

Hypoosmotic Swelling Test of Sperm Supplemented with Pentoxifylline after Preparation by Two-layer Percoll Gradient Method

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Abstract : The aim of this study was to compare the percentage of sperm tail membrane swelling under hypoosmotic conditions with those with and without pentoxifylline supplements in sperm prepared by the two-layer Percoll gradient method. Twenty five normal semen samples were collected from male partners of infertile couples attending the Infertility Clinic at Siriraj Hospital. After the process of sperm preparation by the two-layer Percoll gradient method, the final samples were divided into 2 tubes, 0.5 ml each. Pentoxifylline 0.5 ml was added into one tube and another tube was kept as control. The hypoosmotic swelling test was performed on both specimens. The percentage of swollen spermatozoa in the pentoxifylline supplement group was significantly higher than the control group (82.8 ± 7.7 vs 70.8 ± 12.7 ; $p < 0.001$). It was concluded that the addition of pentoxifylline to the sperm prepared by the two-layer Percoll gradient method can enhance the sperm membrane integrity, and it may be beneficial to add pentoxifylline to sperm preparation for use in IUI or IVF.

Key words : Pentoxifylline, hypoosmotic swelling test, Percoll gradient

เรื่องย่อ : การทดสอบการบวมตัวของหางอสุจิที่เตรียมโดยวิธีเปอร์คอลล์สองชั้นภายหลังการใส่สารเพนทอกซิฟิลลิน

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จุดประสงค์ของการศึกษาเพื่อเปรียบเทียบจำนวนร้อยละของอสุจิที่มีการบวมตัวของหางภายใต้ภาวะไฮโปออสโมติก ภายหลังการเตรียมอสุจิด้วยวิธีเปอร์คอลล์สองชั้น ระหว่างกลุ่มที่ใส่สารเพนทอกซิฟิลลิน และไม่ได้ใส่ ได้นำอสุจิของคู่สมรสที่มีบุตรยากฝ่ายชาย ซึ่งผลการตรวจวิเคราะห์ปกติ ที่มารับการรักษาที่คลินิกผู้มีบุตรยาก โรงพยาบาลศิริราช จำนวน 25 ตัวอย่าง มาเตรียมด้วยวิธีเปอร์คอลล์สองชั้น แบ่งอสุจิที่ผ่านการเตรียม

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แล้วออกเป็น 2 ส่วน ๆ ละ 0.5 มล. ส่วนแรกใส่สารเพนทอกซิฟิลลิน 0.5 มล. ลงไป และส่วนที่สองไม่ใส่สารดังกล่าว หลังจากนั้น นำน้ำอสุจิทั้ง 2 ส่วน ผสมกับสารละลายไฮโปออสโมติก แล้วตรวจดูการบวมน้ำของหางอสุจิ ผลปรากฏว่า ร้อยละของอสุจิที่มีการบวมน้ำของหางในกลุ่มที่ใส่สารเพนทอกซิฟิลลิน มากกว่าในกลุ่มที่ไม่ได้ใส่สารดังกล่าวอย่างมีนัยสำคัญทางสถิติ (82.8 ± 7.7 และ 70.8 ± 12.7 ; $p < 0.001$) สรุปได้ว่าสารเพนทอกซิฟิลลิน สามารถเพิ่มฟังก์ชันของผนังอสุจิที่ผ่านการเตรียมด้วยวิธีเปอร์คอลลล์สองชั้น สารดังกล่าวจึงอาจมีประโยชน์ในการนำมาใช้กับการเตรียมอสุจิที่ใช้สำหรับการฉีดอสุจิเข้าสู่โพรงมดลูกและการปฏิสนธิในอกร่างกาย

INTRODUCTION

Semen preparation to obtain morphologically normal and progressively motile spermatozoa is an important procedure involving assisted reproductive techniques. In an attempt to improve the motility and, hence, the fertilizing capacity of sperm, different chemical stimulants have been studied extensively¹⁻³. Pentoxifylline is a metabolic stimulant which is used extensively in human assisted reproduction. It is a methylxanthine derivative, which acts as an inhibitor of cyclic 3',5'-adenosine monophosphate (cAMP) phosphodiesterase activity. As a result of this effect, intracellular cAMP concentration increases, leading to a marked augmentation of sperm motility^{4,5}. Pentoxifylline has also been shown to enhance acrosome reaction⁶ and to increase sperm penetration into the zona-free hamster oocyte⁷.

