The Affect of Pentoxifylline on Sperm Parameters in Normozoospermic, Asthenozoospermic and Oligo-Asthenozoospermic Males

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OBJECTIVE: To determine the affect of pentoxifylline (PTX) on sperm parameters in normozoospermic, asthenozoospermic, and oligo-asthenozoospermic males.

STUDY DESIGN: Each semen sample collected from normozoospermic (n=20), asthenozoospermic (n=20), and oligo-asthenozoospermic (n=20) males was divided into two equal parts. One part of the semen sample washed using Puresperm only (NidaCon, Sweden) (After-wash category) and other part was treated with pentoxifylline after washing with Puresperm (PTX-treated category). The semen parameters of the after-wash and PTX-treated categories were compared.

RESULTS: PTX-treated category was demonstrated significant increment of sperm concentration and rate of sperm with intact acrosome (SIA) when compared to after-wash group in both asthenozoospermic and oligo-asthenozoospermic males. In addition, PTX-treated group resulted higher sperm morphology in oligo-asthenozoospermic males when compared to after-wash group. However, PTX did not show any superiority on after-wash group in normozoospermic males.

CONCLUSIONS: Pentoxifylline have beneficial effect on sperm parameters especially in oligo-asthenozoospermic and asthenozoospermic patients.

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Key Words: Pentoxifylline, Asthenozoospermia, oligoasthenozoospermia, male infertility, sperm motility, sperm morphology

The quality of ejaculated semen is the most important factor in male fertility. In vivo, adequate sperm motility is mandatory for sperm to go in the cervical canal and cervical mucus, and to immigrate through the uterine cavity into the fallopian tubes. Intact acrosome and adequate acrosome reaction are also needed for sperm to penetrate into the oocyte in order for fertilization to take place.[1]

Pentoxifylline (PTX) is a member of methylxanthins groups that are phosphodiesterase inhibitors. They inhibit phosphodiesterase activity in cells, and prevent breakdown of cyclic adenosine monophosphate (cAMP), and cause a subsequent increase in intracellular cAMP levels.[1] Increased cAMP levels result in increment of intracellular adenosine triphosphate (ATP) and consequently enhancement of sperm motility. It was also demonstrated that PTX stimulated the acrosome reaction (AR)[2], improved the penetration of zona-free hamster oocytes and enhanced binding to the zona pellucida in the hemizona assay.[3] It has been suggested that the positive effect of PTX was encountered especially with oligo-astheno-zoospermic samples rather than normozoospermic ones.[4] However, some authors have demonstrated that PTX could significantly improve sperm motility of both normozoospermic and asthenozoospermic samples.[5] Thus, attention should be given to select sperm samples that will benefit from PTX treatment.

This prospective study was designed to investigate the in vitro effects of PTX on sperm motility, morphology and the rate of spermatozoa with intact acrosome (SIA) in semen samples of normozoospermic, asthenozoospermic, and oligo-asthenozoospermic patients.

Materials and methods

Sixty male patients as a partner of infertile couple had been admitted to our department of OB-GYN were included in the study. No exclusions were made regarding the cause of infertility. An inform consent has been taken from each individual patient. The study was approved by the ethical committee of the University.

Collection of Semen Samples

Semen samples were collected by masturbation after 2-4 days of sexual abstinence. Semen samples were allowed to liquefy for 30 min at room temperature (21 °C), and examined by routine semen analysis. Semen samples were assessed according to the descriptions of World Health Organization (WHO). The strict criteria of Kruger et al were used to evalu-

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ate sperm morphology.[6]

After liquefaction, 1 ml of each samples was analyzed on light microscopy and according to the concentration, and the motility of the samples, the patients were categorized into three groups: Group I (n=20, normozoospermic "NZS"), sperm concentration ≥20x106 /mL and progressive motility ≥50%; Group II (n=20, asthenozoospermic "AZS"), sperm concentration ≥20x106 /mL and progressive motility <50%; and Group III (n=20, oligo-asthenozoospermic "OZS"), sperm concentration <20x106 /mL and progressive motility <50%.

