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ORIGINAL PAPER

Pentoxifylline treatment as a safe method for selecting viable testicular spermatozoa before cryopreservation of a small numbers of spermatozoa in azoospermia individuals

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Summary

Background: *Single sperm cryopreservation (SSC) is a specific technique especially used in individuals with small numbers of sperm who suffered from non-obstructive azoospermia (NOA). Testicular specimens possess poor motility and low population of viable spermatozoa. Therefore, sperm selection methods such as applying pentoxifylline (PTX) may improve motility in these cases. The main aim of this study was to evaluate the protective effects of PTX on testicular spermatozoa before and after performing SSC.*

Methods: *Thirty testicular samples were obtained from men with azoospermia. This study was conducted in two phases. Phase 1 evaluated the effect of PTX for sperm selection before SSC. Twenty testicular samples were divided to two experimental groups: SSC without (I) and with PTX treatment (II). For PTX treatment spermatozoa were incubated with PTX at 37°C for 30 min and only motile spermatozoa were selected for SSC. In phase 2, ten testicular samples were cryopreserved with SSC and warming procedure was carried out in droplet with and without PTX. Motility and viability rates, morphology by motile sperm organelle morphology examination (MSOME), DNA fragmentation by sperm chromatin dispersion test (SCD) and mitochondrial membrane potential (MMP) were evaluated.*

Results: *In phase 1, post warm motility rate was higher in PTX exposed group compared to the unexposed group (25.6 ± 8.13 vs. 0.85 ± 2.1) ($p > 0.00$). Recovery rate, viability and morphology were not significantly different between groups. DNA integrity and MMP were also similar between both groups. In phase 2 although motility increased in PTX group compared to without PTX group (29.30 ± 12.73 vs. 1.90 ± 2.64) ($p > 0.00$), the viability rate was not different (70.40 ± 12.12 vs. 65.30 ± 11.87). All above mentioned parameters were similar between the two SSC groups.*

Conclusions: *Supplementation of testicular spermatozoa with PTX before cryopreservation increases motility and did not have adverse effects on viability, morphology, DNA integrity and MMP. PTX could be used as sperm selection method before single sperm cryopreservation, but PTX could not maintain motile the most of viable testicular sperms.*

KEY WORDS: *Non-obstructive azoospermia; Pentoxifylline; Testicular sperm; Single sperm cryopreservation.*

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INTRODUCTION

Azoospermia, defined as lack of spermatozoa in ejaculate samples, affects 1% of all men and 10-15% of individuals with infertility (1, 2). Moreover, about 60% of men with azoospermia have *non-obstructive azoospermia* (NOA) that occurs by severe defects during spermatogenesis procedure (3). Cryopreservation of spermatozoa after biopsy of testicular tissue prevent from repetitions of biopsy in azoospermic cases (4). Conventional cryopreservation methods are not appropriate in these cases owing to limited numbers of spermatozoa which are lost during freezing and thawing techniques (5). Therefore, the concept of *single spermatozoa cryopreservation* (SSC) was proposed in 1997 by *Cohen et al.* (6) who used empty zona pellucida for freezing.

After that, various cryo-devices and methods were designed to increase fertilization chance in cases with limited number of sperm (7). In this regard, some details of this technique, such as selecting proper spermatozoa before freezing, requir much more attention. In these cases, sperm specimen obtained from testicular tissue usually poses little or no motility. Using spermatozoa with no motility in ICSI procedure negatively affects clinical outcomes (8). Therefore, it is noteworthy to find a method for selecting viable spermatozoa before freezing in order to optimize thawing outcomes (especially sperm motility) with less possible adverse effects on sperm biological characteristics.

In case of absent or poor movement of testicular sperm specimen, different methods including *hypo- osmotic swelling test* (HOS), sperm tail flexibility test and *in-vitro* culturing are available to enhance motility of testicular sperm (9, 10). The mentioned methods might adversely affect sperm functions and biological characteristics. For instance, HOS test causes water entrance to spermatozoa resulting in membrane expansion which finally leads to cell membrane lysis and death (11). *Pentoxifylline* (PTX), sperm activation chemical agent, is a *phosphodiesterase* (PDE) inhibitor that is able to improve sperm motility by increasing *cyclic adenosine monophosphate* (c-AMP) levels and *protein kinase A* (PKA) activity. PTX is a user-friendly and easy method to identify viable spermatozoa from immotile spermatozoa (12).

To our knowledge, PTX as sperm selection method has not been used in SSC technique. The main aim of this study is to investigate the effect of PTX on sperm motility, morphology, viability, DNA fragmentation and mitochondrial membrane potential by incubation of NOA testicular samples in PTX (as sperm selection method) before and after applying SSC technique using Cryo-vial device.

Methods and materials

Study design

This study was performed from 22 June 2023 to 28 September 2023 at Yazd Reproductive Sciences Institute. It was was approved by ethics committee of *Shahid Sadoughi University of Medical Sciences* (IR.SSU.MEDICINE.REC.1401.016). Written informed consents were obtained from the men who referred to Yazd infertility center for treatment before collection of samples.
The study was conducted in two phases.

