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Pentoxifylline increase sperm motility in devitrified spermatozoa from asthenozoospermic patient without damage chromatin and DNA integrity

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ABSTRACT

The freeze–thaw process results in reduced motility, viability and fertilization potential of human spermatozoa. So, a variety of substances were evaluated in order to enhance human sperm resistance to the stress of cryopreservation, such as Pentoxifylline (PTX) for improving the Intracytoplasmic sperm injection (ICSI) outcomes. The aim was to investigate the effect of PTX on sperm parameters and chromatin/DNA integrity of asthenozoospermic semen post vitrification. A total of 30 semen specimens were obtained from infertile men with asthenozoospermia. The cryoprotectant-free vitrification was performed for the samples after assessment of sperm parameters. After warming, each sample was exposed for 30 min to 3.6 mmol/l PTX in experimental group and the control group without any treatment apposing at 37 °C for 30 min in regard, to repeat all in vitro analysis (sperm parameters and DNA integrity assay). Regardless of the vitrification devastating impacts on sperm parameters, incubation of post vitrified samples with PTX increased the rate of progressive motility ($P < 0.01$). Moreover, PTX addition did not significantly damage DNA integrity of asthenozoospermic sperm samples. The data showed that PTX was able to improve sperm movement without any adverse effects on sperm chromatin/DNA integrity in vitrification program.

1. Introduction

Asthenozoospermia is characterized by reduced or absent of sperm motility in the ejaculates. There were several reasons for asthenozoospermia, such as metabolic deficiencies, ultrastructural abnormalities of the sperm flagellum and necrozoospermia [35]. In assisted reproduction technology (ART), sperm cryopreservation has become an important issue. However, the success rate of this technique has been limited when using cryopreserved spermatozoa. The freeze–thaw process results in reduced motility, viability and fertilization potential of human spermatozoa [8]. Many studies indicated that sperm freezing and thawing not only alters sperm parameters, but also causes an increase in sperm DNA damage [5,20,36,41,46,58] that may be related to cold shock, osmotic stress and intracellular ice crystal formation [53].

Some investigations evaluated variety of substances in order to enhance human sperm resistance to the stress of cryopreservation thawing [11–13]. Pentoxifyllin (PTX) as a methylxanthine derivate has been considered as sperm movement enhancer [41], hyperactivation agent [23,47], scavenger of reactive oxygen species (ROS) [57] and acrosome reaction-improving agent [54]. PTX is a cyclic adenosine monophosphate (cAMP) phosphodiesterase enzyme inhibitor that prevents the normal functions of cAMP on human sperm respiration, motility the regulation of the acrosome reaction by increasing the intracellular cAMP concentration [40,42,50]. There were several studies in fresh and frozen-thawed semen [33,47,50] with the main focuses on evaluating the beneficial effects of PTX on progressive motility. The findings of in vivo studies demonstrated higher rats of pregnancy [32]. However, some in vitro studies demonstrated no differences on motility, longevity and acrosome integrity during incubation of frozen-thawed semen with autologous prostatic fluid [39]. Milani et al. (2010) indicated that PTX may be useful for enhancing motility of canine frozen-thawed spermatozoa without affecting sperm longevity [29]

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Other showed that PTX significantly improved semen parameters in cases with oligoasthenoteratozoospermia (OAT) or with a reduced risk of failed fertilization cycles utilizing oligo/asthenozoospermic samples [55]. Furthermore, effect of PTX on sperm motility and fertilization capacity has been approved in asthenozoospermia [17]. But, there is no comprehensive assessment for chromatin/DNA integrity of human spermatozoa after vitrification during PTX administration. Since, sperm DNA chromatin integrity plays an important role on human fertilization; sperm DNA damage may have a great impact on outcome of assisted reproduction [21,25,30,45]. So, the aim of the present study was to investigate the effect of in-vitro application of PTX on the sperm parameters, fine morphology and chromatin/DNA integrity of asthenozoospermic samples post vitrification. To the best our knowledge, this is the first study to evaluate the DNA integrity and chromatin status of post warm asthenozoospermic sperm using different cytochemical techniques following PTX treatment.