The semen sample from an individual was divided into two equal parts after the initial semen analysis (Pre-wash group). First part of the semen sample was washed using Puresperm (NidaCon, Sweden) (After-wash group) and the second part was first treated with PTX then washed with Puresperm (PTX-treated group). The concentration, motility and the rate of rapid progressive forward motility (RPFM) were assessed again by semen analysis for each group (Puresperm wash only and Puresperm wash plus PTX). Spermac stain technique (Fertipro, Belgium) was used in order to determine the sperm morphology and the rate of spermatozoa with intact acrosome (SIA).

A single embryologist performed all sperm analysis.

Puresperm gradients technique

Puresperm gradients of 90% and 40% were prepared from Puresperm stock Nidacon (Gothenburg, Sweden). Purewash washing medium was used as media for dilution. The lower gradient (90%) (1mL) was firstly put into a 15-mL conical Falcon tube (Falcon 2095, Becton Dickinson), and then a 1 mL of the upper gradient (40%) was added to the tube and layered on lower gradient. The semen was stayed on top of the gradients. The sample was incubated at 37°C with 6% CO_2 in incubator for 20 min. After centrifugation at 1800 g for 15 min, the lower gradient layer was carefully collected and transferred to new tubes containing 6-7 mL Purewash washing medium. The sample was washed at 1800 g for 15 min before being resuspended in 0,5mL Purewash washing medium. The sample was incubated at 37°C with 6% CO_2 in incubator for 30-60 min. Finally, swim-up sample of 0.2 ml was obtained into the Falcon tube for semen analysis.

Preparation of pentoxifylline

A stock solution of 30 mg/ml pentoxifylline (PTX) (Sigma Chemical Co., St. Louis, MO, USA) was prepared for each day. PTX from this stock solution was diluted by 3 ml Earle's Balanced Salt Solution (EBSS) or Purewash washing medium to obtain a solution of 1mg/ml (3.6mM). This solution was filtered by a filter which has 0,2 micron pores. This working solution was added to 1 ml of the sperm suspension and wait-ed at room temperature for 20 min. Then above mentioned Puresperm gradients technique was applied to the waited sperm suspension. At last, 0.2 ml of swim-up sample was obtained for semen analysis.

Spermac stain procedure

A drop of semen was smeared on a glass slide. The Spermac kit manufacturer's guidelines (Stain Enterprises, Onderstepoort, South Africa) were used to carry out the Spermac staining procedure. The smear of sperm was allowed to air-dry before being fixed in the FIX solution (Sigma 50/20 ml) provided in the Spermac kit for 5 minutes at room temperature (20°C). After each slide with fixed sperm was washed in tap water, and stain solution A was used to inundate the slide. Stain solution A was kept on the slide for 90 seconds and then rinsed off with tap water. This procedure for same sperm slide was repeated exactly for Stain solution B and C. The stained slides were waited in air to dry for 20 minutes and then analyzed by light microscopy (×1000) to determine the rate of sperm with intact acrosomes.

The assessment of Spermac stain test was carried out according the criteria of Chan et al.[7] Regardless of the shape of the sperm head, sperm was considered to have an intact acrosome when the anterior acrosomal region stained green and dark green, thickened, "rubber-band" border forming a semicircle at the tip of the head remained unbroken or continuous. The posterior postacrosomal region of each sperm head was red-pink in coloration. Sperm that lacked the red-pink counterstain in the posterior head region were inadequately stained and were not counted. Sperm with nonintact acrosomes (reacted or defective acrosomes) showed peeled acrosomal membranes, spotting, irregular thickness in the green band, or partial green coloration. Another type of sperm with nonintact acrosomes (missing acrosomal enzymes) were stained either white or red at the acrosomal region and had no green color at the head. At least 100 sperm cells were assessed for each slide, and the same technician performed all the Spermac stain analyses. For each sperm smear, the percentage of sperm with intact acrosomes (SIA) was calculated by dividing the number of sperm with intact green acrosomes by the total number of sperm analyzed and multiplying by 100. The cutoff value of 40% and higher values were interpreted as normal.