Phase 1:
1. Testicular sperm specimens were collected surgically by TESE and micro-TESE from 20 azoospermic men. 2. Selected spermatozoa were randomly divided into two experimental groups: (I): SSC without PTX treatment (control) and (II): SSC with PTX treatment. 3. Testicular spermatozoa were prepared, and the suspension was divided and poured in two culture dishes (one for control group and the other one for PTX group. 4. In both groups the spermatozoa were selected first based on tail movement and then by tail flexibility gently by a ICSI injection micropipette (*Nikon, Japan*) equipped with a micromanipulator. 5. In control group, twenty spermatozoa were randomly selected and placed in HOS medium and the percentage of viable spermatozoa was determined. 6. The viability and motility of fresh testicular samples were also reported. 7. The remaining spermatozoa were cryopreserved. 8. In PTX group, the selected spermatozoa transferred to the PTX droplets and after that, only motile spermatozoa were selected for SSC.

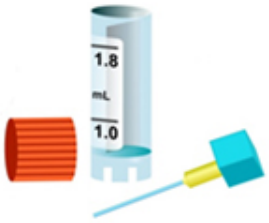
Phase 2:
1. In this phase, spermatozoa were selected from TESE and micro-TESE samples from 10 azoospermic men. 2. About one-hundred testicular spermatozoa or more were selected as previous described and cryopreserved by SSC technique on several cryotops. 3. For warming, Cryotops were randomly divided to be warmed in (I): sperm washing medium without PTX (control) or (II) in sperm washing medium with PTX in the same concentration as phase 1 (Figure 1).

Testicular sperm preparation and processing
Seminiferous tubules fragments were poured in large culture dishes consisting of sperm washing medium (*Bio chrome, Berlin, Germany*) supplemented with 5 mg/mL Human serum albumin (*HSA, Vitrolife, Englewood, CO*). Seminiferous tubules were recognized by applying a stereomicroscope and the stainless blades were used to remove blood clots. After that, extruded testicular tissue was washed in sperm washing medium supplemented with 5 mg/mL HSA to remove the blood and then placed to the central-well dish (*Falcon, USA*) consisting of fresh sperm medium. Tubules mechanical dispersion was performed by mincing repeatedly using stainless blades. The media containing spermatozoa was centrifuged at 300g for 10 min. After removing the supernatant, the collected pellet was resuspended in 1 mL of sperm washing medium supplemented with 5 mg/mL HSA and used for single sperm cryopreservation (13).

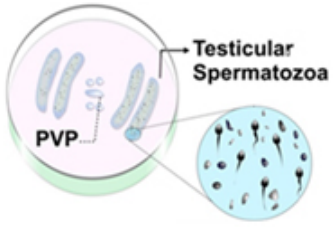
Single sperm cryopreservation and warming procedure
Testicular samples (1-3 µL droplet) were added to 5 µL droplet of sperm washing medium supplemented with 5 mg/ mL HSA on ICSI dish (*Falcon, 1006 dish*) (Figure 1B). Sperm cells were collected using ICSI injection micropipette (*Nikon, Japan*) equipped with a micromanipulator (Figure 1C). For single sperm cryopreservation *Cryotop Vial Device* (CVD) was used. In previous study, we designed a new carrier which contains cryotop (*Kitazato, Japan*) and cryovial (*Nest, China*) (14) (Figure 1A). Selected spermatozoa were placed on 0.5 M sucrose (*Sigma-Aldrich*) diluted 1:1 with sperm washing medium (final concentration 0.25 m sucrose) droplet on the Cryotop strip by using the ICSI pipette (Figure 1D, E). Then, the polypropylene strip was placed into the cryovial and closed carefully (Figure 1F). Cryovial directly plunged into liquid nitrogen (Figure 1G). The samples were maintained in liquid nitrogen at least 72 h pre warming (15). For warming procedure, Cryotops were removed from cryovials and Cryotop strip was placed directly in a pre-warmed 5 µL droplet of sperm washing medium supplemented with 5 mg/ mL HSA on ICSI dish at 37°C. Cryotop was washed with 10 µL droplets to remove all spermatozoa remain on Cryotop strip (Figure 1I). Pre-warmed oil (Ovoil; vitrolife, Sweden) was poured on droplet with spermatozoa. After 30 min incubation at 37°C, to aspirate sperm cells a micropipette equipped with micromanipulator on an inverted microscope was used. Finally, retrieved spermatozoa were transferred to a new droplet of sperm washing medium supplemented with 5 mg/mL HSA (15). In phase 2, in addition to warming in sperm washing medium, warming was also performed in PTX droplet (Figure 1J).

