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The usefulness of pentoxifylline for the recovery of human spermatozoa in assisted reproductive technologies

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ABSTRACT

Objective: To evaluate the effects of pentoxifylline (PF) on sperm motility and yield of low quality spermatozoa recovered via various sperm preparation techniques.

Design: Human spermatozoa were treated with Ham's F-10 medium containing 0 or 1.0 mg/mL PF and processed via a variety of sperm preparation techniques, followed by quantitative and qualitative assessment of the recovered spermatozoa. Semen specimens were prepared via sperm wash, swim-up, Percoll density gradient centrifugation and Sephadex (SpermPrep™) filtration.

Setting: Andrology Institute of Lexington, Lexington, Kentucky.

Patients: Patients (N=25) referred for male infertility workup, whose ejaculates exhibited various spermatogenic deficiencies, were selected for this study.

Main Outcome Measures: Improvements in the sperm number and quality of spermatozoa following semen processing via various methods used in assisted reproductive technology.

Results: Spermatozoa separated via a variety of sperm preparation methods were qualitatively superior to either fresh or washed specimens. Additional improvements were noted when spermatozoa were processed with media containing 1.0 mg/mL PF.

Conclusions: The use of pentoxifylline, as applied in this study, enhanced the recovery of spermatozoa with improved qualitative characteristics. The sperm quantitative and qualitative improvements were more noticeable in sperm preparation techniques that required sperm progressive motility as the mode of separation for these spermatozoa.

Key Words: Spermatozoa, sperm preparation, sperm selection, pentoxifylline, motility.

Fertilization failure is common in cases of severe oligozoospermia, especially when associated with asthenozoospermia or other spermatogenic deficiencies (1).

Stimulants of sperm metabolic activity have been used successfully to improve sperm motility in such cases (2-5), and pentoxifylline (PF) has increased the incidence of pregnancy in cases with previous fertilization failure (6,7). Also, treatment of spermatozoa with PF appears to be a valuable treatment option in IVF related procedures (8). A number of laboratory techniques have been developed to select and improve the quality of spermatozoa (i.e. swim-up, Percoll density gradient, Sephadex filtration, etc.) and subsequent fertilization rates in vitro for males with sperm disorders, particularly those with oligoasthenozoospermia (Moderate < 5 million/mL; very severe < 1 million/mL with <30% motility) and

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teratozoospermia (<30% normal morphology) (9). In addition, epididymal sperm collections can be performed in cases of congenital absence of the vas or vaso-epididymal obstructions (10-12). Such specimens usually contain considerable debris, blood cells and degenerated spermatozoa, hence sperm preparation using a variety of techniques to improve those spermatozoa may be preferred (13,14,15). Many of these manipulative techniques often increase sperm quality at the expense of numbers of recovered spermatozoa which may not be especially advantageous for men with seminal deficiencies such as oligozoospermia or asthenozoospermia (1). Thus, the utilization of a sperm motility stimulant may increase the yield and quality of spermatozoa recovered by a variety of sperm preparation techniques and possibly increase fertilization rates by increasing the contact rates between gametes (16,17).

The objectives of this study were 1) to study the effects of PF, a synthetic xanthine derivative, on possible enhancement of sperm progressive motility, and 2) to evaluate the effects of such enhancement on the qualitative and quantitative parameters of sperm recovered via a number of sperm preparation techniques. It was believed that via the use of phosphodiesterase inhibitors, such as PF, which elevates intracellular levels of cyclic adenosine 3'5'-monophosphate (cAMP), we could enhance the progressive motility of the spermatozoa which could subsequently bring about improvements in the rate of recovery and quality of spermatozoa recovered via various sperm preparation techniques (18).

MATERIALS AND METHODS

Twenty-five ejaculates with various spermatogenic deficiencies were collected from 25 men who were referred to our Andrology facilities (Andrology Institute of Lexington, Lexington, KY, USA) for male infertility workup (Table 1). Ejaculates were collected with exactly 4 days of abstinence each time. All patients collected their own ejaculates using the Male-Factor Pak™ (ZBL, Inc., Lexington, KY 40523, USA). The Male-Factor Pak™ is a condom-shaped device (made from biologically inert polyurethane) designed for semen collection at intercourse (19).