Data analyses

Data was analyzed using the SPSS, INC. for Windows (version 12; SPSS, Inc., Chicago, IL). Normally and non-normally distributed independent variables confirmed with Kolmogorov-Smirnov test were tested by ANOVA and Kruskal-Wallis test, respectively. The dependent variables were analyzed by paired sample t-test. Values are expressed as mean±SD. p value was set to 0.05.

Table 1. Results of sperm concentration, motility, morphology, rate of SIA and rate of RPFM in Group I, II and III according to categories of pre-wash, after-wash and PTX-treated groups.

	Sperm concentration (x10⁶ /mL)	Sperm motility (%)	Sperm morphology (%)	Rate of SIA (%)	Rate of RPFM (%)
Groups					
Group I; NZS					
Pre-wash	95.6±27.5[a]	76.8±11.6[b]	4.3±2.6[b]	38.5±11.3[b]	65.4±9.6[b]
After-wash	123.5±48.3[c]	100.0±0.0	7.3±4.1	48.7±11.8[c]	100.0±0.0
PTX-treated	104.0±42.3	100.0±0.0	6.9±2.6	46.5±10.9	100.0±0.0
Group II; AZS					
Pre-wash	60.2±24.5	56.2±17.3[d]	4.0±4.0[d]	24.2±11.3[d]	36.6±11.9[d]
After-wash	60.2±40.2	100.0±0.0	6.1±4.6	33.2±11.6	100.0±0.0
PTX-treated	65.1±37.9[e]	100.0±0.0	6.4±3.9	38.7±12.5[e]	100.0±0.0
Group III; OZS					
Pre-wash	14.2±5.9	58.4±14.5[f]	1.1±0.9[f]	14.8±5.3[f]	37.7±12.6[f]
After-wash	16.9±9.0	100.0±0.0	2.6±1.6	23.0±6.8	100.0±0.0
PTX-treated	20.1±11.2[g]	100.0±0.0	3.4±1.9	29.3±7.2[g]	100.0±0.0

NZS= Normozoospermic, AZS= Asthenozoospermic, OZS=Oligoasthenozoospermic, PTX= Pentoxifylline
Values were expressed as mean± SD
[a] *Statistically different from after-wash group in Group I.*
[b] *Statistically different from after-wash and PTX-treated groups in Group I.*
[c] *Statistically different from PTX-treated group in Group I*
[d] *Statistically different from after-wash and PTX-treated groups in Group II.*
[e] *Statistically different from pre-wash and after-wash groups in Group II.*
[f] *Statistically different from after-wash and PTX-treated groups in Group III.*
[g] *Statistically different from pre-wash and after-wash groups in Group III.*

Results

Table 1 presents the results of sperm concentration, motility, morphology, rate of spermatozoa with intact acrosome (SIA) and rate of rapid progressive forward motility (RPFM) of group I, II and III in pre-wash, after-wash and PTX-treated categories.

In group I (NZS, n=20):

The both after-wash and PTX-treated groups had higher sperm concentration, sperm motility, sperm morphology, rate RPFM, and rate of SIA when compared to pre-wash group (p<0.05). In group I, PTX-treated category had higher sperm concentration and rate of SIA when compared to after-wash group.

In group II (AZS, n=20):

The both after-wash and PTX-treated categories showed significant differences of sperm motility, sperm morphology, the rate of RPFM, and the rate of SIA when compared to pre-wash group (p<0.01). However, they both did not make any difference of sperm concentration. PTX-treated group were demonstrated significant increment of sperm concentration and the rate of SIA when compared to after-wash group

(65.1±37.9 vs 60.2±40.2 and 38.7±12.5 vs 33.2±11.6 respectively, p<0.05)

In group III (OZS, n=20):

After-wash and PTX-treated categories were demonstrated significant increment of sperm motility, sperm morphology, the rate of SIA, and the rate of RPFM when compared with pre-wash group (p<0.01). Besides mentioned differences, PTX-treated group had also higher sperm concentrations when compared to pre-wash group (20.1±11.2 vs 14.2±5.9; p<0.01). PTX-treated group were demonstrated significant increment of sperm concentration (20.1±11.2 vs 16.9±9.0), sperm morphology (3.4±1.9 vs 2.6±1.6) and the rate of SIA (29.3±7.2 vs 23.0±6.8) when compared to after-wash group (p<0.01)