Treatment of testicular spermatozoa with PTX
For PTX preparation, 3.6 mM stock solution of PTX (*Sigma, St. Louis, MO, USA*) was provided by adding 1 mg PTX powder to 1 mL sperm washing medium supplemented with 5 mg/ml HAS. This solution can be stored at 4°C for up to 7 days.
To prepare working solution, the stock solution was diluted 1:1 with sperm washing medium. The final concentration of PTX in the specimen was 1.76 mM. Selected spermatozoa by ICSI injection micropipette (*Nikon, Japan*) equipped with a micromanipulator were added to the PTX droplet, which was overlaid with pre-warmed oil (*Ovoil; vitrolife, Sweden*). Testicular sperm samples were incubated in PTX droplets at 37°C for 30 min. The droplet was investigated for viable and motile spermatozoa and then, selected by ICSI injection micropipette and transferred to the new droplet for freezing (16). In phase 2, if spermatozoa were not motile, the viability was checked with HOS test.

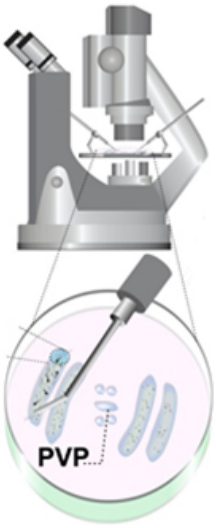
Figure 1.
Steps of study design. CVD: Cryotop vial device, PVP: Polyvinylpyrrolidone, ICSI: intracytoplasmic sperm injection, LN: liquid nitrogen, HSA: human serum albumin (Figure is original and never used before).



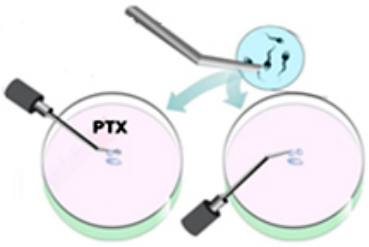
A.
Cryotop vial device (CVD) was used for single sperm cryopreservation. This device consists of vial, a screw cap, Cryotop, soft foam and a fine polypropylene strip.



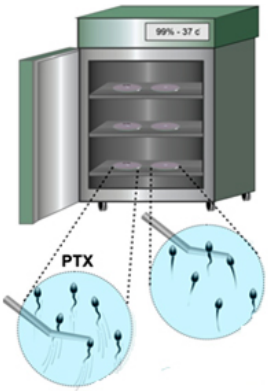
B.
Droplets of testicular spermatozoa and PVP droplet for needle stabilization.



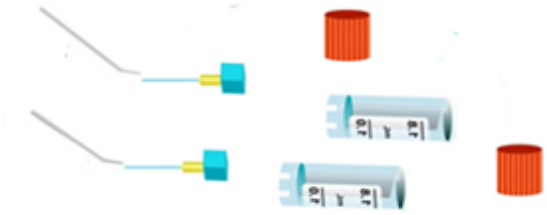
C.
Sperm cells were collected using a ICSI injection micropipette equipped with a micromanipulator.



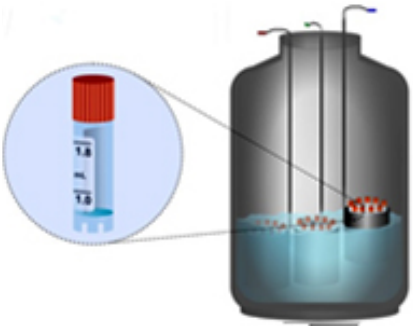
D.
Sperm cells were placed into the PTX droplet and in the droplets without PTX.



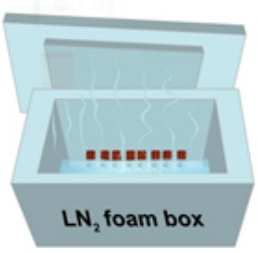
E.
Both culture dishes were transferred to the incubator.



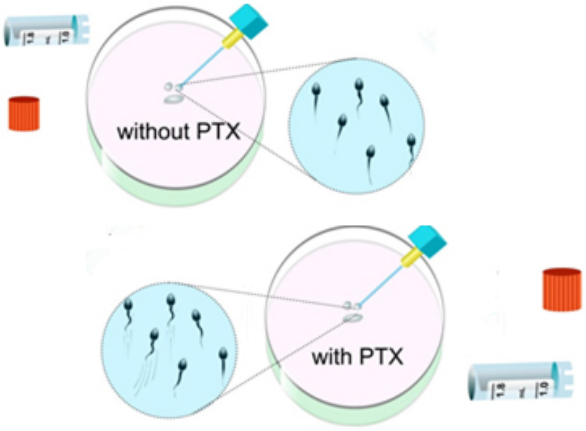
F.
Spermatozoa incubated with and without PTX were placed into the sucrose droplet on the tip of the Cryotop and then, the Cryotops were inserted into cryovial and closed with screw cap tightly.



