

Effects of pentoxifylline treatment before freezing on motility, viability and acrosome status of poor quality human spermatozoa cryopreserved by the liquid nitrogen vapor method

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Abstract

The objective of the present study was to investigate the effects of the direct addition of pentoxifylline (PF) to the ejaculates of men with poor sperm quality before freezing on post-thaw sperm motility, viability, acrosome integrity, and agonist-induced acrosome reaction. Semen specimens from 16 infertile men with impaired sperm count and motility (oligoasthenozoospermia) were divided into two equal aliquots: one received no treatment (control) while the other was incubated with 5 mM PF (treated). Both aliquots were cryopreserved by the liquid nitrogen vapor method. Motility was assessed according to WHO criteria. Acrosome integrity and spontaneous and calcium ionophore-induced acrosome reactions were assessed with fluorescein isothiocyanate-conjugated peanut agglutinin combined with a supra-vital dye (Hoechst-33258). Cryopreservation impaired sperm motility (percentage reduction: 87.4 (interquartile range, IQ: 70.3-92.9) vs 89.1 (IQ: 72.7-96.0%)), viability (25.9 (IQ: 22.2-29.7) vs 25.6 (IQ: 19.7-40.3%)) and acrosome integrity (18.9 (IQ: 5.4-38.9) vs 26.8 (IQ: 0.0-45.2%)) to the same extent in both treated and control aliquots. However, PF treatment before freezing improved the acrosome reaction to ionophore challenge test scores in cryopreserved spermatozoa (9.7 (IQ: 6.6-19.7) vs 4.8 (IQ: 0.5-6.8%); $P = 0.002$). These data show that pre-freeze treatment of poor quality human sperm with pentoxifylline did not improve post-thaw motility or viability nor did it prevent acrosomal loss during the freeze-thaw process. However, PF, as used, improved the ability of thawed spermatozoa to undergo the acrosome reaction in response to calcium ionophore. The present data indicate that treatment of poor quality human sperm with PF may enhance post-thaw sperm fertilizing ability.

Key words

- Acrosome
- Cryopreservation
- Human spermatozoa
- Ionophore
- Pentoxifylline
- Oligoasthenozoospermia

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Part of the data were presented by
S.C. Esteves as a Doctoral thesis
to the Federal University of
São Paulo, Brazil.

Publication supported by
Institutional Funding for Research.

Received September 29, 2006
Accepted May 8, 2007

Introduction

Cryopreservation impairs sperm fertilizing ability by decreasing sperm motility and motion characteristics, penetration into the cervical mucus and into zona-free hamster eggs, viability, membrane integrity and acrosome structure, and activity of acrosome protease and acrosin (1-4). Pentoxifylline (PF), a phosphodiesterase inhibitor, has been used to stimulate the motility and fertilizing capacity of human sperm (5-8). PF inhibits cyclic adenosine monophosphate (cAMP) phosphodiesterase, thus increasing intracellular cAMP concentration (5,7) and tyrosine-phosphorylation at the tail level (8). cAMP has a role in sperm kinematics and in the acrosome reaction second-messenger system (7,9). Treatments that increase intracellular cAMP concentrations often cause an increase in sperm motility and kinematics as well as in the agonist-induced acrosome reaction (9) and fertilization rates (5,6). Also, it has been postulated that PF has the ability to scavenge reactive oxygen radicals (10,11).

It has been reported that pre-freeze sperm treatment with PF decreases acrosome loss during the freeze-thaw process and increases the post-thaw agonist-induced acrosome reaction rate in normal semen (12). This strategy may be clinically useful because it can improve the fertilizing potential of cryopreserved human semen. One of the major advantages of sperm banking is the fertility preservation in cancer patients prior to potential gonadotoxic treatments, such as radiotherapy and chemotherapy. Although specimens of cancer patients freeze as effectively as those from normal men, the semen quality at the time of cryopreservation is often poor in such individuals, especially in testicular cancer patients (13). In the present study, we investigated if the beneficial effects of the addition of PF directly to the seminal plasma before freezing, as seen in normal human semen (12), can also be demonstrated in poor quality specimens, such as

those obtained from infertile men with decreased sperm count and motility (oligoasthenozoospermia).

