human spermatozoa. *Hum. Reprod.*, **8**, 727–731.

Lewis, S.E., Moohan, J.M. and Thompson, W. (1993) Effects of pentoxifylline on human sperm motility in normozoospermic individuals using computer-assisted analysis. *Fertil. Steril.*, **59**, 418–423.

Mack, S.R. and Zaneveld, L.J. (1987) Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res.*, **18**, 375–383.

Matson, P.L., Yovich, J.M., Edirisinghe, W.R. *et al.* (1995) An argument for the past and continued use of pentoxifylline in assisted reproduction. *Hum. Reprod.*, **10**, 67–71.

Mbizvo, M.T., Johnston, R.C. and Baker, G.H.W. (1993) The effect of the motility stimulants, caffeine, pentoxifylline, and 2-deoxyadenosine on hyperactivation of cryopreserved human sperm. *Fertil. Steril.*, **59**, 1112–1117.

McKinney, K.A., Lewis, S.E. and Thompson, W. (1996) The effects of pentoxifylline on the generation of reactive oxygen species and lipid peroxidation in human spermatozoa. *Andrologia*, **28**, 15–20.

McLaughlin, E.A., Ford, W.C. and Hull, M.G. (1992) Motility characteristics and membrane integrity of cryopreserved human spermatozoa. *J. Reprod. Fertil.*, **95**, 527–534.

Moohan, J.L., Winston, R.L.M. and Lindsay, K.S. (1993) Variability of human sperm exposure to immediate and prolonged exposure to pentoxifylline. *Hum. Reprod.*, **8**, 1696–1700.

Mortimer, D., Curtis, E.F. and Camenzind, A.R. (1990) Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. *Hum. Reprod.*, **5**, 99–103.

Negri, P., Grechi, E., Tomasi, A. *et al.* (1996) Effectiveness of pentoxifylline in semen preparation for intrauterine insemination, *Hum. Reprod.*, **11**, 1236–1239.

Pang, S.C., Chan, P.J. and Lu, A. (1993) Effects of pentoxifylline on sperm motility and hyperactivation in normozoospermic and normokinetic semen. *Fertil. Steril.*, **60**, 336–343.

Paul, M., Sumpter, J.P. and Lindsay, K.S. (1995) Action of pentoxifylline directly on semen. *Hum. Reprod.*, **10**, 354–359.

Paul, M., Sumpter, J.P. and Lindsay, K.S. (1996) The paradoxical effects of pentoxifylline on the binding of spermatozoa to the human zona pellucida. *Hum. Reprod.*, **11**, 814–819.

Polanski, F.F. and Lamb, E.J. (1988) Do the results of semen analysis predict future fertility? A survival analysis study. *Fertil. Steril.*, **49**, 1059–1065.

Rizk, B., Fountain, S., Avery, S. *et al.* (1995) Successful use of pentoxifylline in male-factor infertility and previous failure of *in vitro* fertilization: a prospective randomized study. *J. Assist. Reprod. Genet.*, **12**, 710–714.

Sharma, R.K., Tolentino, M.V., Jr, Thomas, A.J., Jr and Agarwal, A. (1996) Optimal dose and duration of exposure to artificial stimulants in cryopreserved human spermatozoa. *J. Urol.*, **155**, 568–573.

Sikka, S.C. and Hellstrom, W.J. (1991) The application of pentoxifylline in the stimulation of sperm motion in men undergoing electroejaculation. *J. Androl.*, **12**, 165–170.

Tesarik, J. and Mendoza, C. (1993) Sperm treatment with pentoxifylline improves the fertilizing ability in patients with acrosome reaction insufficiency. *Fertil. Steril.*, **60**, 141–144.

Tesarik, J., Mendoza, C. and Carreras, A. (1992a) Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertil. Steril.*, **58**, 1185–1190.

Tesarik, J., Thébault, A. and Testart, J. (1992b) Effect of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. *Hum. Reprod.*, **7**, 1257–1263.

Topfer-Peterson, E., Friess, A.E. and Schill, W.B. (1988) The acrosome reaction in boar spermatozoa. *Hum. Reprod.*, **3**, 319–326.

Tournaye, H., Janssens, R., Camus, M. *et al.* (1993a) Pentoxifylline is not useful in enhancing sperm function in cases with previous *in vitro* fertilization failure. *Fertil. Steril.*, **59**, 210–215.

Tournaye, H., Van der Linden, M., Van der Abbeel, E. *et al.* (1993b) Effects of pentoxifylline on implantation and post-implantation development of mouse embryos *in vitro*. *Hum. Reprod.*, **8**, 1948–1954.

Tournaye, H., Janssens, R., Verheyen, G. *et al.* (1994) *In vitro* fertilization in couples with previous fertilization failure using sperm incubated with pentoxifylline and 2-deoxyadenosine. *Fertil. Steril.*, **62**, 574–579.

Wang, R., Sikka, S.C., Veeraragavan, K. *et al.* (1993) Platelet activating factor and pentoxifylline as human sperm cryoprotectants. *Fertil. Steril.*, **60**, 711–715.

Wittemer, C., Ohl, J., Bettahar-Lebugle, K. *et al.* (1996) Could *in vitro* fertilization with a modified sperm preparation technique be an option to micromanipulations. *J. Assist. Reprod. Genet.*, **13**, 726–730.

World Health Organization (1992) *Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 3rd edn. Cambridge University Press, New York.

Yovich, J.L. (1993) Pentoxifylline: actions and applications in assisted reproduction. *Hum. Reprod.*, **8**, 1786–1791.

Yovich, J.M., Edirisinghe, W.R., Cummins, J.M. and Yovich, J.L. (1990) Influence of pentoxifylline in severe male factor infertility. *Fertil. Steril.*, **53**, 715–722.

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