G.
Cryovials were transferred to the LN tank.



H.
For warming cryovials were removed from LN tank and placed into the LN foam box.



I.
Cryotops were removed from cryovials and Cryotop strip was placed directly in a pre-warmed 5 µL droplet of sperm washing medium supplemented with 5 mg/mL HSA on ICSI dish at 37°C.

Sperm parameters evaluation
To assess sperm recovery, motility and viability an inverted microscope system was used. Sperm recovery rate was recorded by below formula: number of post-warm sperm /number of cryopreserved sperm × 100. Sperm motility and viability were determined by post-thaw motility and viability, respectively (15). For viability assessment after thawing, motile spermatozoa were considered viable, while for immotile spermatozoa hypo-osmotic swelling test (HOS) was performed. The sum of motile and non-motile sperms in the medium was calculated.

Hypo-osmotic swelling test (HOS)
The spermatozoa were selected by using a ICSI injection micropipette (*Nikon, Japan*) equipped with a micromanipulator and then the tail of spermatozoa was placed in 5µl of hypo-osmotic droplet. Coiled tail patterns Viable spermatozoa indicated. For preparing HOS medium, the sperm washing medium was diluted 1:1 with distilled water (17).

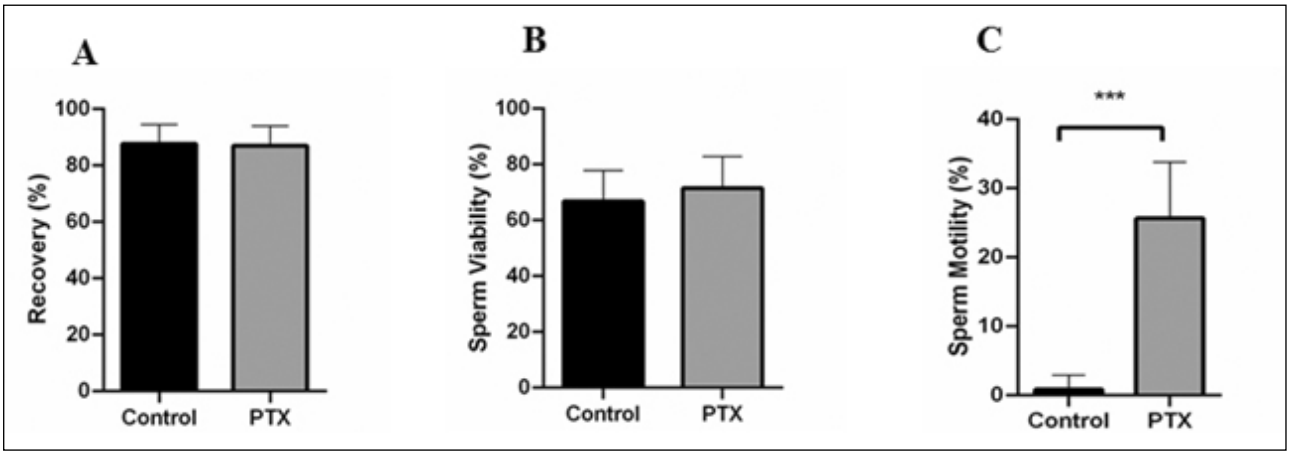
Fine sperm morphology evaluation
The morphology of spermatozoa was evaluated applying an inverted microscope at high magnification using *motile sperm organelle morphology examination* (MSOME) technique (*Nikon ECLIPSE TE300*). Medium droplets were placed into the glass-bottom dish (*GWSt 1000; Will Co.*) and sperm cells were transferred to it by using a ICSI pipette equipped with a micromanipulator. Sperm cells were then assessed under high magnification (6600 ×) using an inverted microscope with high-power differential interference contrast optics. Morphological assessment was carried out on monitor and spermatozoa were categorized in to three groups (high, medium and low-quality) according to the shape of the spermatozoa (sperm head, vacuoles, and base) (18).

DNA fragmentation evaluation
The SDFA kit (*Tehran, Iran*) was used to assess sperm DNA fragmentation by *Sperm Chromatin Dispersion* (SCD) test. Briefly, low-melting-point agarose gel droplet (5 µl) was placed onto the pre-coated slide. Selected spermatozoa by ICSI injection micropipette (*Nikon, Japan*) equipped with a micromanipulator were gently added to the agarose gel droplet and small coverslips were placed on the slide. After that, the staining procedure was done according to the kit instructions. Then, slides were assessed by a bright field microscope according to the halo size. Spermatozoa with no or small halos showed DNA fragmentation while, spermatozoa with medium or large halos considered as intact DNA. Percentage of spermatozoa with DNA fragmentation was recorded (16).

Sperm mitochondrial membrane potential (MMP) evaluation
Sperm *mitochondrial membrane potential* (MMP) was assessed by tetraethylbenzimidazolylcarbocyanine iodide (JC-1) mitochondrial membrane potential assay kit (*Cayman Chemical Co, Ann Arbor, MI, USA; cat #10009172*). The sperm cells were placed in JC-1 working solution droplets by the ICSI pipette equipped with micromanipulator. After that, samples were incubated at 37°C for 30 min in dark environment. Then, the cells were examined by fluorescence microscope (*Olympus BX51, Japan*) according to manufacturer's instructions. Spermatozoa with an orange fluorescence dye were considered as having high mitochondrial membrane potential (active mitochondria, JC-1+). The percentage of cells with a high mitochondrial membrane potential were recorded (15).

