In-Vitro Application of Pentoxifylline Preserved Ultrastructure of Spermatozoa After Vitrification in Asthenozoospermic Patients

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Purpose: To evaluate the effect of in vitro application of pentoxifylline (PX) on sperm parameters and ultrastructure after vitrification in asthenozoospermic patients.

Materials and methods: A total of 30 asthenozoospermic semen samples (aged 25-45 years) were divided into four groups before vitrification, after vitrification, control (without PX) and experimental (with PX). In experimental group, each sample was exposed for 30 min to 3.6mmol/l PX and the control group without any treatment apposing in 370C for 30 min. After incubation, the samples were washed and analyzed again. Vitrification was done according to straw method. Eosin–nigrosin and Papanicolaou staining were applied for assessment of sperm viability and morphology, respectively. The samples without PX and post treatment with PX were assessed by transmission electron microscopy (TEM).

Results: A significant decrease in sperm motility ($P \leq .001$), morphology (11.47 ± 2.9 versus 6.73 ± 2.01) and viability (73.37 ± 6.26 versus 54.67 ± 6.73) was observed post vitrification, but sperm motility (19.85 ± 4.75 versus 32.07 ± 5.58, $P \leq .001$) was increased significantly following application of PX. This drug had no significant (P >.05) detrimental neither negative effect on ultrastructure acrosome, plasma membrane and coiled tail statues of spermatozoa.

Conclusion: Vitrification had detrimental effects on sperm parameters, but PX reversed detrimental effects on sperm motility. However, PX had no alteration on ultrastructure morphology of human spermatozoa after vitrification.

Keywords: asthenozoospermia; pentoxifylline; vitrification; ultrastructure.

INTRODUCTION

Cryopreservation of human spermatozoa is widely used in many assisted conception units to preserve male fertility, for example before cytotoxic chemotherapy, radiotherapy or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction[(1)]. Vitrification is a method that can be useful to achieve the same purpose and does not use the special extenders. It is based on the ultrarapid freezing of the cell by quick immersion in liquid nitrogen. Moreover, it is a simple procedure that requires less time and probably will become safer and more cost-effective than conventional freezing[(2,3)]. A normal motility pattern in ejaculated spermatozoa is a basic condition for male fertility. Because adenosine triphosphate (ATP) supports the chemical–mechanical coupling catalyzed by dyneins (specific ATPases of the flagellum), it is usually accepted that both glycolysis and oxidative phosphorylation are required for optimal sperm function[(4)]. Thus, it is supposed that impaired motility might follow a bioenergetic shortage, although other mechanisms are still possible[(5)]. Mohamed et al. showed the significant affection of the progressive motility, viability and mitochondrial membrane potential of human spermatozoa by cryopreservation. Although, both conventional slow and the vitrification techniques had similar results, vitrification is faster, easier with less toxicity and costs[(6)]. Kuznyetsov showed that vitrification was an alternative method for freezing human spermatozoa without toxic permeable cryoprotectants (CPAs). It can be used to optimize the vitrification and post thaw recovery of a small number of spermatozoa in normozoospermic and severe oligozoospermic samples[(7)].

Pentoxifylline (PX) is a non-specific phosphodiesterase (PDE) inhibitor of the methylxantine group, inhibiting the breakdown of cyclic adenosine monophosphate (cAMP). It is known that intracellular cAMP concentration plays a central role in sperm motility. It is considered to be a sperm movement enhancer, hyperactivation agent, inhibitor of reactive oxygen species formation and acrosome reaction-improving agent[(8)].

Electron microscope has been used for examining the ultrastructure of spermatozoa. Studies by means of TEM showed that cryopreservation has deleterious effects on spermatozoa, especially on plasmalemma, acrosomes and tails[(9,10)]. After thawing, the rate of spermatozoa that were considered normal by means of TEM evaluations decline. Among defined abnormalities, the defects of acrosomal change and subacrosomal swelling increase.

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Table 1. The results of sperm analysis between before vitrification and after warming

Variables*	Before vitrification (group III) Mean ± SD	After warming (group IV) Mean ± SD	*P* value
Progressive motility (%)	40.57 ± 6.49	21.03 ± 4.57	<.001
Non progressive motility (%)	14.57 ± 3.23	15.63 ± 3.22	.097
Immotile (%)	43.47 ± 9.33	63 ± 5.32	<.001
Normal morphology (%)	11.47 ± 2.9	6.73 ± 2.01	<.001
Viability (%)	73.37 ± 6.26	54.67 ± 6.73	<.001

Abbreviations: PX, pentoxifylline; SD, Standard Deviation.
*Values are mean ± SEM.

