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Pentoxifylline treatment had no detrimental effect on sperm DNA integrity and clinical characteristics in cases with non-obstructive azoospermia

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Research Article

Cite this article: Mahaldashtian M *et al*. (2023) Pentoxifylline treatment had no detrimental effect on sperm DNA integrity and clinical characteristics in cases with non-obstructive azoospermia. *Zygote*. **31**: 8–13. doi: 10.1017/S0967199422000247

Received: 2 February 2022
Revised: 31 March 2022
Accepted: 1 May 2022
First published online: 19 October 2022

Keywords:
Chemical pregnancy; Cryopreservation; DNA fragmentation; Non-obstructive azoospermia; Pentoxifylline

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Summary

The aim of this study was to assess the consequences of treatment with pentoxifylline (PTX), an inducer of sperm motility, on sperm DNA fragmentation (SDF) and clinical characteristics in non-obstructive azoospermia (NOA) patients. The pilot study included 15 NOA patients. Half of each sperm sample before and after rapid freezing, was treated with PTX (3.6 mM /l, 30 min) as the PTX group and the remaining samples were considered as the control. SDF and sperm motility were assessed in each group. The clinical study comprised 30 fresh testicular sperm extractions (TESE) and 22 post-thawed TESE intracytoplasmic sperm injection cycles. Half of the mature oocytes from each patient were injected with PTX-treated spermatozoa and the remaining oocytes were injected with non-treated spermatozoa. Fertilization was assessed at 16 h post injection. Embryo transfer was carried out on day 2 after fertilization. Chemical pregnancy was assessed 2 weeks after transfer. PTX was found to significantly increase ($P < 0.05$) sperm motility. There was an insignificant difference in SDF rates between the groups ($P > 0.05$). In patient ovaries given fresh TESE, there was not any significant difference in clinical characteristics ($P > 0.05$). In patient ovaries given post-thawed TESE, there was a significant difference in the number of 2PN and in embryo formation ($P < 0.05$). Differences in the results of chemical pregnancy were insignificant ($P > 0.05$) between the groups. In addition, there was not any correlation between DNA fragmentation index and sperm motility and laboratory outcomes. Therefore, obtaining viable spermatozoa using PTX was more effective in post-thawed TESE regime patients in terms of 2PN and in embryo formation, deprived of damaging effects on sperm DNA integrity.

Introduction

Almost 1% of all men and 10–15% of infertile males are azoospermic, which is indicated by the lack of sperm cells in ejaculate (Cocuzza *et al*., 2013; Cetinkaya *et al*., 2015). In addition, ~60% of azoospermia cases are the result of non-obstructive azoospermia (NOA), which is triggered by intense damage during spermatogenesis (Modarresi *et al*., 2015). Sperm cells that are recovered from testicular samples usually have little or no motility (Dutra *et al*., 2018). Injection of an immotile spermatozoa into the oocyte negatively affects the intracytoplasmic sperm injection (ICSI) outcome (Mahaldashtian *et al*., 2021). Therefore, it is essential to find a method for selection of viable spermatozoa (Dutra *et al*., 2018). Alternatively, testicular biopsy and cryopreservation of spermatozoa for subsequent use can be considered in azoospermia patients; therefore, re-biopsy and ovulation induction are no longer necessary (Griveau *et al*., 2006). Although the consequences of freezing procedure on motility, morphology and viability of spermatozoa are recognized, the consequences of any probable change in the spermatozoa genome after cryopreservation are still controversial (Rahiminia *et al*., 2017).

At present, there has been no strong validation of whether the freezing process induces sperm DNA damage (Rahiminia *et al*., 2017; Le *et al*., 2019). The freezing process, especially for NOA, has led to complete immobility of the sperm cells (Griveau *et al*., 2006). The selection of living spermatozoa for ICSI from frozen–thawed samples of NOA patients is very time consuming, difficult and sometimes impossible (Griveau *et al*., 2006). Pentoxifylline (PTX), a phosphodiesterase inhibitor, increases c-AMP levels and protein kinase A activity, and can increase sperm motility (Manetta, 1998). Nevertheless, a concern has been voiced by some investigators to the possible effects on the genome (Meseguer *et al*., 2009; Asokan *et al*., 2015) and clinical (Mahaldashtian *et al*., 2021) damage induced by PTX.