Statistical analysis
Data were analyzed by the *Statistical Package for the Social Sciences* (SPSS) version 20 (*IBM, California, United States*). Data were expressed as mean ± SD. Kolmogorov-Smirnov test was used to test the normality of data. Independent sample T test and Mann-Whitney Test were used for comparing the data with normal and abnormal distribution, respectively between two cryo-groups groups. Paired sample T test was used for comparing the fresh group with cryo-groups. Plotted graphs were carried out with GraphPad Prism 8.4.2 (*GraphPad Software, Inc., San Diego, CA, USA*). $P < 0.05$ was considered significant.

Figure 2.
*Sperm parameters. Recovery (A), viability (B) and motility (C) of testicular spermatozoa incubated with PTX before single sperm cryopreservation (Phase 1). Data are presented as mean ± SD. ***P<0.001 (analysis by Mann-Whitney Test).*
A and B analysis by Independent Samples T Test.



Results

Phase 1 (PTX treatment before cryopreservation)

Post-warmed spermatozoa parameters

In phase 1 results of sperm parameters showed that recovery rate after SCC was similar between PTX and control groups (87.00 ± 7.07 *vs.* 87.70 ± 6.74) (Figure 2A). The viability of fresh testicular samples, which was at first selected by tail flexibility and twitching movement and then confirmed by HOS test, was about 98%. The percentage of viable spermatozoa decreased in PTX and control groups compared to the fresh group (71.55 ± 11.36 *vs.* 66.80 ± 11.09 and *vs.* 98.8 ± 3.15, $p > 0.001$). No remarkable changes were observed in viability rates between the two SSC groups (Figure 2B). The percentage of fresh motile testicular spermatozoa was about 11.75%. After incubation with PTX, only the motile spermatozoa (approximately, 100% motile spermatozoa) were selected for SSC in the PTX group. Exposure of testicular samples with PTX before freezing significantly increased the motility rate of post-thawed spermatozoa compared to the unexposed group (25.65 ± 8.13 *vs.* 0.85 ± 2.10) (Figure 2C $p > 0.001$).

Post-warmed sperm DNA fragmentation and mitochondrial membrane potential

In phase 1, comparison of DNA fragmentation results between cryopreservation groups showed that PTX did not significantly alter DNA fragmentation percentage (32.6 ± 4.18 *vs.* 31.10 ± 4.15) (Figure 3A).

The percentage of spermatozoa with active mitochondrial membrane potential in phase 1 was not different between two cryopreservation groups (50.20 ± 9.15 *vs.* 49.25 ± 9.12) (Figure 3B).

Post-warmed fine sperm morphology

The percentages of high (10.40 ± 6.85 *vs.* 9.30 ± 6.30), medium (53.30 ± 15.71 *vs.* 51.30 ± 15.52) and low (33.90 ± 14.48 *vs.* 38.00 ± 13.91) quality spermatozoa in phase 1 were not different between PTX and control groups (Figure 4A-C).

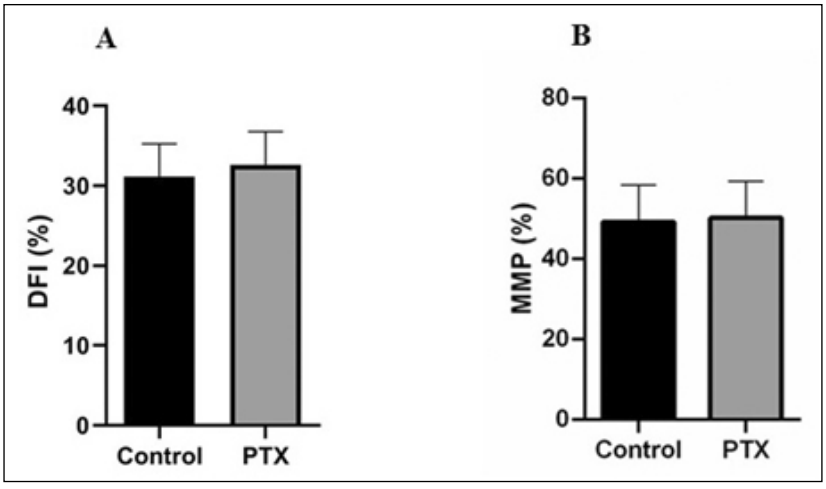


Figure 3.
DNA fragmentation index (DFI) (A) and mitochondrial membrane potential (MMP) (B) after incubation with PTX before single sperm cryopreservation (Phase 1).
Data are presented as mean ± SD and analysis by Independent Samples T Test.