Patients and Methods

Chemicals

TEST yolk-buffer freezing medium, sperm-washing medium (modified Biggers-Whitten-Whittingam; BWW) and Dulbecco's phosphate-buffered saline (PBS) were purchased from Irvine Scientific (Santa Ana, CA, USA). Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), calcium ionophore A23187, PF, and bis-benzimide (Hoechst-33258) were obtained from Sigma (St. Louis, MO, USA).

A stock solution of FITC-PNA was prepared by dissolving 2 mg FITC-PNA in 1 mL PBS and stored in 600- μ L aliquots at -20°C . Hoechst-33258 was prepared by dissolving 1 mg H33258 in 1 mL PBS and stored in 10- μ L aliquots at -20°C .

Semen collection, sperm processing and cryopreservation procedures

Sperm specimens were obtained by masturbation into sterile specimen cups from men seeking infertility evaluation at a University-based hospital. All subjects were asked to abstain from ejaculation from 2 to 3 days before collection. After liquefaction at 37°C , a small aliquot was removed from each specimen and the sperm parameters were determined according to World Health Organization (WHO) guidelines (14). A total of 16 oligoasthenozoospermic semen samples were selected according to WHO criteria for sperm count of $<20 \times 10^6/\text{mL}$ and total motility of $<50\%$. The etiology of male infertility was not taken into consideration in this study. The median values of sperm count (million/mL), percent motility and seminal plasma leukocytes, as assessed by the Endtz test (14), were 12.0 (25-75% inter-

quartile range: 9.0-14.0), 40.5 (25-75% interquartile range: 30.0-59.0), and 0.2 (interquartile range: 0.1-0.4), respectively. Informed consent from all subjects included in the study and approval by the Institution Ethics Committee were obtained. The experimental design and methods were identical to those reported in a previous study (12). Briefly, after initial analysis each specimen was divided into two equal aliquots. The first aliquot received no treatment (control) and the second was treated by adding a 10-mM PF solution directly to the liquefied ejaculate (1:1, v/v). The final concentration of PF in the mixture was 5 mM. Control and treated aliquots were incubated for 30 min at 37°C and then evaluated for percent and progressive motility according to WHO criteria (14), and for acrosome integrity (12,15).

Control and treated specimens were then cryopreserved by the liquid nitrogen vapor method (12). TEST-yolk buffer with glycerol was used as a freezing agent for cryopreservation (15). Briefly, an aliquot of the freezing medium equal to 25% of the original specimen volume was added to the specimen. This process was repeated until the ratio of freezing medium to ejaculate was 1:1. Cryovials were loaded with 1 mL freezing medium/ejaculate mixture, and kept at -20°C for 8 min. Next, the cryovials were exposed to liquid nitrogen vapor at -79°C for 2 h before being plunged into liquid nitrogen for storage at -196°C. After at least 48 h in liquid nitrogen, the samples were thawed at room temperature for 5 min and transferred to a 37°C incubator for 20 min (4). In order to remove the cryomedium, the thawed specimens were diluted with BWW medium at a ratio of 1 volume of freezing medium/ejaculate to 3 volumes of medium and washed by centrifugation at 300 g for 7 min. The supernatant was removed and the pellet resuspended in 0.7 mL BWW medium with 0.3% human serum albumin. A small aliquot (0.1 mL) was removed from each washed specimen for the assessment of percent motility,

progressive motility and the frequency of spontaneous acrosome reaction.

Sperm incubation under capacitating conditions and ionophore challenge test

After thawing, the motile sperm population from each specimen was isolated by the swim-up method from a washed sperm preparation (15). The motile spermatozoa were then capacitated for 3 h by incubation in BWW medium with 3% human serum albumin at 37°C in a 5% CO₂ atmosphere (4). After capacitation the specimens were divided into two equal aliquots: one was incubated with 2.5 µmol/L calcium ionophore A23187 solution, and the other was treated with dimethylsulfoxide (10%, v/v) solution to serve as a control (12). Both aliquots were then incubated at 37°C for 1 h under the same experimental conditions.