Another concerning issue in male-assisted reproduction is fragmentation of the sperm chromatin, which may have a harmful effect on fertility rates (Muriel *et al*., 2006;

Meseguer *et al.*, 2009). Designation of sperm DNA fragmentation (SDF) may be a special option for male infertility (Nabi *et al.*, 2021). SDF seems to be associated with the capacity of sperm to fertilize an oocyte (Muriel *et al.*, 2006). One reliable test for assessment of DNA fragmentation is the sperm chromatin dispersion (SCD) test. In this test, spermatozoa with fragmented DNA are unable to form a 'halo' (dispersed DNA loops), whereas halos are formed in spermatozoa with intact DNA (Setti *et al.*, 2021b). The evaluation of DNA fragmentation via the SCD test is a good predictor of embryo development and clinical outcome (Tandara *et al.*, 2014). As mentioned previously, the effectiveness of PTX on DNA fragmentation and clinical outcomes is doubtful (Mahaldashtian *et al.*, 2021). Some research has shown that PTX could improve sperm motility without any adverse effects on genome integrity (Asokan *et al.*, 2015; Salian *et al.*, 2019) and clinical outcomes (Amer *et al.*, 2013; Navas *et al.*, 2017). To our knowledge, there has been only one study that applied SCD analysis to the assessment of the effect of PTX on SDF in azoospermia cases (Meseguer *et al.*, 2009). The aim of present study was two-fold: (1) Pilot study: Evaluation of the effects of PTX treatment on sperm motility and DNA fragmentation in both fresh and post-thawed testicular samples. (2) Clinical study: Assessment of the role of PTX in clinical outcomes of both fresh and post-thawed ICSI cycles of NOA patients.

Materials and methods

Study population

The pilot section of this prospective study included 15 NOA patients with an average age of 29.60 ± 2.13 years. Each sample was divided into fresh and frozen groups. In each group, half of the sample was treated with PTX and the remaining half was considered as the control (Figure 1). The clinical phase of the study included 52 patients and included fresh TESE ($N = 30$) and post-thawed TESE ($N = 22$) groups. The mean ages of men and women participants and duration of infertility were 36.17 ± 6.54, 31.34 ± 5.10 and 6.75 ± 3.98 ($N = 52$), respectively. The mean levels of anti-mullerian hormone (AMH) and luteinising hormone (LH) were 4.17 ± 2.94 ($N = 47$) and 3.490 ± 4.62 ($N = 31$) pg/ml respectively. The oestradiol levels before hCG injection were 1771.14 ± 1453.49 ($N = 47$) pg/ml. Metaphase II (MII) oocytes from each patient were divided and half of the oocytes were injected with non-treated sperm cells (control group) and the rest of oocytes were injected with PTX-treated sperm cells.

All patients had been referred to the Yazd Reproductive Institute for infertility treatment from November 2020 to August 2021. Inclusion criteria were as follows: male and female ages less than 50 and 40 years, respectively. The cases had at least 1 million spermatozoa in their samples, no history of varicocele or diabetes or tobacco addiction. Exclusion criteria were cases with no motile spermatozoa. The diagnosis of azoospermia was confirmed using the centrifugation of a semen sample for 15 min at 3000 *g* followed by high-powered examination of the pellet (Cocuzza *et al.*, 2013). This research was approved by the Scientific and Ethics Committee of our institution (IR.SSU.RSI.REC.1398.013). All patients signed informed consent to participate in the research.

Ovarian stimulation and ICSI

Ovarian stimulation was carried out using a GnRH agonist or antagonist co-treatment procedure with recombinant urinary–follicle stimulating hormone (Gonal-FSH, Serono, Geneva, Switzerland). Human recombinant chorionic gonadotropin (hCG) (Pregnyl®; Organon, Oss, the Netherlands) was injected when at least three follicles were ≥17 mm diameter. Ovum pickup was performed 38 h post hCG trigger (Mangoli *et al.*, 2020).

Laboratory handling of testicular specimens

Testicular tissue was rinsed in a Petri dish containing 1–2 ml of Ham's F10 medium (Biochrome, Berlin, Germany) supplemented with 5 mg/ml human serum albumin (HSA; Vitrolife, Englewood, CO, USA) and finely crushed and dissected mechanically using two sterile slides. Afterwards, the presence of sperm cells was evaluated at ×400 magnification under an inverted microscope (Diaphot, Nikon Corporation, Tokyo, Japan). Cell suspensions were aspirated and transferred to a sterile centrifuge tube and diluted with 3 ml of fresh culture medium and centrifuged at 300 *g* for 10 min. The supernatant was discharged and the pellet was gently resuspended in 0.5–1.0 ml culture medium (Esteves and Schneider, 2011).

