ISSN: 2573-9506

Research Article

International Journal of Women's Health Care

# Sperm Activation with Pentoxifylline Is Beneficial For Non-Obstructive Azoospermia Patients Using Testicular Sperm before Intracytoplasmic Sperm Injection

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**Submitted:** 21 Mar 2022; **Accepted:** 28 Mar 2022; **Published:** 04 Apr 2022

***Citation:*** *Yang Luo, Fang Peng, Lei Li, Yuling Mao, Shiping Chen. (2022). Sperm Activation with Pentoxifylline Is Beneficial For Non-Obstructive Azoospermia Patients Using Testicular Sperm before Intracytoplasmic Sperm Injection. Int J Women's Health Care, 7(2), 73-80.*

## Abstract

***Purpose:*** *This study aimed to assess the efficacy and safety of sperm activation with pentoxifylline (PF) for non-obstructive azoospermia (NOA) patients that underwent testicular sperm aspiration (TESA) for intracytoplasmic sperm injection (ICSI) in in vitro fertilization (IVF) program.*

***Methods:*** *A total of 457 patients with 457 TESA- ICSI cycles who identified no motile spermatozoa under through scrutiny were included and divided into two groups. 121 cycles without PF treatment were recorded as control group. The other 336 cycles with PF treatment were recruited as PF group. The two groups were compared for demographics, routine parameters of IVF treatment, pregnancy rates, and neonatal outcomes.*

***Results:*** *The rates of fertilization, 2PN fertilization, D3 oocyte utilization and good-quality embryo in PF group were significantly higher than the control group ($P < 0.05$). Moreover, PF significantly reduced the total fertilization failure cycle rate, rate of cycles with no transferable embryo and the mean ICSI time per oocyte when compared with the control group ($P < 0.05$). Although no significant differences were observed between the two groups in the implantation rate, clinical pregnancy rate, miscarriage rates and live birth rate ($P > 0.05$), significantly increased cumulative live birth rate were found in the PF group ($P > 0.05$). Neonatal outcomes were comparable between the two groups, and no congenital anomalies were reported in the two groups.*

***Conclusion:*** *Sperm activation with pentoxifylline is beneficial for NOA patients who underwent TESA- ICSI treatment. However, further research with larger groups of couples to assess the long-term effect were still required.*

**Keywords:** Testicular Sperm Aspiration, Non-Obstructive Azoospermia, Sperm Activation, Pentoxifylline

## Introduction

Infertility is considered as a disease by the World Health Organization (WHO), which affects nearly 16% of couples around the world [1-2]. Some studies indicated that male-factor infertility contributes to almost 50% of the disease in these couples [3-4]. Non-obstructive azoospermia (NOA) is the most severe form of male-factor infertility, which is due to defective spermatogenesis because of genetic abnormalities or idiopathic causes, and accounting for about 15% of male infertility [5].

The introduction of the intracytoplasmic sperm injection (ICSI) with testicular sperm aspiration (TESA) played a significant role

in the treatment of severe male-factored infertility patients, especially for the NOA patients [6-7]. However, TESA samples are usually characterized by immotile spermatozoa, which often attach to residual tissue. As a result, it is time-consuming and very difficult for embryologists to select motile spermatozoa for TESA-ICSI. Indeed, the selection of motile spermatozoa for ICSI had a better treatment outcome as compared to the immotile spermatozoon, included the fertilization rate and clinical pregnancy rate, when compared to the immotile spermatozoon [8-9].

Many strategies have been used to selecting the motile spermatozoa, including hypo-osmotic swelling test (HOST), laser strikes the tail of a sperm, and extended in-vitro culture of testicular biopsies [10-11]. However, the above-mentioned methods were not user-friendly and might be harmful to sperm. For example, HOST allows water to enter into viable spermatozoa and causes membrane expansion, even might induce lysis of the cell membrane and cell death. Pentoxifylline (PF) is a phosphodiesterase inhibitor, and inhibits the breakdown of cyclic adenosine monophosphate, leading to augmented generation of cyclic nucleotides, which is known to play a pivotal role in sperm motility [12].