2. Materials and methods

2.1. Experimental design

Thirty asthenozoospermic specimens were obtained from men (aged 25–45 years) referred to Yazd Research and Clinical Center for Infertility. The exclusion criteria included smoking and varicocoel disorder. Samples selection were based on sperm total motility of <32% according to WHO guidelines [34]. Informed consents were obtained from patients, and the study was approved by author's institute ethics committee (IR.SSU.RSI.REC.1394.14). All seminal samples after collection were prepared using density gradient method [32]. Assessments of sperm parameters were carried out before and after the vitrification. After the warming and administration of PTX and without PTX treatment, final analysis of sperm parameters and chromatin/DNA integrity were done (Fig. 1).

2.2. Collection of sperm samples and analysis

Semen analysis was carried out according to WHO guidelines [34] after liquefaction at 37 °C. Sperm count and motility were assessed by Mackler chamber, and motility was categorized as percentages of progressive, nonprogressive and immotile spermatozoa [30]. Eosin-Nigrosin and Papanicolaou staining tests were done for sperm viability and morphology evaluation, respectively [33].

2.3. Vitrification and warming of sperm

Vitrification (close system) and warming of sperm samples were performed at room temperature (RT) using straw to straw method [20] and modified in some aspect. Briefly, the suspension was mixed with Ham's F10 medium supplemented with 5% HSA/0.5 mol/l sucrose (1:1) at RT. 100 μl of the sperm suspension was transferred on a 0.25 ml sterile straw maintained horizontally. Then, it was placed into 0.5 ml straws and sealed at the end, and straws were immersed directly into liquid nitrogen and stored for one week.

For warming, one end of the straw was cut and the samples were vacated in pre-warmed Ham's F10 with 5% HSA. The simple-wash method was used at post-warming and sperm parameters were evaluated for second time according to WHO criteria [34]. Moreover, the survival rate, morphology and sperm parameter were done immediately after washing process.

2.4. Pentoxifylline administration

A stock solution of PTX (Sigma, USA) in distilled water was prepared each week and kept at 4 °C until use. The samples were divided into two groups of control and experiment. In experimental group, each sample was exposed to 3.6 mmol/l PTX for 30 min and the control group without any treatment apposing at 37 °C in incubator for 30 min. After incubation, the samples were washed utilizing simple wash method and analyzed again (Fig. 1) [24].

2.5. TUNEL assay

The percentage of apoptotic spermatozoa in each sample was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay by In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) using fluorescent microscopy (BX51, Olympus, Tokyo, Japan) [26]. Briefly, the slides were fixed with 4% paraformaldehyde for 1 h at RT, and then they were washed three times with PBS, before treating with 3% H2O2 in methanol. In the next step, they were immersed in 0.1% triton X-100 in 0.1% sodium citrate for 5 min. After rinsing with PBS, the slides were treated with 5 μl enzyme solution plus 45 μl label solution and incubated for 1 h [26]. The apoptotic cells with DNA fragmentation exhibited intensive and brilliant florescent green (TUNEL + as apoptotic sperm); while, the normal cells displayed pale and opaque green (TUNEL-as non-apoptotic sperm). The apoptotic sperm cells were presented as percentage in each sample [27].

2.6. CMA3 staining

Regarding CMA3 staining for evaluation of the degree of protamination of sperm chromatin [18], smears were dried and fixed in Carnoys solution (methanol/glacial acetic acid, 3:1) at 4 °C for 10 min. Each slide was stained with 100 μl of CMA3 (Sigma, St Louis, MO, USA) (0.25 mg/ml in McIlvaine buffer; 7 ml citric acid, 0.1 M + 32.9 ml Na2HPO4 7H2O 0.2 M, pH 7.0 containing 10 mM MgCl2) for 20 min. After washing in buffer and mounting with buffered glycerol (1:1), at least 200 spermatozoa were counted under florescent microscopy with a 460-nm filter. The spermatozoa with bright yellow heads (CMA3 +) and without brightness (CMA3 -) were determined as percentage results [2].

2.7. Aniline blue (AB) staining

Fresh sperm smear of each case was air dried and fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at RT. Each smear was treated with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min. At least 200 sperm cells were evaluated in each slide by light microscopy (Olympus Co., Tokyo, Japan) and unstained or pale blue stained cells and dark blue cells were considered as normal (AB-) and abnormal spermatozoa (AB+), respectively [56].

2.8. Toluidine blue (TB) staining

TB stain binds to phosphate groups of DNA strands and reveal the chromatin condensation status [38]. Pale blue sperm cells were considered as normal and dark blue or violet purple spermatozoa were categorized as abnormal cells. At least 200 spermatozoa were checked for each slide and the normal (TB-) and abnormal (TB+) sperm cells were reported as percentage [44].