Discussion

Many substances including serum, peritoneal fluid and follicular fluid or other chemically defined pharmacological substances like progesterone, adenosine analogues or methylxanthins have been proposed to stimulate human sperm functions.[8] Pentoxifylline (PTX) is a methylxanthine derivative, which is also, like caffeine and theophylline, a non-specific

inhibitor of phosphodiesterase. Since PTX inhibits the cyclic adenosine monophosphate (cAMP) phosphodiesterase, the intracellular cAMP content increases consecutively. cAMP is known as a major source for human sperm glycolysis and endogenous adenosine triphosphate (ATP). Endogenous ATP produced in numerous sperm mitochondria may be the energy source for their movements. PTX is the most preferable substance among the methylxanthines group since PTX is superior to others such as caffeine, 2-deoxy-adenosine, and kallikrein in motility stimulation of both fresh and cryopreserved sperm. In addition, PTX is more water soluble and less cytotoxic then caffeine, and this increases its usability.[9]

The beneficial effect of pentoxifylline on sperm motility and motion characteristics like sperm velocity or hyperactivity has repeatedly been described for both fresh[10-13] and cryopreserved spermatozoa.[14,15] However, there is conflict about in which group of semen samples and in which parameter the PTX will be beneficial. Some authors found no effect of PTX in normozoospermic patients[10,16] but they just observed a significant increase in motility and the number of progressively motile spermatozoa in patients with only oligo-asthenozoospermia.[16-18] On the other hand, some authors have demonstrated that, PTX could improve sperm motility of both normozoospermic and oligo-asthenozoospermic males.[5,19] It is also suggested that PTX could increase the sperm motility but could not improve sperm count and morphology since sperm motility is more amenable to pharmacologic manipulations than sperm count and morphology, which are difficult to be modified once sperm is formed.[20] On the contrary in some publications, it was demonstrated that the PTX significantly improved the motility, sperm count and morphology in oligo-asthenozoospermic men.[20,21] In our study, the use of PTX improved the total sperm concentration, the sperm morphology and the rate of SIA when compared to both pre-wash and after-wash group in group III. Beside this, PTX also improved the sperm concentration and the rate of SIA in group II, when compared to after-wash group. However, these superior effects of PTX to Purewash gradient technique were not noticed in group I. Thus, it seems that the beneficial effect of PTX was only restricted to oligo-asthenozoospermic and asthenozoospermic males rather then normozoospermic males.

Besides the sperm motility, an intact acrosome and acrosome reaction are essential for sperm to penetrate through the zona pellucida in vivo. It is logical that sperm cells with low percentages of intact acrosomes would not fertilize oocytes, and this will be resulted in low percentages of fertilization rates. It is obvious that integrity and the functionality of the sperm membrane are essential for progressive sperm motility and fertilization.[22] The presence of intact acrosome is usually associated with a good quality sperm which are necessitating

a functional and intact sperm membrane. Stanic et al demonstrated that the adding PTX after thawing the sperm increased the rate of sperm with intact membrane.[14] Thus, the reason of improvement in rate of SIA with PTX in our study may be that, the PTX increased the membrane integrity. There is no consensus about the cut-off value of SIA. In a study, the mean percentage of fertilization in the abnormal acrosome group was significantly lower than in the group with >40% intact sperm acrosomes.[7] In our study PTX improved the rate of SIA in both asthenozoospermic oligo-asthenozoospermic samples. However, this effect was not noticed in normozoospermic samples.

Today, the most medical centers including us perform intrauterine insemination (IUI) in treatment of male subfertility. In oligo-asthenozoospermic and asthenozoospermic males, PTX can be used for preparation of IUI semens since PTX may stimulate sperm functions that are critically involved in fertilization.

In conclusion, PTX have beneficial effect on sperm parameters especially in oligo-asthenozoospermic and asthenozoospermic patients. Puresperm gradients technique can be used adequately in normozoospermic patients.

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