As a chemical agent of sperm activation, PF could activate sperm motility, allows us to observe motile sperm, thus easily identify the vital sperm from immotile spermatozoa population. There have been many studies about the clinical applications of PF that could be added to human spermatozoa to enhance their motility [13]. PF has been used for intrauterine insemination (IUI) and in many in vitro fertilization (IVF) programs. Stone reported higher pregnancy rates following sperm activation with PF combined with IUI [14]. Amer found that pentoxifylline can be used for improving ICSI outcomes including fertilization, embryo quality and pregnancy rates in cases of asthenozoospermia [15].

However, there were few studies reported about the efficacy and safety of sperm activation with PF in NOA patients. Thus, this study aims to assess the effect of pentoxifylline treatment in the TESA-ICSI of NOA patients in terms of laboratory and clinical outcomes, including fertilization, early embryo development, pregnancy and neonatal outcomes.

## Materials and Methods

### Study Population

Data of patients who underwent TESA-ICSI treatment due to NOA between January 2015 and December 2018 at Reproductive Medicine Center of the Third Affiliated Hospital of Guangzhou Medical University were retrospectively analyzed. All procedures were performed under the supervision and guidance of the National Health and Family Planning Commission. Informed consents were obtained from all couples, and the Institutional Review Board of the Ethical Committee of the Third Affiliated Hospital of Guangzhou Medical University approved the study protocol. Diagnosis of azoospermia was confirmed with at least three semen samples. All men had a physical examination, hormonal analysis and genetic studies (Y chromosome deletions as well as peripheral karyotype analysis) before surgical sperm retrieval.

Finally, 457 patients who identified no motile spermatozoa under through scrutiny in TESA-ICSI procedure were included. 121 cycles without PF treatment before the initiation of sperm activation with PF in our IVF center were recorded as control group. The other 336 cycles of TESA-ICSI with sperm activation with PF were recruited as PF group.

### Ovarian Stimulation and Oocyte Retrieval

Long protocol treatment with gonadotrophin-releasing hormone (GnRH) agonist (Triptorelin; Diphereline, Ipsen, France) or GnRH antagonist (Cetrorelix; Cetrotide, Merck, Germany) protocol for ovarian stimulation were provided for infertile women. Human chorionic gonadotrophin (HCG, Merck Serono) was administered when at least three follicles reached a mean diameter of 17 mm or at least two follicles reached a mean diameter of 18mm. Ultrasound-guided transvaginal oocyte retrieval was performed 34-36 hours later, and oocytes were collected and incubated in incubators until insemination.

### Testicular Sperm Preparation

Testicular tissue specimens were obtained from NOA patients undergoing TESA. A needle attached to a syringe was inserted through the scrotal skin into the testis. The needle was usually inserted into the anterolateral portion of the superior testicular pole at an oblique angle toward the medium and lower poles. The testicular parenchyma was aspirated by creating negative pressure, and the specimen was sent to the laboratory.

The extracted testicular tissue was placed in a Petri dish with G-MOPS-Plus (Vitrolife, Sweden), minced into testicular cell suspensions, and transferred to a conical test tube. These tubes were then centrifuged at 400g for 5 minutes, and the supernatant was discarded. The pellet was suspended with G-IVF Plus medium (Vitrolife, Sweden). The specimen was then incubated at 37°C in 6% $CO_2$ under humidified conditions for use in TESA-ICSI.

### ICSI and Sperm Activation Treatment

Immediately after retrieval, oocytes were placed in G-IVF Plus medium and maintained in incubators 6% $CO_2$ and 5% oxygen at 37°C for 2 -3 h before cumulus cells were removed by hyaluronidase and repeated pipetting. In control group, oocytes were injected with spontaneously motile sperm, or immotile sperm were injected under through scrutiny for more than 10 minutes. In PF group, PF solution was added to the micro droplets of the sperm to give a final concentration of 1.5 mmol/L PF. After incubation for 10 min, embryologist picked the motile sperms, and transferred them into washing droplet using the microinjection needle. The sperms were then washed and immobilized before transferred to the oocyte-placed drop for ICSI procedure.