Acrosomal change defect, which is described by natural equatorial acrosomal content but an altered apical acrosomal region. Apical head alterations in acrosomal change defect are lack of stability, loss of acrosomal content and appearance of vesiculations. Another abnormality was subacrosomal swelling, which is characterised by detachment of the inner acrosomal membrane from the nuclear envelope and filamentous widening of the subacrosomal space[10,11]. So far, some studies evaluated the effects of PX on cryopreserved spermatozoa and were focused mainly on post thaw sperm motility and characteristics like velocity, head movements, and linearity[1,8,12] rather than ultrastructure of it. In this study, we attempt to evaluate the effect of In-vitro application of PX on sperm parameters and ultrastructure after vitrification in asthenozoospermic patients.

MATERIAL AND METHODS

Study Population

A total of 30 asthenozoospermic semen samples were selected according to WHO criteria for sperm total motility of <32%. Sperm specimens were obtained by masturbation into sterile cups. All subjects were asked to abstain from ejaculation from 2 to 3 days before collection. After liquefaction at 37ºC, a small aliquot was removed from each specimen and the sperm parameters were determined[1].

Inclusion and exclusion criteria

In this study, smokers, patient with varicocele and patients with history of varicocelectomy were excluded. The patients agreed to participate in this project via filling out the consent forms. The ethics committee of the institution approved this study.

Evaluation of sperm parameters

The seminal samples were prepared using the density gradient technique. Sperm parameters of motility, viability and normal morphology (%) were evaluated for 100 spermatozoa in each sample. Sperm motility was assessed using Makler chamber and light microscopy (Olympus, Tokyo, Japan). Motility was expressed as % of progressive and non-progressive. Sperm viability and morphology were evaluated by Eosin and Papanicolaou staining tests, respectively[13]. Assessments of sperm parameters were carried out before vitrification (group I), after warming (group II), after the warming and without PTX treatment (group III), and PTX treatment (group IV). Final analysis of sperm parameters and ultrastructure were undertaken (**Figure 1**).

Vitrification and Warming

Vitrification was done by straw to straw method according to Isachenko et al[14]. In this process, the suspension was mixed with Ham's F10 medium supplemented with 5% HSA/0.5 mol/l sucrose (1:1) at room temperature, 100 µl of the sperm suspension was transferred into a 0.25 ml plastic straw maintained horizontal. Then, it was placed into 0.5ml straws and sealed at the end, and were immersed directly into liquid nitrogen. At least after one week for warming, after cutting one end side of the straw, the samples were vacated in pre-warmed Ham's F10 with 5% HSA. The simple-wash method was applied and sperm parameters were evaluated according to WHO criteria[14].

Incubation with pentoxifylline

A stock solution of PX (Sigma, USA) in distilled water was prepared each week and kept at 4°C until used. The samples were divided into two groups of control and

Table 2. The results of sperm analysis between Without PX treatment and PX treatment

Variables*	Without PX treatment (group III) Mean ± SD	PX treatment (group IV) Mean ± SD	*P* value
Progressive motility (%)	19.87 ± 4.75	32.07 ± 5.58	<.001
Non progressive motility (%)	15.63 ± 3.76	14.97 ± 3.7	.219
Immotile (%)	64.3 ± 5.37	53.23 ± 6.16	<.001
Normal morphology (%)	6.63 ± 1.97	6.53 ± 1.97	.083
Viability (%)	53.07 ± 5.71	53.2 ± 5.85	.161

Abbreviations: PX, pentoxifylline; SD, Standard Deviation.
*Values are mean ± SEM.