Cryopreservation and warming procedure

Cryopreservation and warming were performed as described previously (Desai and Rambhia, 2017). In brief, the testicular sample was diluted with an equal amount of cryopreservation medium (Sperm Freezing Medium, ORIGIO, USA), mixed thoroughly, and allowed to equilibrate at room temperature (RT) for 10 min. The specimen was distributed among prelabelled cryovials (Cryo Bio System, Saint-Ouensur-Iton, France). Then, each vial was placed in a horizontal position 4–5 cm above the surface of the liquid nitrogen. After 1 h, the vial was immersed in liquid nitrogen (–196°C) and secured in a cryocane for storage. For warming, the sample was held at RT for 2–3 min and the cap was loosened to release any gas pressure from trapped liquid nitrogen. Then, the sample was placed in a warmer at 37°C for 20 min until it was completely thawed. After that, 0.5–1.0 ml of culture medium was slowly added to the sample, which was centrifuged at 300 *g* for 5 min. Following removal of the supernatant, the sperm pellet was resuspended in fresh medium.

Treatment of spermatozoa with PTX

The testicular cell suspension was added to the droplets of culture medium containing 1 mg/ml of PTX (Sigma, St. Louis, MO, USA) under paraffin oil (Kovacic *et al.*, 2006) at the ratio of 1:1. So, the final concentration of PTX in the sample was 1.76 mM. Droplets were incubated at 37°C for 30 min. Then, the sample was examined for identification and isolation of viable motile spermatozoa.

Assessment of sperm motility

The droplets were observed under an inverted microscope for assessment of sperm motility. Some spermatozoa displayed twitching motility, whereas some sperm cells acquired an adequate grade of motility to reach the periphery of the droplet (Mangoli *et al.*, 2011).

Sperm DNA fragmentation test

Sperm DNA fragmentation was assessed using the SDFA kit (Tehran, Iran) based on a previous study (Anbari *et al.*, 2020). In brief, low-melting-point agarose gel was placed at 90–100°C for 5 min and transferred to an incubator at 37°C for equilibration.

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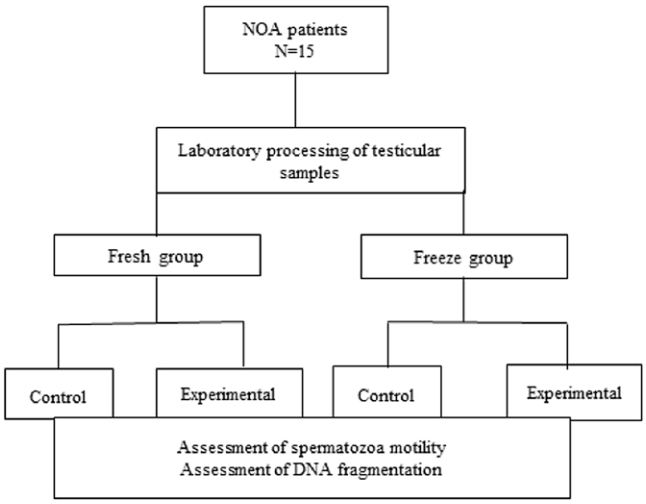

Figure 1. Diagram of pilot study design.

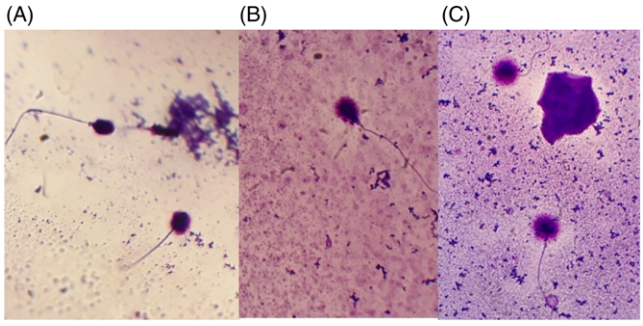

Figure 2. Sperm DFI detected in NOA sperm cells. (A) Spermatozoa without halo and small halo. (B) Spermatozoa with medium-sized halo. (C) Spermatozoa with large halo assigned as without fragmentation.