## Fertilization Check, Embryo Transfer

Observation of fertilization were performed 16-18 h after the ICSI insemination. The normally fertilized embryos which clearly displayed two pronuclei (2PN) were cultured in G1 Plus medium (Vitrolife, Sweden) under mineral oil (Vitrolife, Sweden) until evaluation on day 3(D3). A transferable embryo on D3 was defined as more than 5 cells with less than 20% cellular debris; A good-quality embryo on D3 was defined as 7-9 cells with less than 20% cellular debris, and uniformity in cell size.

After embryo transfer and vitrification on D3, the remaining embryos exposed to extended culture to either day 5 or day 6, and the expanded B4-B6 blastocysts displayed with inner cell mass (ICM) and/or trophectoderm (TE) of at least one B grade or better were vitrified for later transfer. One or two embryos were transferred, depending on women's age, embryo availability and quality. Luteal phase support was continued with progesterone for at least two weeks after embryo transfer (ET). The fertilization rate/2PN fertilization rate were defined as the number of total fertilized oocytes/2PN fertilized oocytes divided by the total number of retrieved oocytes. The cleavage rate was defined as the number of cleaved embryos on D3 divided by the number of total fertilized oocytes. The rate of D3 oocyte utilization was defined as the number of transferable embryos on D3 divided by the number of retrieved oocytes. The rate of D3 good-quality embryo was defined as the number of good-quality embryos on D3 divided by the number of 2PN-fertilized oocytes.

The implantation rate was calculated by dividing the total number of fetal-cardiac-activity events detected by the total number of transferred embryos. The clinical pregnancy rate was defined as the number of visible sacs with fetal heartbeat in the fifth gestational week via ultrasonography examination divided by the number of fresh ET cycles. The miscarriage rate was defined as the number of cases with pregnancy loss before 28 weeks of gestation divided by the number of clinical pregnancies. The live birth rate was defined as the number of the cases with at least one live born infant after 28 weeks of gestation divided by the total number of fresh ET cycles. The cumulative live birth rate (CLBR) was calculated as the first live birth achieved after all ET cycles having an embryo (fresh and thawing ET cycles) or all embryos were used between the two groups. All delivered infants were evaluated for complications during pregnancy or at delivery, including gestational age at delivery, birth weight, neonatal length, and neonatal defects.

## Statistical Analysis

Data are reported as mean ± standard deviation (SD) or the corresponding percentage and the number of cases. Statistical comparisons between the groups were performed with a t-test or chi-squared test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the SPSS software 19.0 (SPSS Inc., USA).

## Results

In the present study, clinical data of 457 NOA patients received 457 TESA-ICSI treatment cycles were analyzed. In the control group, the testicular sperms of 121 patients injected without sperm activation. Of 336 patients in the PF group, motile sperms were found and injected in all of the 336 TESA-ICSI cycles after sperm activation with PF treatment.

The basic clinical characteristics of patients in two groups are shown in Table 1. In the control group, the mean ages of the male and female partners were 34.60 years old and 31.05 years old, respectively; the body mass index (BMI) of male and female partners were 24.25 and 21.72 kg/m$^2$, respectively. In the PF group, the mean ages of the male and female partners were respectively 34.54 years and 31.29 years old; the BMI of male and female partners were 23.96 and 21.85 kg/m$^2$, respectively. No significant differences were observed between the two groups considering the couples' age and BMI. The characteristics of the female regarding infertile duration, infertile type and infertile factors, female anti-mullerian hormone (AMH), the basal levels of steroid hormones (LH, FSH, E2), ovarian stimulation protocols, duration of gonadotropin (Gn) stimulation and total Gn dose showed no evident differences between the two groups ($P > 0.05$). Overall, Table 1 demonstrated that the basic clinical features were comparable between the two groups.