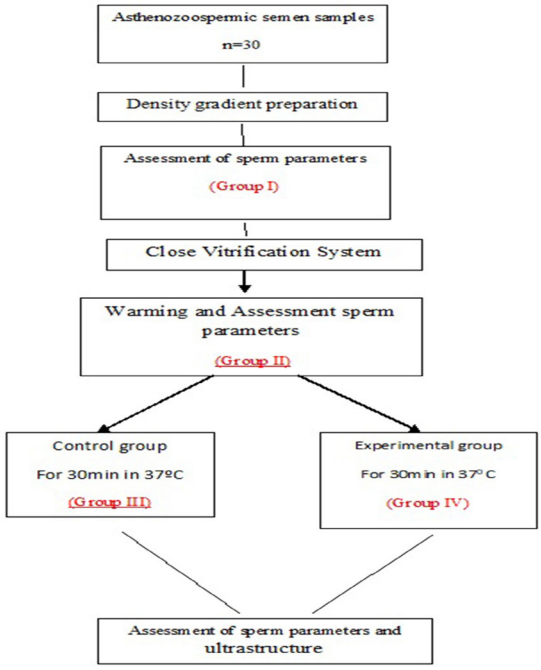

Figure 1. Schematic diagram for study design.

experimental. In experimental group, each sample was exposed for 20 minutes to 3.6mmol/l PX at 370C and the control group in 370C for 30 minutes. After incubation, the samples were washed and analyzed again[1].

Transmission Electron Microscopy (TEM)

The samples without PX (group III) and post treatment with PX (group IV) were assessed by TEM. For this goal, spermatozoa were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 2 h at 4°C. The sperm samples were washed three times in PBS by centrifugation suspension at 1000×g for 10 minutes and post fixed in 1% osmium tetroxide for 1 h. The samples were then centrifuged at 1000×g for 10 minutes and the pellets were dehydrated in graded acetone. Finally, the pellets were embedded in Epon. Ultra-thin sections (50-70 nm) were obtained with Leica EM UC7 ultra-microtome and stained with uranyl acetate and lead citrate. The samples were observed with the TEM (Zeiss, EM 10) to evaluate intact (with acrosome), reacting (with ruptured and irregular outer acrosomal membrane), reacted sperm (without acrosome), Nucleus , Vacuole and coil tail[10,15]. Evaluations were according to Visual Atlas[16]. Different parts of hundred sperm samples were studied and illustrated as the percentage of healthy sperm in **Table 2**.

Statistical analysis

Data were analyzed using SPSS version 20 software (SPSS, Inc., Chicago, IL, USA).The data were shown as mean ± SD. Paired-samples T test was used for comparison of sperm parameters between before vitrification and after warming, also this test was used for evaluated of sperm ultrastructure between without PTX treatment and PTX treatment. The term 'statistically significant' was used to signify a two-sided P value < .05 for sperm parameters and ultrastructure.

RESULTS

Table 1 shows sperm parameters between before vitrification and after warming samples. This table reveals that sperm motility, morphology and viability revealed significant differences (P < .001) between before vitrification and after warming. **Table 2** shows the sperm parameters between without PX treatment and PX treatment. This table reveals that sperm motility had significant differences (19.85 ± 4.75 vs. 32.07 ± 5.58, $P \leq$ .001) between without PX treatment and PX treatment. The data showed that vitrification impaired sperm parameters and PX can reverse detrimental effects of vitrification on sperm parameters. Figure 2 shows the results of sperm analysis between after warming and PX treatment. The results of sperm ultrastructure evaluation between groups III and groups VI are presented in **Table 3**. There were insignificant differences (P >.05) between the groups with regards to acrosome, plasma membrane, vacuole, nucleus and coiled tail statues (**Figure 3**).

DISCUSSION

The findings of present study showed that almost all sperm parameters revealed significant decrease following vitrification. The decline in sperm parameters, especially motility after cryopreservation, is a matter of current study, since this is a vital factor affecting post freezing–thawing success rate. PX indirectly increases intracellular cAMP concentration and plays a critical role in sperm motility improvement[17]. The technique of vitrification is based on the ultra-rapid freezing of the cell by quick immersion in liquid nitrogen. Thereby, formation of intracellular ice crystals is avoided with-

Table 3. The results of sperm ultrastructure evaluation between without PX and with PX groups.

Variables*	Without PX treatment (group III) Mean ± SD	PX treatment (group IV) Mean ± SD	*P* value
Acrosome (%)	38 ± 5.71	37.5 ± 5.56	.914
Plasma membrane(%)	32.5 ± 3.31	31 ± 3.46	.620
Nucleus (%)	46.75 ± 7.89	43.5 ± 8.06	.655
Vacuole (%)	48.5 ± 13.8	47.5 ± 15.1	.929
Coiled tail(%)	64.5 ± 9.39	67.25 ± 9.17	.621

Abbreviations: PX, pentoxifylline; SD, Standard Deviation
*Values are mean ± SEM.