In each droplet of testicular tissue 10–15 motile sperm cells were selected. Next, 5 µl of low-melting-point agarose gel was deposited onto the pre-coated slide. Immediately, selected motile sperm cells were gently released onto the agarose gel. Slides were placed on a cold plate in the refrigerator (4°C) for 5 min. Then, the slides were exposed to acid solution and lysing solution for 15 and 25 min, respectably. After rehydration, the slides were washed and allowed to dry and were evaluated under a bright field microscope. Depending on the halo size, the sperm cells were categorized as no halo, small (halo width is slighter than one-third of the minor diameter of the core), medium (halo size is between large and small halos), and large (halo width is similar to or larger than the minor diameter of the core) halos by observing under a light microscope (Meseguer *et al.*, 2009). Spermatozoa with no or small halos were considered to contain DNA damage and results were expressed as a percentage (Figure 2).

ICSI procedure, embryo culture, selection, and transfer

After washing selected spermatozoa in droplets of HEPES buffer medium, the sperm were moved in a central droplet of polyvinyl pyrrolidone (PVP) solution. A single spermatozoon was aggressively immobilized and then injected using a microinjection pipette into the cytoplasm of an MII oocyte. Poor quality oocytes such as large polar body oocytes and those containing smooth endoplasmic reticulum clusters and large vacuoles (≥14 µm) within the ooplasm were discarded. Injected oocytes were cultured in SAGE 1-Step medium (Origio/Cooper Surgical) enclosed within mineral oil at 36.8°C, 7% O_2 and 6% CO_2 (Mangoli *et al.*, 2019). Fertilization was evaluated 16 h post ICSI. Embryo selection was performed according to Hill categories: grade A, embryo with even blastomere size and no fragmentation; grade B, embryo with slightly uneven size blastomere and up to 10% fragmentation; grade C, embryo with uneven blastomere size and 10–50% cytoplasmic fragmentation; and grade D, blastomere with significantly uneven size, large black granules and more than 50% fragmentation. Grades A and B are considered as good quality, and C and D as poor quality embryos (Hill *et al.*, 1989). Embryo transfer was carried out 2 d post fertilization and chemical pregnancy (positive β-hCG in the serum/transfer) was detected 2 weeks after transfer.

Statistical analysis

Assessment of data distribution was implemented using the Kolmogorov–Smirnov test. Pilot study design considered the dependencies between the groups. The DNA fragmentation index (DFI) was analyzed by repeated measurements test and the Mauchly's sphericity test was used to validate a repeated measures analysis of variance. Sperm motility was analyzed using the Friedman test. In the clinical phase of the study, quantitative variables were compared between the groups using the Mann–Whitney test. Assessment of dichotomous parameters between the groups was accomplished by means of Pearson's chi-squared (χ^2) test, as well as Fisher's exact test. Statistical analysis was performed using the Statistical Package for the Social Sciences 20 (SPSS Inc., Chicago, IL, USA) with significance level at $P < 0.05$. Correlations were examined using Pearson's test. The results are presented as mean ± standard deviation (SD).

Results

Pilot study

The mean sperm motilities before PTX treatment were 1.13 ± 2.99 and 0.33 ± 0.89 in fresh and frozen groups, respectively. After PTX treatment, the rates of motile spermatozoa in both fresh sperm (21.06 ± 7.92) and frozen sperm (11.46 ± 4.96) groups were significantly increased ($P < 0.05$) (Figure 3). There were no significant differences between the rates of motile spermatozoa in the fresh control and frozen control groups ($P = 1.00$), also between the fresh PTX and frozen PTX ($P = 0.28$) groups. Also, Mauchly's sphericity test for DFI data gave a value of 0.574 ($P = 0.217$). According to the sphericality assumed test, there were no significant differences between the rates of DNA fragmentation in fresh and frozen samples in both control and PTX-treated groups ($P = 0.291$) (Figure 3). In addition, sperm DFI was not correlated with sperm motility ($P > 0.05$) (Pearson correlation: 0.01, $P = 0.9$).

Clinical phase

In both the fresh and post-thawed patient groups, there were no significant differences in DNA fragmentation between the control and the PTX groups ($P > 0.05$) (Table 1). As shown in Table 1, in both classes of patients, there was an insignificant difference in the number of oocytes injected between control and PTX groups. Overall, in the fresh TESE groups, there was no significant difference in the mean number of 2PN and embryo formation, total 2PN

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Table 1. Results of the Mann–Whitney test comparing DFI and clinical characteristics of embryos resulting from ICSI with non-treated and PTX-treated sperm cells