**Table 1: Basic Clinical Characteristics of Control Group and PF Group**

| | Control group | PF group | P value |
|---|---|---|---|
| No. of cycles | 121 | 336 | -- |
| Male age(y) | 34. 60±6. 86 | 34. 54±6. 33 | 0. 898 |
| Male BMI(kg/m$^2$) | 24. 25±3. 69 | 23. 96±3. 76 | 0. 315 |
| Female age(y) | 31. 05±4. 83 | 31. 29±4. 88 | 0. 513 |
| Female BMI(kg/m$^2$) | 21. 72±3. 36 | 21. 85±3. 29 | 0. 581 |
| Infertile duration (y) | 4. 95±3. 49 | 4. 88±3. 60 | 0. 798 |
| Infertile type (%) | | | 0. 498 |
| Primary infertility | 83. 47(101/121) | 86. 01 (289/336) | |
| Secondary infertility | 16. 53(20/121) | 13. 99(47/336) | |
| Infertile factors (%) | | | 0. 079 |
| NOA alone | 88. 43(107/121) | 93. 45(314/336) | |
| NOA with female factors | 11. 57(22/121) | 6. 55(22/336) | |
| Female AMH (ng/ml) | 5. 25±1. 52 | 5. 38±1. 64 | 0. 359 |
| Female basal serum LH level mIU/ml) | 3. 23±0. 98 | 3. 56±1. 03 | 0. 301 |
| Female basal serum FSH level (U/L) | 5. 76±1. 70 | 5. 58±1. 91 | 0. 201 |
| Female basal serum E2 level (pmol/ml) | 134. 09±50. 40 | 135. 73±52. 87 | 0. 122 |
| Ovarian stimulation protocol (%) | | | 0. 258 |
| GnRH agonist | 89. 26(108/121) | 85. 12(286/336) | |
| GnRH antagonist | 10. 74(13/121) | 14. 88(50/336) | |
| Duration of Gn stimulation(d) | 11. 34±1. 93 | 11. 68±2. 06 | 0. 689 |
| Total Gn dose (IU) | 2409. 22±1014. 79 | 2281. 53±997. 33 | 0. 086 |

Note: Data are reported as mean ± standard error of the mean or percentage. PF= pentoxifylline; BMI = body mass index; AMH= anti-mullerian hormone; LH = luteinizing hormone; FSH = follicle stimulating hormone; E2= Estradiol; Gn= gonadotropin

As presented in Table 2, 1754 oocytes were retrieved in the control group, and the average number of oocytes obtained was 14.00. The PF group obtained 4898 oocytes, and the average number of oocytes obtained was 14.08; the rates of metaphase- II (MII) oocyte between the two groups were 84.04% and 83.07%, respectively. The average number of oocytes per cycle and MII oocyte rate were similar between the two groups (P>0.05). The fertilization rate and 2PN fertilization rate in PF group were significantly higher than the control group (75.96% versus 62.21%; 70.07% versus 60.52%, respectively, P<0.001). The cleavage rate between the two groups was similar (96.51% versus 95.96%, P>0. 05). Moreover, the PF group yields higher rates of D3 oocyte utilization and good-quality embryo when compared with the control group (32.12% versus 28.96%; 21.71% versus 10.65%, respectively, P<0.05). After sperm activation with PF, it significantly reduced the total fertilization failure cycle rate, rate of cycles with no transferable embryo and the mean ICSI operation time per oocyte when compared with the control group (1.79% versus 5.79%, 4.17% versus 9.92%, 3.12±1.28 versus 8.37±3.62 , respectively, P<0.05).

**Table 2: Laboratory Outcomes of Control Group and PF Group**

| | Control group (n=121) | PF group (n=336) | P value |
|---|---|---|---|
| No. of retrieved oocytes per cycle | 14. 00±7. 52 | 14. 08±7. 24 | 0. 883 |
| MII oocyte rate (%) | 84. 04(1474/1754) | 83. 07(4069/4898) | 0. 354 |
| Fertilization rate (%) | 62. 21(917/1474) | 75. 96(3091/4069) | 0. 000 |
| 2PN fertilization rate (%) | 60. 52(892/1474) | 70. 07(2851/4069) | 0. 000 |
| Total fertilization failure cycle rate (%) | 5. 79(7/121) | 1. 79(6/336) | 0. 016 |
| Cleavage rate (%) | 96. 51(885/917) | 95. 96(2966/3091) | 0. 673 |
| Rate of D3 oocyte utilization (%) | 28. 96(508/1754) | 32. 12(1573/4898) | 0. 015 |
| Rate of D3 Good-quality embryos (%) | 10. 65(95/892) | 21. 71(619/2851) | 0. 000 |
| Rate of cycles with no transferable embryo (%) | 9. 92(12/121) | 4. 17(14/336) | 0. 000 |
| ICSI operation time per oocyte (min) | 8. 37±3. 62 | 3. 12±1. 28 | 0. 000 |
| Note: MII = metaphase- II; PN = pronuclei; D3=day 3; ICSI = intracytoplasmic sperm injection | | | |