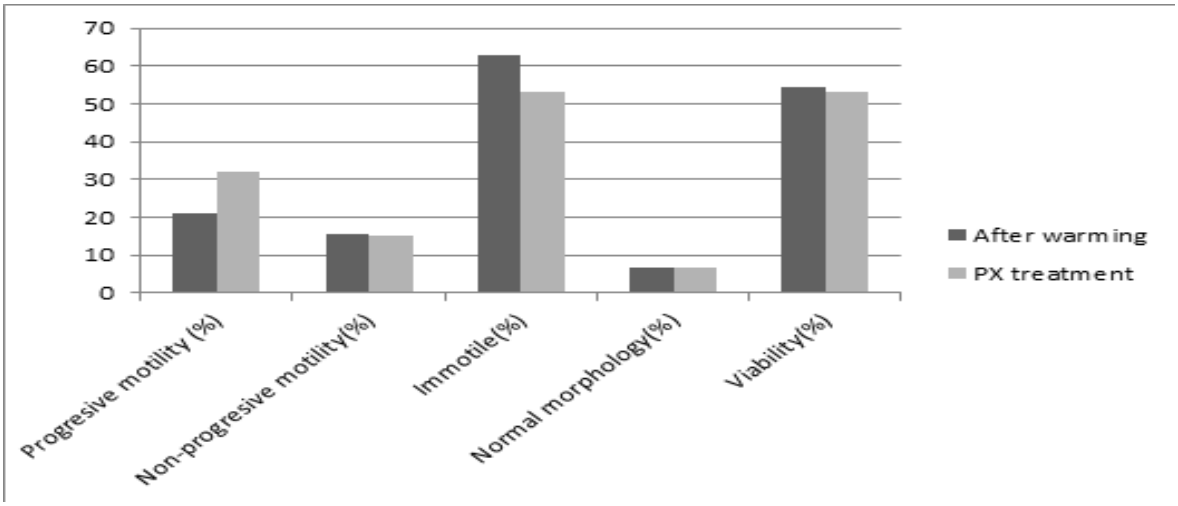

Figure 2. The results of sperm analysis between after warming and PX treatment
Abbreviations: PX, pentoxifylline

in sperm cells[2]. Morris discussed that the intracellular compartment of the cell will vitrify during cooling and will not devitrify until approx-30ºC during warming. This caused the outer surface of the plasmalemma being exposed to the stresses of osmotic shock, therefore kills the sperm cell. Ultrastructure analysis of rapidly cooled samples during warming showed wide ice recrystallization occurred around the cell during the warming phase. At 40 ºC, the solution around the cell would be fluid, but the intracellular compartment would still be a glass, this occasion cause extensive membrane impairment. It is described that rapid rates of cooling has frequently been elated to the formation of intra cellular ice and cellular damage[18].

Iasachenko et al. reported function of mitochondria decreased after vitrification[19]. Assuming that oxidative phosphorylation and proton transport are membrane-bound phenomena, it is possible that ATP pro-