Groups		DFI	Oocyte injected	2PN formation	Embryo formation
Fresh control	Mean ± SD	39.75 ± 17.44	5.20 ± 8.85	2.80 ± 2.10	2.53 ± 1.87
	N	30	30	30	30
Fresh PTX	Mean ± SD	39.93 ± 17.05	5.03 ± 3.71	3.30 ± 1.93	3.06 ± 1.85
	N	30	30	30	30
P-value		0.965	0.868	0.238	0.201
Post-thawed control	Mean ± SD	44.59 ± 12.05	3.50 ± 1.01	1.72 ± 0.984	1.40 ± 0.959
	N	22	22	22	22
Post-thawed PTX	Mean ± SD	44.34 ± 11.71	3.77 ± 1.50	2.45 ± 1.01	2.27 ± 1.12
	N	22	22	22	22
P-value		0.888	0.824	0.041[a]	0.016[a]

[a]Significance between groups ($P < 0.05$).

Table 2. Results of the chi-squared test comparing pregnancy outcome of all transferred embryos resulting from ICSI with non-treated and PTX-treated sperm cells

Groups		Total 2PN arrest	Embryo transferred	Chemical pregnancy rate
Fresh control	*n* (%)	2 (6.7%)	11 (39.3%)	1 (9.1%)
	N	30	28	11
Fresh PTX	*n* (%)	0 (0.0%)	12 (40.0%)	2 (17.7%)
	N	30	30	12
P-value		0.492[b]	0.956[a]	1.00[b]
Post-thawed control	*n* (%)	4 (18.2%)	8 (44.4%)	2 (25%)
	N	22	18	8
Post-thawed PTX	*n* (%)	0 (0.0%)	12 (54.5%)	5 (41.7%)
	N	22	22	12
P-value		0.108[b]	0.525[a]	0.642[b]

[a]Pearson chi-squared test.
[b]Fisher's exact test ($P < 0.05$).

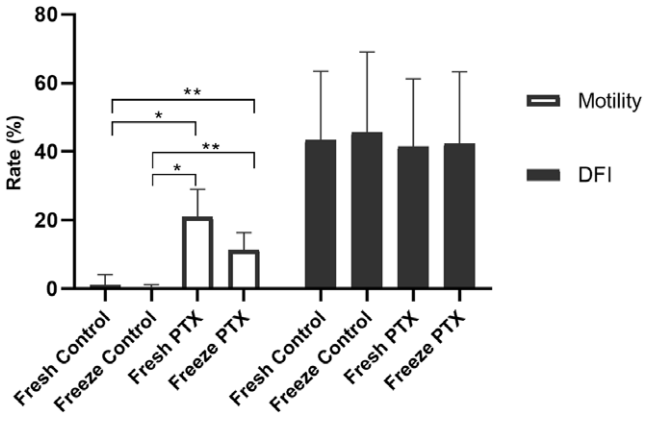

Figure 3. Rates of sperm motility based on the Friedman test, there was a significant difference between groups ($P < 0.05$). Also, repeated measurements analysis of the DNA fragmentation, showing no significant differences between all groups ($P > 0.05$). Data are shown as bar graphs representing mean ± SD. Graphs were plotted using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA). Significance is shown as $^{*}P < 0.001$ and $^{**}P < 0.05$.

arrest and chemical pregnancy outcome of ICSI cycles between groups ($P > 0.05$). However, in the post-thawed TESE patients, there were significant differences in the number of 2PN and in embryo formation between groups ($P < 0.05$). Nevertheless, there was no significant differences in total 2PN arrest and chemical pregnancy of ICSI cycles ($P > 0.05$) (Table 2). Furthermore, there was no correlation between DFI and the number of 2PN and embryo formation, 2PN arrest, as well as chemical pregnancy.

Discussion

The findings showed that a 30-min incubation of testicular samples with PTX significantly increased sperm motility in both fresh and post-thawed samples without any detrimental effect on SDF, which agreed with findings of a previous study (Meseguer *et al.*, 2009). Cryopreservation of testicular samples has many difficulties due to the very low concentration and motility of the sperm cells (Griveau *et al.*, 2006). Furthermore, the success rate of this procedure has been limited because sperm freezing and thawing affect the vital parameters of spermatozoa (Nabi *et al.*, 2017). Motility is

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one of the parameters that is extremely affected by cryopreservation. Reduction of sperm motility may be due to osmotic stress, intracellular ice crystal creation (Nabi *et al.*, 2017) and damage to the mitochondrial membrane (Le *et al.*, 2019). Application of PTX post freezing has been shown to recover the post-thaw motion of testicular specimens (Griveau *et al.*, 2006; Xian *et al.*, 2022), which is consistent with our findings. The mechanism of PTX on sperm movement has not been fully recognized, but it appears that PTX increases the action of creatine kinase (Mahaldashtian *et al.*, 2021).