Figure 4.
Fine sperm morphology. Morphology of sperm classified in three groups high (A), medium (B) and low (C) quality after incubation in PTX before single sperm cryopreservation (phase 1).
Data are presented as mean ± SD and analysis by Independent Samples T Test.

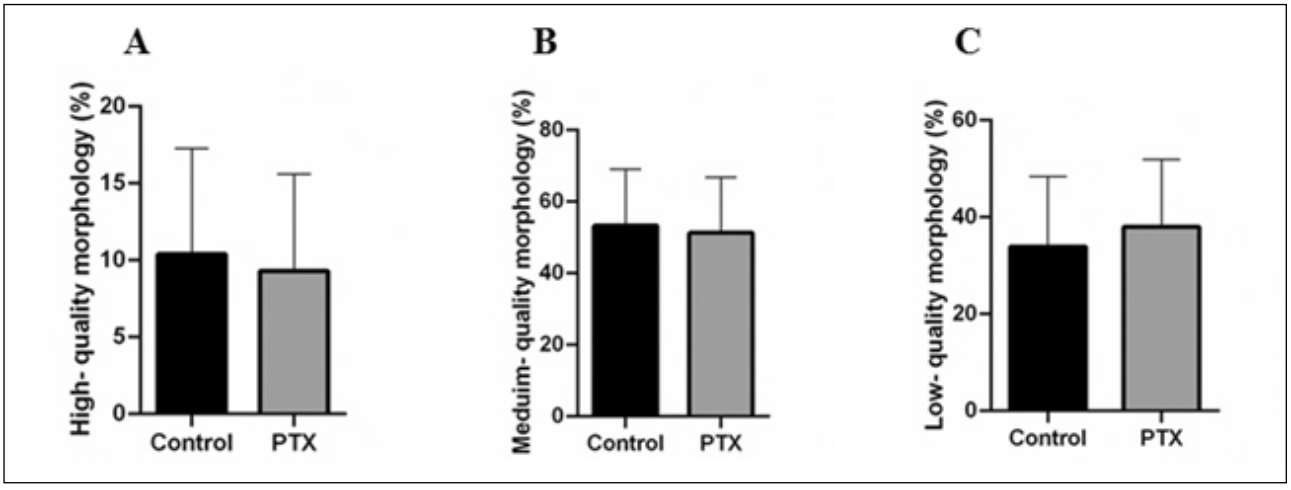
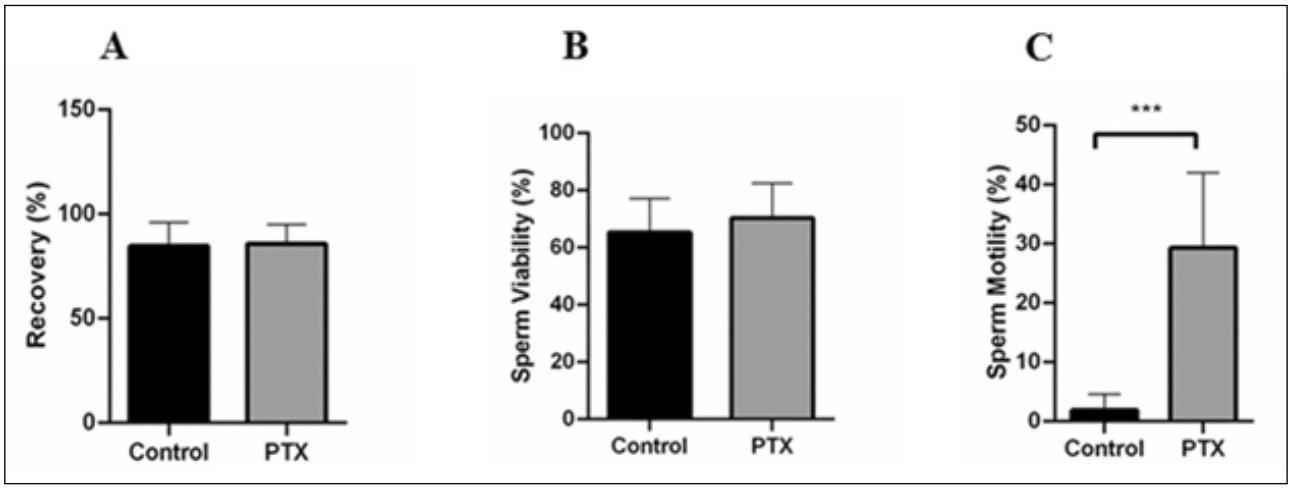


Figure 5.
Sperm parameters. Recovery (A), viability (B) and motility (C) of testicular spermatozoa incubated with PTX after single sperm cryopreservation (phase 2). Data are presented as mean ± SD and analysis by Independent Samples T Test.
****P < 0.001 versus control and PTX groups.*