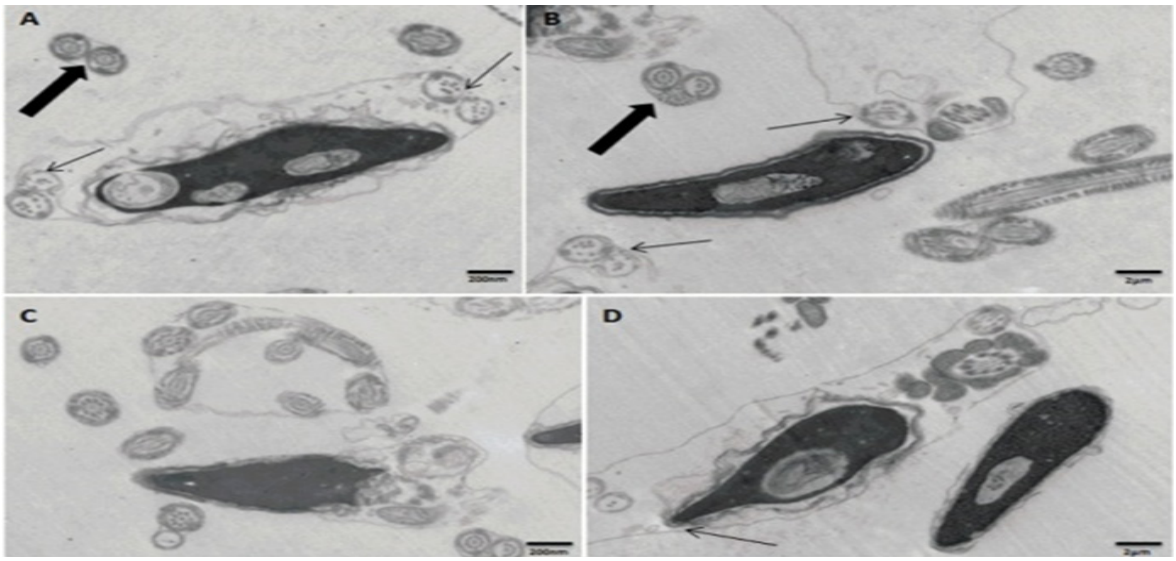

Figure 3. Ultra micrograph of human spermatozoa
(A) Without PX, shows acrosome discharged completely and vesiculating, the remnant of plasma membrane was seen. The midpiece disrupted. Thin arrow shows the arrangement of tubulin in was section of tail disrupted and two sections of tail adherent together that means rolled tail. Thick arrow shows two sections of tail adherent together that means rolled tail. The sperm was seen morbid.
(B) Without pentoxifylline (PX), Nucleolus show with large vacuoles, acrosome intact. Thick Arrow shows several sections of tail adherent together that means rolled tail. Thin arrow shows the arrangement of tubulin in was section of tail disrupted and several sections of tail adherent together that means rolled tail.
(C) Addition with PX, Acrosome shows partly intact. Disrupted midpiece and tail and shows several sections of different area of tail meaning rolling tail.
(D) Treatment with PX, acrosome discharged completely, the remnant of plasma membrane was seen. The plasma membrane midpiece was swollen. One vacuoles is seen in chromatin. Thick arrow shows a vesiculated acrosome.

duction would be reduced. Also it is probable that change of the energy availability or damage to the axonemal components could contribute to the cause of motility failure after cryopreservation[20]. In this study we observed some nuclear defect that were not statistically significant. After cryopreservation, the structure of chromatin is less condenses. The number of nuclear vacuole was increased and chromatin may have granular view. In some spermatozoa, a vacuole may expand and cause chromatin to marginalize. There are two factors may cause these expansions: ice crystal formation and osmotic shock. Ice crystal in nuclear was not formed, because this structure is highly viscose and intracellular ice was not occurred. But, osmotic shock is more probable. The marginalized chromatin is the sign of apoptosis according to Baccetti□s study[21]. No significant differences in the DNA integrity of prepared spermatozoa was reported related to the freezing method or presence of a cryoprotectant[19]. Recently, reported vitrification did not show any adverse effect on DNA integrity of human spermatozoa with TUNEL test[2]. Simultaneously, more study should be done to confirm that the shape of chromatin causes DNA damage or not.

Our results confirm the findings of Moein et al.[22] that patients with poor sperm motility can benefit from PX added straight to the washed semen specimens. Johanson and colleagues studied the role of PX-treated cryopreservation ejaculates on pregnancy rate. The higher pregnancy rate in the PX group proved that stimulated sperm motility is good predictor of fertility potential in an infertility treatment program[23]. There are some studies that are in line with our results. Esteves et al. showed that the use of PX improves the capability of thawed spermatozoa to undergo the acrosome reaction in reaction to calcium ionophore. Also, the treatment of poor quality human sperm with PX may enhance post-thaw sperm fertilizing ability[1]. Also, Rashidi et al. observed an increase of motile spermatozoa and fertilization rate after addition of 3 mmol/L PX to mouse sperm cells[24]. PX has been used as a useful compound prior to oocytes injection for improving ICSI outcome in asthenozoospermic patients[17]. Since, the motility of sperm cells is reduced after freezing, adding PX as a stimulant factor can improve it. To the best of our knowledge, this is the first investigation on the relationship between sperm ultrastructure and administration of the PX after vitrification in asthenozoospermic patients. Our results showed that treatment of spermatozoa with PX after thawing did not have significant detrimental effect on sperm ultrastructure (acrosome, plasma membrane and coiled tail statues).

CONCLUSIONS

PX had no alteration on ultrastructure morphology (acrosome, plasma membrane and coiled tail statues) of spermatozoa post vitrification of asthenospermia samples. However, vitrification has detrimental effects on human sperm parameters of motility, morphology and viability, but PX reversed detrimental effects on sperm motility.

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CONFLICT OF INTEREST

None declared

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