Semen Evaluation

After semen samples were produced and completely liquefied (within 30 min.), each semen specimen was evaluated using phase-contrast microscopy according to methods described by the World Health Organization (WHO; 9). Semen parameters included volume (mL), sperm count ($x10^6$ spermatozoa/mL), percentage and grade of motility (0 to 4;20), percentage of morphologically normal spermatozoa, and the percentage of spermatozoa reactive to the hypoosmotic swelling (HOS) test (21). After assessment of sperm parameters, the number of total motile sperm (TMS) and the total functional sperm fraction (TFSF) were calculated (13). The TMS is the product of total sperm count x % motility. The TFSF is the product of TMS x % morphologically normal spermatozoa x % swollen spermatozoa. Seminal specimens were evaluated by the same technician under blind conditions (Table 1).

Semen Preparation

Following semen evaluation each ejaculate was split into 2 aliquots (Aliquot 1 and 2). Aliquot 1 was diluted 1:2 (v/v) with Ham's F-10 medium supplemented with 30.0 mg/mL bovine serum albumin (control media; ZBL, Inc.). Aliquot 2 was diluted with the same medium containing 1.0 mg/mL PF (experimental media). Semen specimens were washed by centrifugation (400 x g for 10 min.) and reconstitution of the sperm pellets with control or experimental media. Washed specimens (control and experimental) were split into 4 (1.0 mL) aliquots. One aliquot from each of the washed specimens was maintained for assessment and the others were used for processing via swim-up, Percoll density gradient centrifugation and Sephadex (SpermPrep™ II) filtration method. Semen specimens were assessed for quantitative and qualitative characteristics, as previously described, following preparation via the various techniques employed. Data from the various treatments applied were compared to each

Table 1. Seminal characteristics of sperm specimens (N=25) deficient in various qualitative characteristics collected at intercourse*

Seminal parameters assessed	Values
Volume (mL)	3.5±0.6
Concentration (x10^6/mL)	38.6±31.1
Motility (%)	30.0±5.7
Grade (0 to 4)	1.2±0.4
Normal Morphology (%)	40.0±8.7
†HOS-test(%)	41.0±8.1

*Values are mean ± SD.
†HOS-test(%): Hypoosmotic swelling test; % of swollen spermatozoa.

other using ANOVA followed by the Student's t-test (22).

Swim-up Procedure

The swim-up procedure was performed by gently overlayering 1.0 mL of the corresponding media over the sperm specimen. The culture tube was held at a 45° angle for 60 min. (37 °C) to allow the maximal number of motile spermatozoa to migrate into the overlayered medium. Following incubation, 0.8 mL of the medium was carefully removed. The recovered spermatozoa were centrifuged and reconstituted with 1.0 mL of control or experimental media.

Percoll Density Gradient

A discontinuous two-layer Percoll density gradient technique was used to separate motile spermatozoa. In brief, an isotonic, 90% (v/v) solution of Percoll was prepared by adding 10X control or experimental media. This solution was diluted further with the corresponding media to obtain 40% and 70% (v/v) Percoll solutions. Two mL of each solution were carefully layered in increasing concentrations, starting with 70% at the bottom, then a layer of 40% on the top. The washed sperm specimens were layered onto the top of the 40% Percoll fraction and were centrifuged for 20 min. at 300 x g. The 70% Percoll fraction (1.75 mL) that contained the sperm pellet was collected with a disposable Pasteur pipette, diluted with 2.0 mL of either control or experimental medium and recentrifuged for 5 min. at 300 x g. The sperm pellets were reconstituted to a final volume of 1.0 mL with the control or experimental media.

SpermPrep™ II Filtration Procedure

The SpermPrep™ filtration method consist of a filtration column and a Sephadex matrix. Dead, abnormal or immotile spermatozoa are retarded by the filter matrix while motile spermatozoa pass or penetrate the pores between the hydrated Sephadex beads (differential filtration) and end up in the filtrate first (13). The SpermPrep™ II column (ZBL, Inc.) was used very similarly as previously described for the SpermPrep™ I filtration process (13). Before use, the SpermPrep™ II column was hydrated by placing 6.0 mL of control or experimental media in the barrel of the column. Sperm filtration was begun by mixing the washed specimens with the media in the barrel of the column and was continued for 5 min. The recovered sperm specimens were centrifuged and reconstituted into a final volume of 1.0 mL with control or experimental media.