Conversely, the integrity of sperm chromatin is a valuable index of fertility potential (Zini *et al.*, 2008). The SCD test provides a prognostic assessment for SDF in addition to terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and sperm chromatin structure assay (SCSA) (Chohan *et al.*, 2006). Whereas TUNEL measures DNA damage, the SCD test measures damage after denaturation (Panner Selvam and Agarwal, 2018). Our results indicated that PTX did not inflict any harmful effects on DNA fragmentation, which agreed with the findings in recent studies, indicating that PTX had no adverse effect on sperm chromatin/DNA fragmentation in TESA (Dutra *et al.*, 2018) and asthenozoospermic (Asokan *et al.*, 2015; Nabi *et al.*, 2017) patients. This may be due to the antioxidant characteristics of PTX (Chehab *et al.*, 2015) that, by preventing xanthine oxidase, decrease the levels of intracellular ROS formed by defective spermatozoa (McKinney *et al.*, 1996). As mentioned previously, the consequences of cryopreservation on DNA fragmentation are still controversial (Di Santo *et al.*, 2012; Le *et al.*, 2019). After the freezing process, DFI increased in both control and PTX groups, but this increase was not significant. These results were similar to studies that indicated that cryopreservation does not impair the sperm genomic structure (Duru *et al.*, 2001; Rahiminia *et al.*, 2017). Contradictory statements may be the result of different freezing methods and different tests used to assess DNA fragmentation (Di Santo *et al.*, 2012). Recently, Rahimnia and colleagues compared different methods of cryopreservation in normal samples. Their data indicated that the vapour phase freezing method did not impair the sperm genome (Rahiminia *et al.*, 2017). Spermatozoa have a high compartmentation structure, which results in a limited separation of proteins (Engel *et al.*, 2018). Furthermore, DNA in sperm cells is mostly protamine bound (Engel *et al.*, 2018), which could avoid DNA fragmentation to some degree.

When comparing clinical characteristics, a better outcome was surprisingly obtained in the post-thawed TESE group in terms of number of 2PN and embryo formation. We hypothesized that PTX can compensate for the detrimental effects of cryopreservation on spermatozoa. Use of frozen–thawed TESE specimens is usual in ART clinics, as it avoids multiple testicular surgeries (Griveau *et al.*, 2006). However, the success rate of this procedure has been limited, as freezing and thawing expose the spermatozoa to various stresses that could lead to the loss of fertilization potential (Setti *et al.*, 2021a). Griveau and co-workers reported that the 'use of PTX in frozen–thawed testicular spermatozoa produces the same outcome after ICSI as that observed with fresh ejaculated spermatozoa'. This enhancement may be due to the selection of viable sperm cells (Griveau *et al.*, 2006). Also, addition of PTX has led to improved spermatozoa–zona pellucida (ZP) binding and enhanced the acrosome reaction (Mehta and Sigman, 2014).

However, Dutra and colleagues (2018) reported that there was no correlation between spermatozoa movement and DNA fragmentation from TESA samples (Dutra *et al.*, 2018). Some studies have reported that fragmentation in sperm chromatin is repaired to some extent by the oocyte, but negatively affects the clinical outcomes (Ferrigno *et al.*, 2021). Nevertheless, there was no correlation between DFI and clinical characteristics and chemical pregnancy in our work. Our finding is in accordance with the Setti and colleagues (2021b) study that stated that when spermatozoa were injected into the oocytes of women less than 40 years old, there were insignificant differences in the laboratory and clinical results for cycles with SDF <30% or SDF ≥30%.

In conclusion, PTX enhanced sperm motility with no harmful effects on spermatozoa DNA integrity and ICSI outcomes. Therefore, it is recommended that the use of PTX is considered a safe method for sperm selection in NOA patients, especially in post-thawed ICSI cycles. This strategy provides an opportunity for patients to have their own children.

Acknowledgements. The authors thank the personnel of the andrology, research and ART laboratories at the Yazd Reproductive Sciences Institute for their excellent assistance throughout this research.

Conflict of interest. The authors state that no conflict of interest exists in this research.

Funding information. This study was supported by funding from Shahid Sadoughi University of Medical Sciences.

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https://doi.org/10.1017/S0967199422000247 Published online by Cambridge University Press