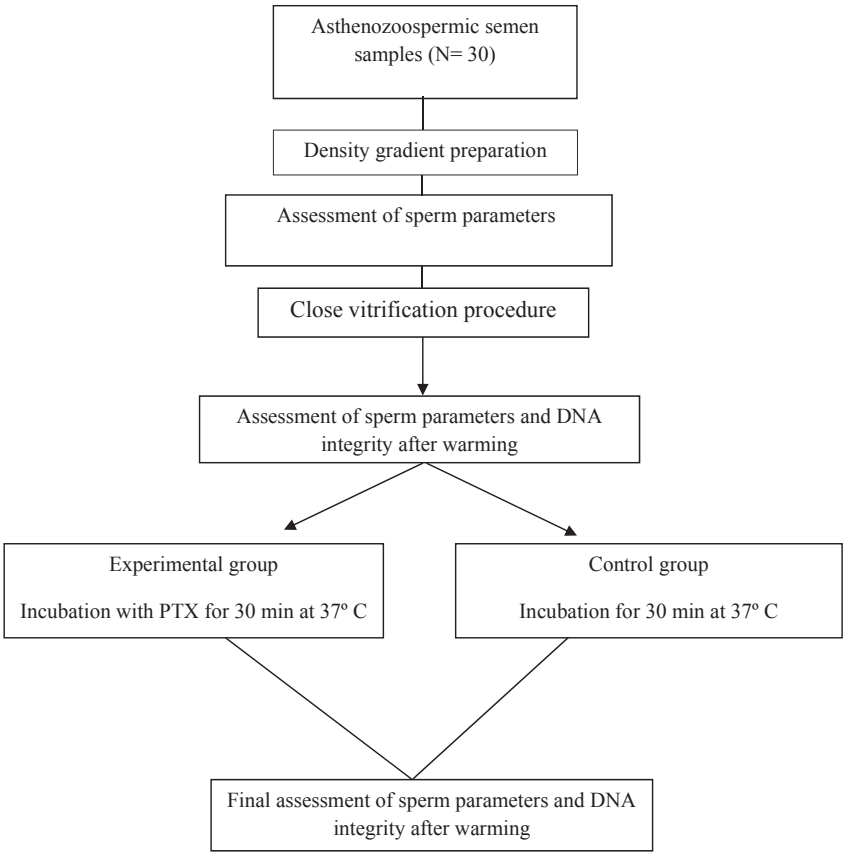

Fig. 1. Study design flowchart.

2.9. Sperm chromatin dispersion (SCD) test

The SCD test was performed according to the previous study [30]. Spermatozoa with large or medium sized halos were considered as no DNA fragmentation, while sperm cells with DNA fragmentation showed either a small or no halo. At least 200 spermatozoa were checked and sperm DNA damage was calculated by dividing the abnormal to the total spermatozoa and reported as percentage.

2.10. Statistical analysis

The data were shown as mean ± SD. One way ANOVA test was used to evaluate the significant differences between the groups and the Tukey post-test was operated for determination of differences between the groups. The term 'statistically significant' was used to signify a two-sided P-value <0.05.

3. Results

3.1. Sperm parameters

The vitrification had negative effects on progressive motility and induced immotile sperm cell percentages and non-progressive motility of fresh samples in comparison with post vitrified sperm cells ($P < 0.01$). But, incubation of post vitrified samples with PTX increased the rate of progressive motility and immotile sperms ($P < 0.01$). Also, there was no significant demonstration based on improvement of non-progressive motility between three post vitrified groups.

There was also a significant reduction regarding normal morphology and viability between before and after vitrification assessments. While, the duration time of incubation with or without PTX treatment had no beneficial effects on morphology and viability after vitrification ($P < 0.05$) as well as progressive, non-progressive and immotile sperm rates (Table 1).

3.2. Chromatin/DNA status

In contrast, PTX addition did not significantly improve DNA integrity of asthenozoospermic sperm samples according to three steps of evaluation including after vitrification, PTX treatment and without addition of PTX as control group (Tables 2 and 3).