Assessment of viability, acrosome status and ionophore-induced acrosome reaction

Acrosome status was assessed with FITC-PNA in combination with the nuclear stain bis-benzimide to monitor viability. Viability and acrosome status were assessed simultaneously by mixing 100 µL of sperm specimens with 100 µL of a 2-µg/mL Hoechst-33258 solution (12). The mixture was incubated for 10 min in the dark. Excess stain was removed from the spermatozoa by dilution with PBS and centrifugation at 300 g for 5 min, and the pellet was resuspended in 100 µL BWW. A 20-µL aliquot of this solution was smeared on a microscope slide and allowed to dry in the dark at room temperature. Air-dried slides were immersed in ice-cold methanol for 30 s to permeabilize the sperm membranes. The fixed smears were immersed in a solution of 40 µg/mL FITC-PNA for 20 min, and washed gently in PBS to remove excess label.

A fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with

an epi-illumination module and a mercury ultraviolet source was used to examine the slides at 1000X magnification. The B2A filter cube was used for FITC-PNA, which fluoresces apple-green, and the UV2A cube for Hoechst-33258, which fluoresces a bright medium blue. Hoechst-33258 stains the nuclei of damaged cells (dead spermatozoa), which show a bright blue-white fluorescence, and is excluded from viable cells (live spermatozoa), which show a pale blue fluorescence. The same spermatozoon was examined for FITC-PNA labeling and for Hoechst-33258 staining by interchanging the two filters (12).

Staining patterns showing sperm viability and acrosome status

In viable spermatozoa, the sperm head showed a pale-blue fluorescence, and in dead spermatozoa, the sperm head showed a bright blue-white fluorescence. In an intact acrosome, the acrosomal region of the sperm head exhibited a uniform apple-green fluorescence, and in a reacted acrosome, only the equatorial segment of the acrosome was stained. A total of 200 spermatozoa per sample were scored. The calculated frequency of spermatozoa with intact and reacted acrosomes was based on viable cells only. The acrosome reaction to ionophore challenge (ARIC) scores was calculated by subtracting the frequency of acrosome reaction occurrence following ionophore challenge from the frequency of occurrence before it.

Statistical analysis

On the basis of previously published data showing that the average percent decrease in sperm motility, viability and acrosome integrity after the freeze-thawing process is about 15% (2,4,12,13,15,16), and that *in vitro* sperm treatment with PF increases sperm motility and acrosome response to ionophore challenge from 10 to 20% (12,17,

18), we expected that similar results would be achieved with the experimental conditions used in the present study. Assuming a level of significance of 0.05 and a power of 80%, it was calculated that 15 subjects were needed in each experimental group to detect differences of $\geq 10\%$ in the main outcome measures (sperm motility, viability, acrosome integrity, and acrosome reaction in response to ionophore challenge). Data were assessed for homogeneity and normality by the Kolgomorov-Smirnov test. Statistical differences in sperm motility, viability and acrosome status between control and treated specimens before or after cryopreservation were assessed by the Wilcoxon signed-rank test. Data are reported as median and interquartile range. The level of significance was set at $P < 0.05$. Statistical analysis was performed using the SAS statistical software package (Cary, NC, USA).

Results

Pre-freeze and post-thaw sperm motility and viability in control and PF-treated specimens are compared in Table 1. Before freezing, PF treatment significantly increased both the percentage of motile spermatozoa ($P < 0.01$) and the percentage of spermatozoa exhibiting progressive motility ($P = 0.006$). However, PF treatment did not alter pre-freeze sperm viability. The freeze-thaw process significantly decreased sperm motility ($P < 0.001$), progressive motility ($P < 0.001$) and viability ($P < 0.001$) in both control and treated specimens. The percent change in these parameters from before freezing to after thawing was not statistically different between treated and non-treated groups.