Phase 2 (PTX treatment after cryopreservation)
Post-warmed spermatozoa parameters
In phase 2, recovery rate was similar between PTX and control groups (84.70 ± 11.26 *vs.* 85.80 ± 9.15) (Figure 5A). The percentage of fresh viable testicular spermatozoa, which were selected as in phase 1, was about 98.5% and decreased in both SSC groups compared to the fresh group (70.40 ± 12.12 *vs.* 65.30 ± 11.87 and *vs.* 98.5 ± 2.10, p > 0. 001). Moreover, there were no significant changes between the two SSC groups in viability rate (Figure 5B). the percentage of fresh motile testicular spermatozoa was about 10%. After warming, the percentage of motile spermatozoa which were incubated with PTX was significantly increased compared to the spermatozoa without PTX incubation (29.30 ± 12.73 *vs.* 1.90 ± 2.64) (Figure 5C p > 0. 001).Comparison of motility (29.30 ± 12.73) and viability (70.40 ± 12.12) in PTX group in phase 2 were significantly different (p > 0. 001). This result showed that after PTX not all viable spermatozoa could be motile.

Post-warmed sperm DNA fragmentation and mitochondrial membrane potential
In phase 2, DNA fragmentation was similar between the two SSC groups (28.70 ± 4.64 *vs.* 29.50 ± 3.97) (Figure 6A).

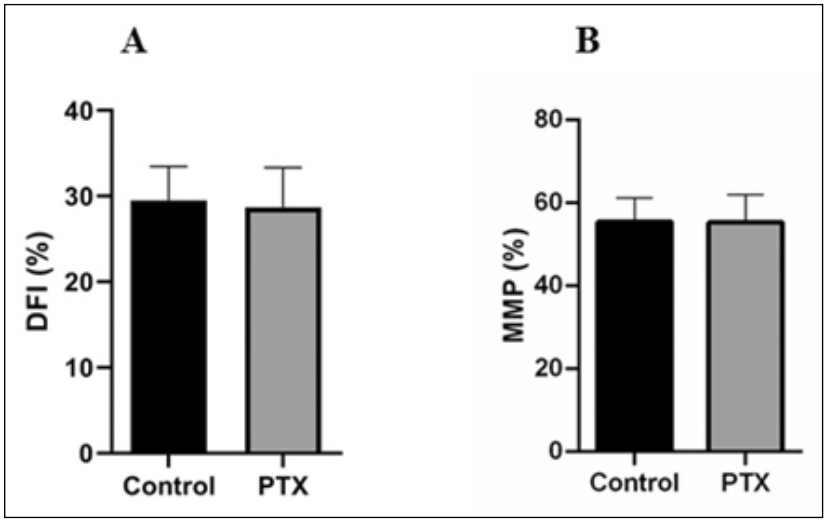
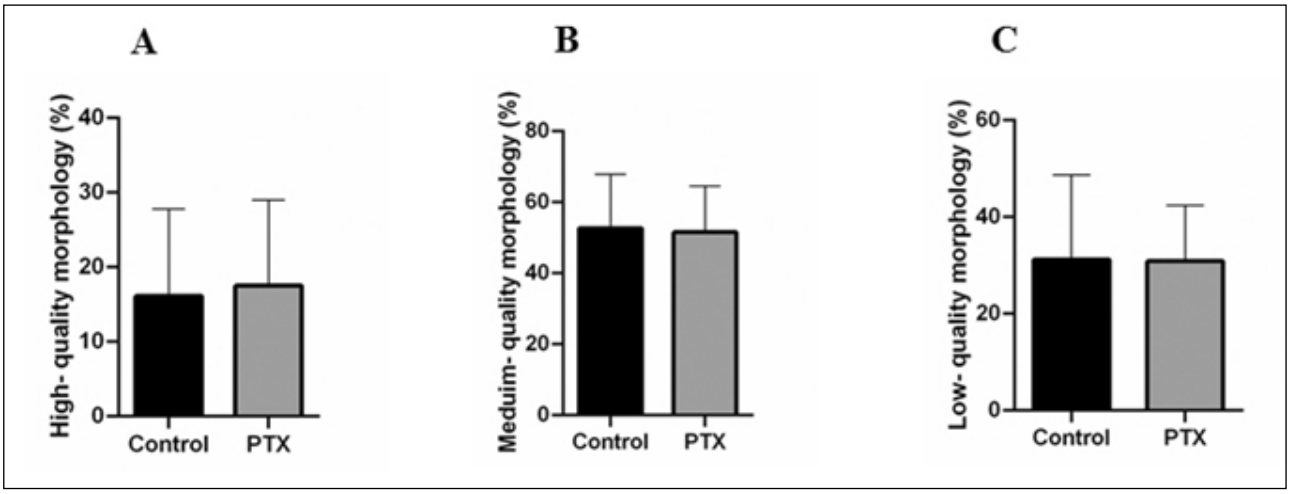


Figure 6.
DNA fragmentation index (DFI) (A) and mitochondrial membrane potential (MMP) (B) after incubation with PTX before single sperm cryopreservation (Phase 1). Data are presented as mean ± SD and analysis by Independent Samples T Test.