4. Discussion

The sperm cryopreservation as a valid technique in ART has been applied for different purposes, such as storage or fertility preservation. Although, sperm parameters including motility, morphology, viability and DNA integrity usually were descended following thawing procedures [9,41]. Motility is among the parameters most seriously affected by freezing [52]. It can also be a strong predictor of the capability of confirmed sample to attain fertilization in vitro [10]. Despite its importance, the mechanism through which motility is reduced hasn't been elucidated. Sperm motility is partially determined by mitochondrial function [22]. Hence, various investigations have been performed in order to improve the quality of post thawed spermatozoa and clinical outcome. In this regards, the present study selected an intensive aseptic method for cryoprotectant-free vitrification of human spermatozoa. The vitrification of human spermatozoa in the absence of conventional cryoprotectants is feasible and its quality

Table 1
Comparison of motility, morphology and viability between four groups.

Sperm variables	Before vitrification	After vitrification	PTX treatment	Without PTX treatment	P-value
Progressive motility (%)	41.73 ± 6.81	23.10 ± 6.84	33.93 ± 7.02	21.97 ± 6.90	0.000[a,b,c,d,f,g] 0.526[e]
Non progressive motility (%)	13.83 ± 3.11	15.77 ± 3.92	14.73 ± 4.22	15.80 ± 3.63	0.048[a] 0.354[b] 0.044[c] 0.288[d] 0.973[e] 0.273[f] 0.000[g]
Immotile (%)	44.23 ± 6.94	61.13 ± 7.30	51.53 ± 6.93	62.43 ± 7.45	0.000[a,b,c,d,f,g] 0.483[e]
Normal morphology (%)	11.93 ± 3.25	7.30 ± 2.33	7 ± 2.31	7.07 ± 2.21	0.000[a,b,c,g] 0.652[d] 0.725[e,f]
Viability (%)	73.67 ± 5.97	55.60 ± 6.87	54.33 ± 6.13	54.07 ± 6.05	0.000[a,b,c,g] 0.436[d] 0.346[e] 0.930[f]

Note: Data presents as percentage ±SD.
[a] Difference between before vitrification and after vitrification.
[b] Difference between before vitrification and PTX treatment.
[c] Difference between before vitrification and Without PTX treatment.
[d] Difference between after vitrification and PTX treatment.
[e] Difference between after vitrification and Without PTX treatment.
[f] Difference between PTX treatment and Without PTX treatment.
[g] Difference between four states.

Table 2
Comparison of sperm DNA status between three groups.

Variables	After warming	PTX addition	Without PTX addition	p-value
TUNEL (+) (%)	15.47 ± 4.49	17.63 ± 4.30	15.73 ± 4.37	0.810[a] 0.089[b]
SCD(+) (%)	17.43 ± 4.98	19.10 ± 5.03	23.33 ± 13	0.875[a] 0.284[b]

Abbreviations; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling, SCD: sperm chromatin dispersion.
[a] Difference between after vitrification and PTX treatment.
[b] Difference between PTX treatment and Without PTX treatment.

Table 3
Comparison of sperm chromatin status between three groups.

Variables	After warming	PTX addition	Without PTX addition	p-value
CMA3 (+) (%)	46.93 ± 14.57	49.07 ± 15.19	47.20 ± 14.80	0.942[a] 0.613[b]
AB (+) (%)	56.27 ± 15.94	58.37 ± 16.10	56.60 ± 15.83	0.933[a] 0.658[b]
TB (+) (%)	54.47 ± 14.93	55.60 ± 13.36	53.60 ± 13.35	0.801[a] 0.562[b]

Abbreviations; CMA3: chromomycin A3, AB: aniline blue, TB: toluidine blue.
[a] Difference between after vitrification and PTX treatment.
[b] Difference between PTX treatment and Without PTX treatment.

was compatible with that of standard slow-frozen and thawed spermatozoa [18,20]. This new method is quick and simple, not requiring special cryobiological equipment or chemicals. In addition, the vitrified human spermatozoa are ready to use immediately after warming without any additional treatment, and may be conducted in reasonably equipped laboratory [20].

Iasachenko et al. reported that sperm mitochondria function was decreased after vitrification [19]. During vitrification with assuming that oxidative phosphorylation and proton transport are membrane-bound phenomena, ATP production might be reduced. Also, it is not implausible that any changes in the energy availability or damage to the axonemal components could be probable reason for motility failure after cryopreservation [52]. Recently, Agha Rahimi and collogues [3] reported the vitrification did not have any adverse effects on DNA integrity of human normozospermia samples using TUNEL test and that was in contrast with our findings.