The semen parameters widely used for evaluation of male infertility are sperm concentration, motility, and morphology, but these parameters cannot accurately determine the fertilizing potential of the spermatozoa⁸. The hypoosmotic swelling (HOS) test, introduced by Jeyendran et al⁹, has added another measure for the evaluation of human spermatozoa. The HOS test is easy to score and gives information on the functional integrity of the human sperm membrane. There have been a number of reports that show a positive correlation between the HOS test and other sperm parameters, such as the sperm zona-free hamster ovum penetration assay and IVF outcome¹⁰.

The purpose of this study is to compare the percentage of sperm tail membrane swelling under hypoosmotic conditions with those with and without pentoxifylline supplements in sperm prepared by the two-layer Percoll gradient method.

MATERIALS AND METHODS

A total of 25 semen samples were collected from men attending our Infertility Unit between June and August 2002. The mean ($\pm$ SD) age of the men was 35.0 ± 4.5 years. The ejaculates were collected by masturbation after 3 to 5 days abstinence and allowed to liquefy at room temperature. Semen analyses were performed within 2 hours of collection and all samples were normal according to standard WHO parameters¹¹.

The discontinuous Percoll gradient was prepared in two layers. The lower layer was 2 ml 80% Percoll (Pharmacia, Uppsala, Sweden) overlaid by 2 ml 40% Percoll. Then 1 ml of undiluted semen was layered carefully on top of the column, and centrifuged at 600 xg for 20 min. The supernatant was discarded, and the precipitate washed twice with 3 ml of human tubal fluid (HTF) medium followed by centrifugation at 300 xg for 10 min. The final sperm pellet was resuspended in 1 ml HTF medium, then divided into 2 tubes, 0.5 ml each. An aliquot in one tube was mixed with 0.5 ml of pentoxifylline (1 mg/ml) solution and another tube was kept as a control. Both tubes were placed in an incubator at 37°C for 1 hour.

A 0.1 ml aliquot of sperm sample from each tube was mixed with 1 ml hypoosmotic solution (equal parts of 150 mOsmol fructose and 150 mOsmol sodium citrate) and then incubated at 37°C for 1 hour. Spermatozoa were examined for typical tail abnormalities (Figure 1) under microscope (x400). At least 100 spermatozoa were evaluated, and the percentage of swollen tails was calculated.

Statistical analysis was performed using program SPSS 9.05 for windows. For the comparison of percentages, paired t-test was applied. A p-value < 0.05 was taken to be indicative of a statistically significant difference.

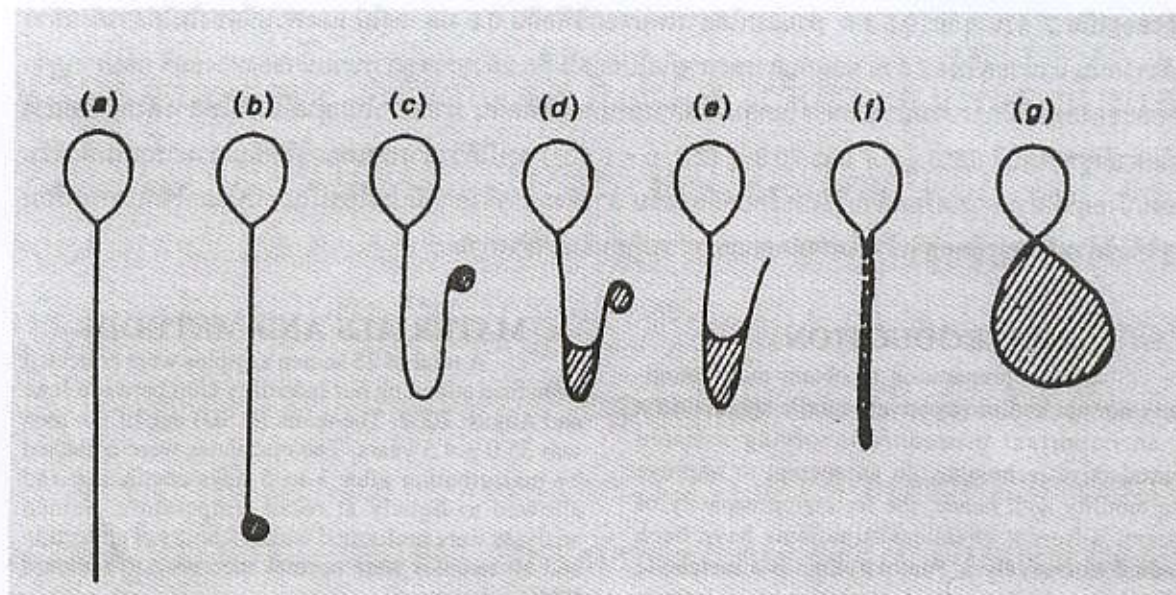


Figure 1. Schematic representation of swelling of sperm tails : (a) = no change, (b-g) = various types of tail changes

RESULTS

Semen characteristics of 25 samples derived from 25 men with a mean age of 35 years (range 25-44 years) are shown in Table 1.