Percentages of viable spermatozoa with intact acrosomes in control and PF groups before and after cryopreservation are shown in Table 1. Acrosome integrity did not differ between control and treated groups before freezing. The freeze-thaw process significantly decreased the percentages of live

sperm with intact acrosomes in both the control and PF groups ($P < 0.001$; Table 1). The percentage of spermatozoa that lost acrosomal contents during freezing and thawing, assessed by subtracting the percentage of viable-intact acrosome spermatozoa post-thaw from the pre-freezing percentage, was 18.9 and 26.8% in the control and PF groups, respectively (Table 1). However, the differences were not statistically significant.

The effects of calcium ionophore on the acrosome status of thawed viable spermatozoa of the control and PF groups are shown in Table 2. After thawing, the percentage of live spermatozoa exhibiting a spontaneous acrosome reaction in control and treated specimens was 43.9 and 38.0%, respectively (Table 2). Again the differences were not statistically significant.

The ARIC was calculated by subtracting the frequency of acrosome reaction occurrence after ionophore challenge from the frequency of occurrence before it. ARIC scores were significantly increased in both the control ($P = 0.02$) and PF groups ($P < 0.001$). However, ARIC scores were significantly higher in the PF-treated group (median: 9.7% (25-75% interquartile range: 6.6-19.7)) than in control (median: 4.8% (25-75% interquartile range: 0.5-6.8; $P = 0.002$)).

We observed that ARIC scores were higher than 10% in 50% (8 of 16) of the specimens in the PF group. In the remaining specimens, ARIC scores were between 5 and 10% in 6 (6/8), and <5% in only 2 specimens (Figure 1). In the control group, only 19% (3 of 16) of the specimens had ARIC scores >10%. In the remaining 13 specimens, five exhibited ARIC scores between 5 and 10%, and 8 scores <5%. When an individual specimen was compared to its own control, pre-freeze PF treatment was found to improve the ARIC scores in 81.2% of the specimens (13/16; Figure 1). Interestingly, pre-freeze PF treatment improved ARIC scores to >10% in all but 1 (4/5) control specimens with scores between 5

Table 1. Effect of pre-freeze treatment with pentoxifylline on motility, viability and acrosome integrity of sperm from 16 oligoasthenozoospermic men before and after cryopreservation.

Sperm parameters	Control	Pentoxifylline
Percent motility		
Pre-freeze	40.5 (30.0-59.0)	55.5 (41.5-62.5)*
Post-thaw	3.5 (2.5-10.5)	5.0 (2.5-12.0)
Percent change ^a	87.4 (70.3-92.9)	89.1 (72.7-96.0)
P value ^b	<0.001	<0.001
Progressive motility (%grades a + b)		
Pre-freeze	35.5 (24.0-58.0)	47.0 (34.5-59.5)*
Post-thaw	1.5 (0.0-5.0)	2.0 (0.0-6.0)
Percent change ^a	94.5 (74.1-99.9)	94.4 (84.1-100.0)
P value ^b	<0.001	<0.001
Sperm viability (%)		
Pre-freeze	76.2 (73.0-90.0)	74.6 (70.6-92.1)
Post-thaw	54.6 (48.0-61.2)	50.7 (44.7-59.2)
Percent change ^a	25.9 (22.2-29.7)	25.6 (19.7-40.3)
P value ^b	<0.001	<0.001
Acrosome integrity (%)		
Pre-freeze	79.2 (67.7-82.7)	76.2 (73.2-81.5)
Post-thaw	62.8 (52.9-80.2)	58.0 (45.9-71.2)
Percent change ^a	18.9 (5.4-38.9)	26.8 (0.0-45.2)
P value ^b	<0.001	<0.001

Data are reported as median with interquartile range in parentheses. Progressive motility = grade a (spermatozoa exhibiting fast progression) and grade b (spermatozoa exhibiting slow progression) according to WHO criteria.