RESULTS

The semen parameters for specimens processed via various sperm preparation methods are shown in Table 2. Removal of seminal plasma and resuspension of spermatozoa (sperm wash) with control media resulted in a slight improvement of qualitative characteristics. Spermatozoa washed with media containing 1.0 mg/mL PF showed improved percentage and grade of motility. Other parameters were similar between washed and fresh specimens. Significant improvements in qualitative characteristics were evident for spermatozoa prepared via swim-up, Percoll density gradient centrifugation or SpermPrep™ II filtration. Further improvements were obtained when sperm specimens were treated with Ham's F-10 containing 1.0 mg/mL PF. In addition to improvements in qualitative parameters, higher numbers of spermatozoa were recovered for specimens prepared with media containing 1.0 mg/mL PF. Filtration via the SpermPrep™ II method yielded greater numbers of

Table 2. Sperm parameters of specimens (N=25) prepared via various semen processing methods in the absence or presence of 1.0 mg/mL pentoxifylline*

Processing method	Count ($x10^6$)	Motility (%)	Grade (0 to 4)	Morphology (% Normal)	HOS† (%)	TMS‡ ($x10^6$)	TFSF§ ($x 10^6$)
Wash[1]	20.0±0.0	35.0±5.9	103±0.5	40.0±8.0	42.0±7.2	7.0±1.2	1.2±1.3
Wash[2]	20.0±0.0	40.0±6.3	1.5±0.6	39.0±8.9	36.0±6.3	8.0±1.2	1.2±1.4
Swim-up[1]	1.1±0.2	47.0±6.1	2.0±0.3	46.0±6.1	53.0±4.5	0.8±0.2	0.2±0.1
Swim-up[2]	1.5±0.4	53.0±5.6	2.2±0.4	47.0±6.1	53.0±4.5	0.8±0.2‖	0.2±0.1‖
Percoll[1]	2.2±0.4	49.0±7.2	1.9P0.5	44.0±8.1	49.0±5.6	1.1±0.2	0.2±0.1
Percoll[2]	2.4±0.6	54.0±7.4	2.3±0.7	46.0±8.7	51.0±6.3	1.3±0.3	0.3±0.2‖
SpermPrep™II[1]	3.1±0.5	55.0±6.1	2.3±0.3	49.0±6.1	57.0±5.1	1.7±0.2	0.5±0.2
SpermPrep™II[2]	3.5±0.5	74.0±5.6‖	3.1±0.4‖	59.0±5.5‖	66.0±5.0‖	2.6±0.2‖	1.0±0.2‖

[1] Semen processing in the absence of 1.0 mg/mL pentoxifylline
[2] Semen processing in the presence of 1.0 mg/mL pentoxifylline
*Values are mean ± SD.
†HOS(%): Hypoosmotic swelling test; % swollen spermatozoa.
‡TMS: Total motile sperm = sperm count ($x10^6$) x % motility.
§TFSF: Total functional sperm fraction = TMS ($x10^6$) x % normal morphology x % swollen spermatozoa.
‖Significant differences between specimens treated with 0 or 1.0 mg/mL pentoxifylline within sperm preparation techniques ($P<0.05$).

high quality spermatozoa as reflected by the TMS and TFSF, regardless of PF treatment, among the sperm selection techniques employed.