The results of the HOS test are shown in Table 2. The mean percentage of swollen spermatozoa in the semen samples supplemented with pentoxifylline was significantly higher than those without pentoxifylline (82.8 ± 7.7 vs 70.8 ± 12.7 ; $p < 0.001$).

DISCUSSION

Pentoxifylline, a phosphodiesterase inhibitor of the methylxanthine group, inhibits the breakdown of cyclic adenosine monophosphate (cAMP), resulting in intracellular cAMP accumulation. It is believed that the accumulated cAMP, through the activation of cAMP protein kinase, is involved in the calcium ion movement of the spermatozoa membrane, thus regulating its motility¹². It has been shown that cAMP also is involved in the control of the acrosome reaction¹³, playing a major role in improved fertilizing ability after addition of pentoxifylline to semen samples.

There is no doubt that pentoxifylline improves the motility of spermatozoa with a maximum activity after 10 minutes and an activity phase of less than 2 hours¹⁴. However there are some conflicting reports concerning the sperm velocity, VSL (straight line velocity), and VCL (curvilinear velocity)¹⁴⁻¹⁷. These apparently conflicting and not easily comparable results may be ascribed to different doses or to different times of exposure to the drug¹⁷⁻¹⁸. Terriou et al¹⁹ reported that pentoxifylline could initiate motility in spontaneously immotile epididymal and testicular spermatozoa and allow normal fertilization, pregnancy and birth after intracytoplasmic sperm injection.

In addition to sperm motility, sperm membrane function is also useful in assessing male infertility. The HOS test is approved as an optional test for semen by the WHO¹¹. There are limited reports concerning the effects of pentoxifylline on sperm membrane integrity. Ponce et al²⁰ studied the effects of pentoxifylline on membrane functional integrity of mouse spermatozoa and found that there was a positive correlation between percentage of tail swelling and motile spermatozoa. Stanic et al²¹

Table 1. Semen characteristics of the samples.

Semen parameters	Mean $\pm$ SD	(Range)
Volume (ml)	2.7 $\pm$ 0.9	(2.0-5.5)
pH	7.9 $\pm$ 0.1	(7.5-8.0)
Sperm concentration ($\times 10^6$ /ml)	50.9 $\pm$ 25.0	(20-97)
Total sperm count ($\times 10^6$)	135.4 $\pm$ 80.7	(40-407)
Motility (%)	57.6 $\pm$ 6.0	(50-73)
Normal morphology (%)	37.2 $\pm$ 5.3	(31-53)
Viability (%live)	81.1 $\pm$ 3.8	(76-89)
White blood cell ($\times 10^6$ /ml)	0.3 $\pm$ 0.2	(0-0.8)

Table 2. Results of the HOS test in semen samples with and without pentoxifylline supplement.

Semen samples	Swollen spermatozoa (%)*	P-value
With pentoxifylline	82.8 $\pm$ 7.7	< 0.001
Without pentoxifylline	70.8 $\pm$ 12.7	

*Values show Mean $\pm$ Standard deviation

reported that pentoxifylline enhanced post-thaw motility of cryopreserved human spermatozoa, when added after thawing, but no improvement was found by freezing sperm with pentoxifylline. In this study, we found that the percentage of swollen spermatozoa in the semen supplemented with pentoxifylline was significantly higher than those without pentoxifylline. The results encourage the use of pentoxifylline to improve sperm quality in treating infertile couples by assisted reproduction. Tarlatzis et al²² reported that pentoxifylline could increase sperm motility and the fertilizing capacity in oligoasthenospermic samples. Negri et al²³ showed that the addition of pentoxifylline during the preparation of semen for IUI (intrauterine insemination) in oligoasthenosper-

mic samples could improve pregnancy rates when compared to the group without pentoxifylline addition (27.5% vs 11.5%; $p < 0.05$). In regards to the possible toxic effects of pentoxifylline, experimental studies on mammalian cells have demonstrated that cells treated for less than two hours manifested no cytotoxic or genotoxic effects²⁴.

In conclusion, our results showed that the addition of pentoxifylline to the sperm prepared by the two-layer Percoll gradient method can enhance sperm membrane integrity. It may be beneficial to add pentoxifylline to sperm preparation for use in IUI or IVF. However, more research is required in regards to its clinical importance in fertilization rates and pregnancy outcomes.

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