Comparisons of pregnancy outcomes of patients between the two groups are shown in Table 3, no significant differences were found related to the female age, BMI, infertile duration, and maximum endometrial thickness between the two groups. Additionally, the number of transferred embryos between two groups were similar (1.88 versus 1.80, P>0.05), and majority of the women had two embryos transferred (85.96% versus 80.00%). Ultimately, no significant differences were observed between the two groups in the implantation rate, clinical pregnancy rate, miscarriage rate and live birth rate (45.79% versus 46.36%, 61.40% versus 61.38%, 11.43% versus 10.11%, 54.39% versus 53.10%, respectively, P>0.05). However, the PF group achieved a significantly higher cumulative live birth rate compared with the control group (69.94% versus 64.46%, P<0.05).

**Table 3: Pregnancy Outcomes of Control Group and PF Group**

| | Control group | PF group | P value |
|---|---|---|---|
| Cycles with fresh ET | 57 | 145 | -- |
| Female age(y) | 31.32±4.99 | 30.23±4.52 | 0.139 |
| Female BMI (kg/m2) | 21.14±4.12 | 21.69±3.02 | 0.293 |
| Infertile duration (y) | 4.80±3.17 | 4.88±3.43 | 0.817 |
| Maximum endometrial thickness n(mm) | 10.87±1.84 | 10.70±2.00 | 0.563 |
| No. of transferred embryos | 1.88±0.38 | 1.80±0.40 | 0.214 |
| 1 | 14.04(8/57) | 20.00(29/145) | 0.324 |
| 2 | 85.96(49/57) | 80.00(116/145) | |
| Implantation rate (%) | 45.79(49/107) | 46.36(121/261) | 0.851 |
| Clinical Pregnancy rate (%) | 61.40(35/57) | 61.38(89/145) | 0.678 |
| Miscarriage rate (%) | 11.43(4/35) | 10.11(9/89) | 0.459 |
| Live birth rate (%) | 54.39(31/57) | 53.10(77/145) | 0.379 |
| Cumulative live birth rate (%) | 64.46(78/121) | 69.94(235/336) | 0.015 |

When the neonatal outcomes were compared, the PF group and control group did not differ significantly regarding the preterm birth rate, gestational weeks at delivery, neonatal length and birth weight, as well as the sex ratio (male/female). In addition, no congenital anomalies were reported in the two groups (Table 4).

**Table 4: Neonatal Outcomes of Control Group and PF Group**

| | Control group | PF group | P value |
|---|---|---|---|
| Preterm birth rate(<37 weeks) | 16. 67(13/78) | 17. 44(41/235) | 0. 269 |
| Gestational weeks | 38. 20±1. 42 | 37. 64±2. 54 | 0. 254 |
| Neonatal Length(cm) | 48. 83±2. 41 | 48. 62±2. 30 | 0. 696 |
| Birth weight (kg) | 2. 90±0. 51 | 2. 89±0. 55 | 0. 923 |
| Sex ratio(male/female) | 1. 11(41/37) | 0. 96(115/120) | 0. 303 |
| Congenital anomalies | 0 | 0 | -- |

## Discussion

NOA represents a heterogeneous condition, with impaired spermatogenesis ranging from hypo- spermatogenesis and maturation arrest, so the number of spermatozoa derived from TESA sample of NOA patients is very limited, and usually only immotile spermatozoa were obtained [16]. Currently, we use the chemical agent of PF to activate the motility of sperm in TESA-ICSI procedure of NOA patient, it can help to distinguish motile and immotile spermatozoa rapidly followed with an immediate ICSI [17].