DISCUSSION

A variety of methods for sperm preparation have been developed over the course of the years for use in the various forms of assisted reproductive techniques (ART). They all have as their basic concepts, which are common in all, the removal of the seminal plasma, undesirable spermatozoa, debris and other factors and the recovery of a highly superior fraction of spermatozoa for further use. In this study, four sperm preparation methods were employed and a variety of sperm parameters were evaluated in semen specimens with various qualitative deficiencies. The sperm parameters considered as outcome variables in this study were percentage and grade of motility, percentage of normal morphology and response to the HOS test which correlates with fertilization outcome (9,21,23). Also inclusive sperm parameters such as TMS and TFSF were calculated and compared to better understand the overall effects of each sperm preparation treatment associated with the use of PF. Our results indicate that spermatozoa separated from quality deficient specimens via a variety of sperm preparation methods were qualitatively superior to fresh or washed specimens. The overall qualitative/quantitative profile of these spermatozoa improved further when PF was incorporated in the incubation medium. Those improvements were more significant in the separation techniques in which sperm motility characteristics (progressive motility) was the parameter required for spermatozoa separation, i.e., swim-up and SpermPrep™ II filtration. It seems that stimulation of sperm motility by PF was the major factor involved in the enhanced separation of superior spermatozoa via swim-up and SpermPrep™ II filtration. Such stimulation could have been mediated by inhibition of cAMP-phosphodiesterase activity resulting in increased levels of cAMP (18,24). Increased duration and intensity of metabolic activity have been obtained in defective spermatozoa treated with PF (7,17,24,25). Treatment of normozoospermic specimens with PF does not appear to improve the percentage of motility and fertilization potential of the spermatozoa (5,26). Severe reductions in sperm motility, as seen in frozen-thawed semen and low quality specimens, are considered to contribute to low

fertilization rates (9). Various studies tend to support the clinical role of PF in enhancing the fertilization potential of spermatozoa with various deficiencies (6,9,25,26). However, other investigators have reported deleterious effects of PF such as delayed or abnormal embryo development (28). Pentoxifylline can be removed by washing the spermatozoa, after a period of stimulation prior to insemination, if harmful side effects such as delayed or abnormal embryonic development are suspected (6).

This study was limited in scope and the processed spermatozoa were never evaluated for either sperm hyperactivation characteristics, acrosome reaction, sperm penetration assay utilizing the hamster oocyte, IVF or IUI rates in order to more accurately determine their fertilization potentials. Merely the efficacy and superiority of the various techniques assessed in this study were measured and compared in terms of the outcome variables that we used. We realize that none of these variables are highly predictive of IVF or IUI outcome. However, we have selected various sperm quality characteristics for evaluation of the various selection methods because of their low cost, ease of assessment and their ability to identify the most appropriate sperm preparation technique. Evaluation of the fertilizing potential of the recovered spermatozoa will be considered in future studies.

In summary, employment of semen preparation techniques was found to be a necessary step in enhancing the quality of the recovered spermatozoa. The use of 1.0 mg/mL PF, as applied in this study further enhanced the qualitative profiles of the recovered spermatozoa. However, treatment of spermatozoa with PF should be applied very selectively since the additional improvements in the rate of recovery and quality of spermatozoa prepared seems to favor those sperm separation techniques in which sperm motility/progressive motility are the factors that determine successful sperm selection. Stimulation of sperm motility, in conjunction with the use of the most appropriate sperm preparation techniques, could contribute to enhanced fertilization rates, especially those recovered from quality deficient specimens. Finally, no one sperm preparation technique should be the only technique that could be applicable in any one ART laboratory. With the availability of a variety of sperm selection techniques today, one should be able to "tailor" a specific method to better assist the infertile male in achieving selection of high quality spermatozoa that could yield the highest conception rates.