PTX was known as a stimulating agent that influences sperm motion features by inhibiting cAMP phosphodiestrase [43]. Although, use of PTX did not increase the number of motile spermatozoa, but it did improve the sperm movement characteristics in selected sperm population [48]. Enhanced recovery and increased motility of spermatozoa in oligozoospermic and asthenozoospermic samples can improve the outcomes of IVF or intrauterine insemination (IUI) cycles [12]. A clinical study concluded that oral administration of PTX is an effective treatment for improving the semen parameters of infertile men with idiopathic OAT [55]. The results regarding the effects of PTX on improving IVF protocols are controversial, however, the majority of studies demonstrated a significantly higher fertilization rates in oocytes inseminated with PTX treated spermatozoa [4]. Recently, scientists showed a positive effect of PTX on ICSI outcomes including fertilization, embryo quality and pregnancy rates in asthenozoospermic patients [4].

In this study, sperm parameters such as motility, normal morphology and viability were examined at several separate steps, including before vitrification, after vitrification and finally after PTX treatment and without PTX treatment. Similar to other studies, our findings showed a reduction of post-warm motility and viability in comparison to fresh samples [10,29]. Moreover, PTX treatment significantly increased human sperm progressive motility rates than control group. PTX was tested in human spermatozoa in order to stimulate motility in cryopreserved semen after thawing [42]. The results showed an improvement after stimulation with PTX when added in vitro and incubated for 60 min to post thaw normozoospermic without altering viability and membrane integrity that is in agreement with our findings. It is interesting that the type of cryopreservation method did not alter the probable effects of PTX treatment on motion characteristics based on ours as well as other studies [42,46]. Gil and associates (2010) concluded that PTX, as a supplement added to the freezing extender (concentration of 4, 8, 16 and 32 mM), had deleterious effects and it did not improve the

survival or fertilizing efficiency of frozen–thawed boar spermatozoa [15]. The probable reason for detrimental effect of added PTX before freezing on spermatozoa is concentration-dependent toxic effects observed in ovine [28] equine [15,16] and human spermatozoa [50].

By introduction of several techniques for evaluation of sperm DNA status and due to important role of sperm DNA integrity in success of ART program [6,7,22], the new context has been provided to research in this field. Recent studies implied that detecting DNA damage may have more diagnostic and prognostic importances than the standard semen analysis [1,55]. The results of several studies suggested that DNA damage assays may not be the most appropriate method for sperm selection in ART laboratories, either in the subjects with normal sperm parameters or infertile men [14,51,57]. Therefore, we tried to evaluate the effect of PTX treatment on sperm DNA fragmentation using SCD and TUNEL tests along with chromatin and DNA integrity using TB, AB and CMA3 assessments.

In addition to well-known effects of cryopreservation procedures on the fertilization potential, motility, morphology and viability of sperm [31], a relationship exists between cryopreservation and extent of sperm DNA fragmentation in infertile men [49]. The data generated from our study showed significant decreasing rates of chromatin condensation and DNA integrity. In contrast, larger rate of apoptosis in post-warmed spermatozoa was noticed in comparison with fresh samples, which is in line with previous work [41]. Such a reduced post-warm longevity is considered one of the causes of the poor conception rates obtained with frozen sperm than with fresh or natural mating [37]. For instance, Zribi et al. (2010) expressed that cryopreservation caused a significant increasing of sperm DNA fragmentation in samples of infertile men [58]. Furthermore, increasing the oxidative stress during cryopreservation is responsible for human sperm DNA fragmentation, rather than caspase activation and apoptosis [49].

A recent finding confirmed an increased DNA damage following supplementation of fresh sperm samples with PTX using SCD test [51]. The results of the late study, in contrast to our data, may be due to the different study design, sample type, the range of PTX concentrations and exposure time of PTX to spermatozoa. Nevertheless, the significant changes were not observed in regards with sperm DNA fragmentation and integrity of DNA with PTX treatment on post warming sperm samples.

Also, Amer et al. (2013) stated that PTX can be useful compound for improving ICSI outcomes in semen samples preparation of asthenozoospermia regardless of the state of sperm motility or the degree of asthenozoospermia [4]. In general, the hypothesis for inducing the motility of spermatozoa after incubation with PTX either freshly or post warming is to enhance the ability of sperm for saving the extra energy generated by this stimulant agent for next steps of fertilization.

5. Conclusion

The data showed that PTX was able to improve sperm movement without any adverse effect on sperm chromatin/DNA integrity of astenozoospermic samples in vitrification program.

Conflict of interest

There is no conflict of interest in this work.

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