Kovacic et al. divided 77 TESA cycles with only immotile sperm into PF and non-PF groups, they found that the mean time required for ICSI was shortened in the PF group, due to easier identification of motile sperm, the PF group had a significantly higher fertilization rate and mean number of embryos per cycle when compared with the non-PF group [18]. In this study, we noticed that the total fertilization rate and 2PN fertilization rate were significantly increased in the PF group ($P<0.001$). Moreover, the PF group yields higher rate of D3 oocyte utilization and lower rate of total fertilization failure cycle ($P<0.05$). We also found that it significantly improved the rates of D3 good-quality embryo and cycles with no transferable embryo after sperm activation with PF ($P<0.001$) at the same time, it significantly reduced the mean ICSI time per oocyte when compared with the control group ($P<0.001$). Therefore, our data is consistent with the results of Kovacic et al. indicating that PF improved the fertilization rate, and shortened the operation time of TESA-ICSI procedure. Furthermore, our data indicated the sperm activation with PF could improve the embryo development, we speculated that PF improves the sperm motility, leading to better selection of the morphologically normal and motile sperm, and sperm play an important role in the fertilization and preimplantation embryo development [19]. In addition, the mean ICSI time per oocyte also reduced in the PF group, which significantly shorten the exposure time of oocyte during in vitro manipulation, might have a beneficial effect on subsequent embryo development.

As to the pregnancy outcomes, we found no significant differences between the two groups in the implantation rate, clinical pregnancy rate, miscarriage rate and live birth rate ($P>0.05$). This may be explained as follows, the embryos selected for the first ET in two groups were all transferable embryos mainly, so the outcomes did not differ significantly. Indeed, the sperm activation with PF increased the rates of D3 oocyte utilization and good-quality embryo. Consequently, PF increased the overall number of transferable embryos. Finally, the PF group achieved a significantly higher cumulative live birth rate when compared with the control group ($P<0.05$).

It is important to note that adverse effects of PF, such as malformations in animal models had been reported, but these adverse effects could be prevented by spermatozoa washing with medium to avoid exposure of oocyte to PF [20]. Safety is a crucial issue considering the possibility of PF-induced adverse effects on human embryos. Hence, unlike many previous studies, the sperm activation with a very low concentration of PF (1.5mmol/l) and very short-term incubation (10 minutes) were performed in our study, and then the selected motile sperms were washed with medium before ICSI injection [21]. We noticed that the PF group and control group did not differ significantly in the preterm birth rate, gestational weeks, neonatal length, birth weight, as well as the sex ratio. In addition, no congenital anomalies were reported in the two groups. Our results agreed with the published study, which suggested no increase of adverse effects on obstetric and neo-natal outcomes after sperm activation with PF in 102 patients [22]. Therefore, we concluded that brief exposure of sperm to PF has no adverse effects on human embryonic development.

Overall, our results demonstrated the efficacy and safety of PF for NOA patients that underwent TESA-ICSI, so sperm activation with PF is beneficial for NOA patients. However, the safety of sperm activation with PF was of utmost concern. More prospective studies with larger groups of couples to further assess the long-term effects for children were required especially.

## Abbreviations

NOA: non-obstructive azoospermia
ICSI: Intracytoplasmic sperm injection
TESA: Testicular sperm aspiration
PF: Pentoxifylline
IVF: In vitro fertilization
LH: Luteinizing hormone
FSH: Follicle stimulating hormone
GnRH: Gonadotrophin-releasing hormone
BMI: Body mass index

AMH: Anti-mullerian hormone
HCG: Human chorionic gonadotrophin
2PN: Two pronuclei
ET: Embryo transfer

## Statements and Declarations

### Funding

This study was funded by the Guangzhou Municipal/University (High-Level University) Joint Funded Basic Research Program (Grant number: 202102010055) and National Nature Science Foundation of China (Grant numbers: 81871211, 81502507).

### Ethics Approval and Consent to Participate

The Ethics Committee of Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, obtained ethical approval for all experimental procedures. Written informed consent were obtained from all patients involved in this study.

### Competing Interests

The authors declare that they have no competing interests. Acknowledgments The authors thank all of the dedicated and talented embryologists at the Center for Reproductive Medicine, The Third Affiliated Hospital of Guangzhou Medical University.

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