REFERENCES

1. Kofinas GD, Zavos PM. Preparation of sperm patients with spermatogenic deficiencies for IUI using new/improved SpermPrep™ II filtration. Mol Androl 1992;4:57-61.
2. Hammitt DG, Bedia E, Rogers PR, Syrop CH, Donovan JF, Williamson RA. Comparison of motility stimulants for cryopreserved human semen. Fertil Steril 1989;52:495-502.
3. Sikka SC, Hellstrom WJG. Functional evaluation and motility parameters of pentoxifylline-stimulated cryopreserved human semen. J Ass Reprod Technol Androl 1990;1:309-19.
4. Tesarik J, Thebault A, Testart J. Effect of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. Hum Reprod 1992;7:1257-63.
5. Pang SC, Williams DB, Huang T, Wang C. Effects of pentoxifylline on sperm motility and hyperactivated motility in-vitro: A preliminary report. Fertil Steril 1993;59:465-7.
6. Sikka SC, Hellstrom WJG, Naz RK. Pentoxifylline: Role in management of male infertility/mechanisms of action. Mol Androl 1993;5:169-77.
7. Yovich JM, Edirisinghe WR, Cummins JM, Yovich JL. Preliminary results using pentoxifylline in a pronuclear stage tubal transfer (PROST) program for severe male factor infertility. Fertil Steril 1988;50:179-81.
8. Yovich JM, Edirisinghe WR, Cummins JM, Yovich JL. Influence of pentoxifylline in severe male factor infertility. Fertil Steril 1990;53:715-22.
9. World Health Organization. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. 3rd ed. New York: Cambridge University Press, 1992.
10. Hanley HG. Pregnancy following artificial insemination from epididymal cyst. Proc Soc Study Fertil 1956;8:20-4.
11. Bustillo M, Rajfer J. Pregnancy following insemination with sperm aspirated directly from the vas deferens. Fertil Steril 1986;46:144-6.
12. Silber SJ, Balmaceda J, Borrero C, Ord T, Asch R. Pregnancy with sperm microaspiration from the proximal head of the epididymis: A new treatment for congenital absence of the vas deferens. Fertil Steril 1988;50:525-8.
13. Zavos PM, Centola GM. Qualitative and quantitative improvements in human spermatozoa recovered via swim-up and a new semen filtration column (SFC) method. Infertility 1990;13:25-34.
14. Zavos PM, Correa JR, Zarmakoupis PN. Improvements and short-term viability of mouse epididymal spermatozoa

recovered through the SpermPrep™ filtration method. Theriogenology 1994;42:1035-42.
15. Zavos PM, Correa JR, Sofikitis N, Kofinas G, Zarmakoupis PN. A method of short-term cryostorage and selection of viable spermatozoa for use in the various assisted reproductive technologies. Tohoku J Exp Med 1995;176:75-81.
16. Lambert H, Steinleitner A, Eisermann J, Serpa N, Cantor B. Enhanced gamete interaction in the sperm penetration assay after inoculation with pentoxifylline and human follicular fluid. Fertil Steril 1992;58:1205-8.
17. Tesarik J, Mendoza C, Carreras A. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus induced acrosome reactions. Fertil Steril 1992;58:1185-90.
18. Wang R, Bell M, Hellstrom WJG, Sikka SC. Post-thaw sperm, cAMP concentration and membrane lipid peroxidation after stimulation with pentoxifylline and platelet activating factor. Int J Androl 1994;17:169-73.
19. Zavos PM, Kofinas GD, Sofikitis NV, Zarmakoupis PN, Miyagawa I. Differences in seminal parameters in specimens collected via intercourse and incomplete intercourse (coitus interruptus). Fertil Steril 1994;61:1174-6.
20. Zavos PM, Cohen MR. The pH of cervical mucus and the postcoital test. Fertil Steril 1980;34:234-8.
21. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-25.
22. SAS User's Guide: Statistics, SAS Institute, Inc., Cary, NC, 1989.
23. Van der Ven HH, Jeyendran RS, Al-Hasani S, Perez-Pelaez M, Diedrich K, Zaneveld LJD. Correlation between human sperm swelling in hypoosmotic medium (Hypoosmotic Swelling Test) and in vitro fertilization. J. Androl 1986;7:190-6.
24. Garbers DL, Lost WD, First NL, Lardy HA. Effect of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochemistry 1971;10:1825-31.
25. Turner EA, Aparicio NJ, Turner D, Schwarzstein L. Effect of two phosphodiesterase inhibitors, cyclic adenosine 3':5'-monophosphate, and a beta-blocking agent on human sperm motility. Fertil Steril 1978;29:320-31.
26. Tournaye H, Janssens R, Camus M, Staessen C, Devroey P, Van Steirtghem A. Pentoxifylline is not useful in enhancing sperm function in cases with previous in-vitro fertilization failure. Fertil Steril 1993;59:210-5.
27. Hellstrom WJG, Sikka SC. Pentoxifylline stimulates the movement characteristics of cryopreserved sperm. Surg Form 1989;40:648-50.
28. Lacham-Kaplan O, Trounson A. Embryo development capacity of oocytes fertilized by immature sperm and sperm treated with motility stimulants. Reprod Fertil Dev 1994;6:113-6.

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