^aPercent change calculated as median of individual changes; ^bP value comparing pre-freeze to post-thaw (Wilcoxon signed-rank test). * $P < 0.05$ compared to control (Wilcoxon signed-rank test).

Table 2. Effects of pre-freeze treatment with pentoxifylline on post-thaw calcium ionophore-induced acrosome reaction in sperm from 16 oligoasthenozoospermic men.

Post-thaw frequency of live spermatozoa with reacted acrosomes (%)	Control	Pentoxifylline
Before ionophore challenge	43.9 (27.2-54.5)	38.0 (30.1-51.2)
After ionophore challenge	45.6 (25.7-59.1)	52.7 (38.5-67.4)*
Percent change ^a	-11.8 (-32.0-2.9)	-30.2 (-40.8-18.5)*
ARIC score (%) [*]	4.8 (0.5-6.8)	9.7 (6.6-19.7)
P value ^b	<0.001	<0.001

Data are reported as median with interquartile range in parentheses. Acrosome reaction in response to ionophore challenge (ARIC) score was calculated by subtracting the frequency of acrosome reaction occurrence after ionophore challenge from the frequency of occurrence before it.

^aPercent change calculated as median of individual changes; ^bP value comparing frequency of live spermatozoa with reacted acrosomes before and after ionophore challenge (Wilcoxon signed-rank test). * $P < 0.05$ compared to control (Wilcoxon signed-rank test).

and 10%. Similarly, pre-freeze PF treatment improved ARIC scores to >5% in all but 1 (7/8) control specimens with scores <5%. Only 3 specimens were refractive to PF treatment (Figure 1).

Discussion

At a concentration of 5 mM PF has oxygen-free radical scavenging capacities by reducing the superoxide release from human spermatozoa following phorbol myristate acetate (11). Higher PF concentrations, however, may be detrimental to membrane integrity (19). Normal sperm incubated with 5 mM PF prior to cryopreservation are protected from the deleterious effects of the freeze-thaw process (12). Based on these findings, PF may be used as a supplement to the cryomedia currently available. In this study, we examined if the benefits of PF for freezing normal sperm can be extrapolated to poor quality sperm obtained from oligoasthenozoospermic men. Oligoasthenozoospermia is often seen in cancer patients, who are the main patient population seeking sperm banking. Our results indicated that

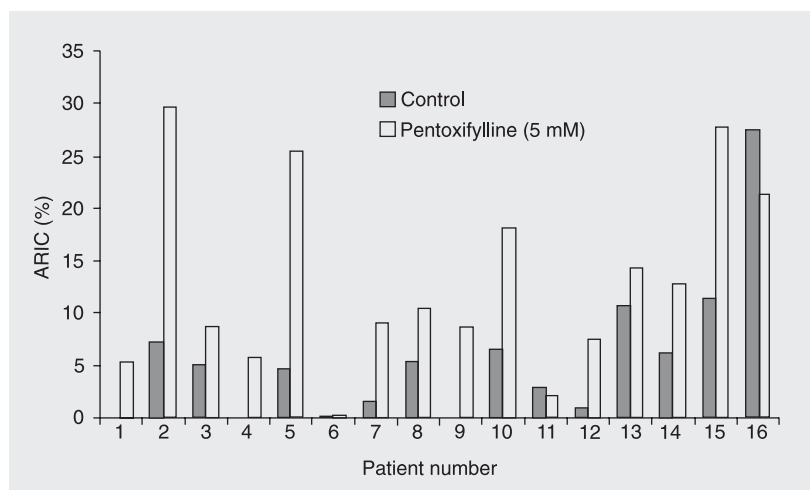


Figure 1. Effects of pre-freeze pentoxifylline treatment on post-thaw acrosome reaction to ionophore challenge (ARIC) in sperm from 16 oligoasthenozoospermic men. Bars represent ARIC scores for each individual: control (filled bars) and 5 mM pentoxifylline-treated (open bars). Filled bars are not seen in controls 1, 4, and 9 because ARIC scores were equal to zero (post-thaw live spermatozoa in these specimens were refractive to stimulation with calcium ionophore).