In phase 2, spermatozoa with active mitochondrial membrane potential were similar in the two cryopreservation groups (55.30 ± 6.73 *vs.* 55.40 ± 5.81) (Figure 6B).

Post-warmed fine sperm morphology
In phase 2, high (17.50 ± 11.50 *vs.*16.10 ± 11.68), medium (51.60 ± 12.86 *vs.* 52.70 ± 15.16) and low (30.90 ± 11.51 *vs.*31.20 ± 17.47) quality testicular spermatozoa rates were similar between PTX and control groups (Figure 7A-C).

Figure 7.
Fine sperm morphology. Morphology of sperm classified in three groups high (A), medium (B) and low (C) quality incubated in PTX after single sperm cryopreservation (phase 2).
Data are presented as mean ± SD and analysis by Independent Samples T Test.



Discussion

The cryopreservation of sperm with SSC technique is beneficial to reduce the times of sperm retrieval techniques by freezing spermatozoa (without testicular tissues, debris and round cells) on a significant number of Cryotop. Therefore, using this method increased the oocyte retrieval times in these groups of patients. We previously reported that SSC using CVD (14) and sucrose medium (15) was a suitable strategy for cryopreservation of testicular sperm.

TESE or micro-TESE samples may have 10 or more than 100 spermatozoa, which it is not practical to place all together on a Cryotop. In this regard, viable spermatozoa with good morphology should be selected. Selecting viable spermatozoa based on sperm tail flexibility depends on skill and experience of the embryologist. Using safe chemical substance like PTX could resolve the weakness of sperm tail flexibility method (19). Although some studies evaluated the safety of PTX on cryopreserved testicular sperms after thawing in conventional freezing method, it is not clear whether testicular sperm exposure to PTX before cryopreservation make it more vulnerable to cryo-damage. *Mahaldashtian et al.* concluded that PTX exerts beneficial effects on post-thawed sperm motility and increased 2PN and embryo formation without detrimental impacts on sperm DNA integrity (16). *Xian et al.* supplemented the sperm freezing media with PTX for assessing testicular sperm motility during cryopreservation and warming procedures and reported that PTX enhances testicular sperm motility (20).

The recovery rate, number of spermatozoa that were retrieved after freezing, is an essential factor that can show the efficacy of cryo-devices. Our recovery rate was about 87% which is in accordance with other researches that applied similar cryo-devices (21, 22). Also, the viability rate after SSC in both phases was about 60% in agreement with our previous report (15). In both phases of this study, at first, tail movement and flexibility method was applied to select viable spermatozoa. The HOS test showed that about 98% of these spermatozoa were viable. This indicated that, if these methods are used properly, a significant number of viable spermatozoa could be selected. Moreover, we showed that the incubation of testicular sperm, before and after SSC, with PTX enhances sperm motility, although no significant difference was observed between groups in sperm viability. The similarity of post-warming viability between groups showed that PTX does not exert detrimental effects on sperm in cryo-warmed process. In phase two, 26% of spermatozoa were motile after warming in PTX, while the viability rate was more than 60%. Probably the cryo-damage to spermatozoa caused that most of viable sperm cannot be motile after PTX exposure. Therefore, using PTX after SSC when the numbers of spermatozoa are limited is not practical for selecting viable spermatozoa.

Exposure of testicular spermatozoa to PTX before and after SSC did not show adverse effect on fine morphology. *Nabi et al.* incubated ejaculated spermatozoa samples with 3.6 mmol/L PTX for 30 min at 37ºC after vitrification warming and showed that PTX did not alter ultrastructural aspects of spermatozoa (23). In another study, *Mahaldashtian et al.* reported that treatment of oligoasthenoteratozoospermic samples with PTX did not change the vacuole status of sperm head (24).

Sperm chromatin integrity and structure are a determining factor in fertility potential. It is vital to inseminate the oocytes with spermatozoa that have intact DNA. Our results showed that PTX did not alter DNA integrity of testicular samples when applied before and after freezing between groups. This finding is in agreement with other studies that showed PTX does not have harmful impacts on DNA/chromatin status in TESE (16) and asthenozoospermic cases (25, 26).

MMP is related to normal sperm parameters including motility and viability. Mitochondria are the main source of adenosine triphosphate generation that is vital for sperm motility and cellular phenomena including capacitation, hyper activation and acrosome reaction (27). According to our results, MMP was similar between PTX exposed and non-exposed group before and after SSC. Previously it was

reported that PTX did not exert any detrimental effect on sperm MMP (24).

Conclusions

Adding PTX at safe concentration as sperm selection method before SSC of testicular samples, could select the viable sperm without intensifying the adverse effects of cryo-damage. However, it seems that using PTX after warming, when numbers of spermatozoa are limited, is not practical for selecting viable spermatozoa, because the most of viable sperm cannot be motile after PTX exposure during warming procedure.

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