the cryopreservation process was detrimental to sperm motility and viability, irrespective of whether or not spermatozoa had been treated with PF before freezing, corroborating previous reports (12). Others have demonstrated an enhancement in post-thaw sperm motility when PF was added prior to cryopreservation (17,18). It is possible that at the concentration used in our study, post-thaw spermatozoa were unable to respond effectively to PF in terms of motility, either because of membrane damage caused by cryopreservation, or because they may be sensitized and require different concentrations for stimulation. Moreover, we observed a significant decrease in the post-thaw percentage of viable spermatozoa with intact acrosomes in both control and PF-treated specimens. In normal semen, a PF-induced protective effect on acrosome loss during the freeze-thaw process was observed (12). In the present study, we evaluated poor quality semen, and it is possible that normal semen would resist cryoinjury better than semen obtained from oligoasthenozoospermic men. We observed that preefreeze treatment of sperm from such individuals with PF improved the ability of cryo-thawed spermatozoa to undergo the acrosome reaction in response to stimuli. Interestingly, the ability of cryo-thawed sperm from abnormal specimens to respond to ionophore challenge appears to be lower than that of normal sperm, irrespectively of treatment with PF before freezing (12). In our study, post-thaw acrosome reaction stimulated by calcium ionophore was 11.8 and 30.2% for control and treated specimens, respectively, while values of 34.8 and 53.3% have been reported for normal men (12). A possible explanation for these differences is that PF, as used, may afford cryoprotection in normal semen while a different concentration is necessary to neutralize excessive reactive oxygen species (ROS) emanating from the already compromised spermatozoa from oligoasthenozoospermic individuals. To our

knowledge, this is the first study to suggest that the benefits of using PF for freezing human sperm may be related to sperm quality.

Although we did not examine ROS levels in the seminal plasma, it has been shown that ROS levels are significantly higher, and seminal plasma antioxidant capacity is significantly reduced in poor quality sperm, independent of the clinical diagnosis of male infertility (20). We speculate that cryopreserved spermatozoa from men with defective spermatogenesis may have higher levels of ROS than those from normal men. Consequently, peroxidation of unsaturated fatty acids in the acrosomal membranes may be more extended, thus decreasing the response to calcium influx signals that trigger the acrosome reaction.

Sperm function tests such as the acrosome reaction are better fertility predictors than traditional semen parameters (21-28). The mechanism of the acrosome reaction induced by ionophores differs from the physiological acrosome reaction induced by the zona pellucida (22). While the physiological acrosome reaction involves activation of several signal transduction pathways, the ionophore-induced acrosome reaction mainly involves a chemical effect on calcium influx that partially reflects the physiological acrosome reaction process (22). We did not test

other physiological agonists on the acrosome reaction, such as progesterone or recombinant zona pellucida. Instead, we elected to use the ionophore-induced acrosome reaction because it has been extensively validated as a sperm function test with excellent predictive values for outcomes in assisted reproduction (23-28). Using an ARIC cutoff of 10%, the sensitivity of the test in predicting subfertility was 54%, with a positive predictive value of 64% and specificity of 85% (24-26). In the present study, ARIC scores were higher than 10% in 50% of the cryopreserved PF-treated specimens, and in only 19% of the controls. In normal men, ARIC scores higher than 10% were seen in 80 and 60% of the cryo-thawed PF-treated and control specimens, respectively (12). We observed that PF improved ARIC scores in 81.2% of our subjects, whereas improvement in all individuals was seen in normal semen (12).

We have shown that pre-freeze treatment of poor quality human sperm with PF improved the ability of thawed spermatozoa to undergo the acrosome reaction in response to calcium ionophore. Further investigation is needed to determine whether treatment of poor quality human sperm with PF before freezing improves fertilization and pregnancy outcomes in assisted reproduction.

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