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Effect of pentoxyifylline on sperm functions post cryopreservation and thawing in men with Asthenozoospemia

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ABSTRACT

Background. Asthenozoospermia is a 32% progressive sperm motility and Pentoxifylline (P.X) is inhibit phosphodiesterase which prevent cyclic adenosine monophosphate (cAMP) from degrading lead to elevate sperm motility.

Purpose. The study aimed to investigate the effect of adding P.X in different concentrations to thawing solutions on percentage of progressive motility, immotile sperm %, viability % and DNA Fragmentation Index (DFI) % for Asthenozoospermia and normozoospermia.

Methods. This study is performed in the Laboratory Unit of Faculty of Medicine and Center Clinic and the Advanced Researches. The range age of men ranged from 22 to 51 years. The study included 100 individuals (60 Asthenozoospermia and 40 Normozoospermia). After taking semen samples from both Asthenozoospermia and Normozoospermia, the semen was analyzed before cryopreservation and measured sperm progressive motility (%) and immotile sperm (%) by sperm analyzer. The percentage of Sperm viability was determined by Eosin Nigrosin stains method. The percentage of DNA Fragmentation Index (DFI) was measured using Acridine Orange (AO) fluorescence stain. Both Asthenozoospermia and Normozoospermia were divided depend on adding pentoxyifylline in certain concentrations to the thawing solutions, into three groups:.G1: without pentoxifylline, G2: contain low concentration of pentoxifylline (5mg/mL) and G3: contain high concentration of pentoxifylline (10mg/mL). All tests that were conducted pre cryopreservation will be repeated on all groups post thawing.

Results. The percentage of progressive and viability % was decreased significantly ($p<0.05$). While noted immotile sperm and DFI increased significantly ($p<0.05$) after thawing in all groups (G1, G2 and G3) comparing with pre cryopreservation in both normozoospermia and asthenozoospermia. A significant decrease ($p<0.05$) in progressive motility % and viability %, besides a significant increase ($p<0.05$) in immotile sperm% and DFI% in G1 compared with G2 and G3 of both normozoospermia and asthenozoospermia. A high concentration of P.X (10mg/mL) has a better effect than low concentration (5mg/mL). A significant increase ($p<0.05$) in (progressive motility % and viability %) and a significant decrease ($p<0.05$) in immotile sperm% and DFI% in G3 compared with G2 in both normozoospermia and asthenozoospermia.

Conclusions. The rapid cryopreservation has numerous detrimental effects on sperm, including decreased progressive motility % and sperm viability %, increased immotile sperm %, and increased DFI %. Additionally, we indicate that adding pentoxifylline at certain concentration to the thawing solution that reduces immotile sperm% and DFI% for Asthenozoospermia and normozoospermia.

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Keywords: Rapid cryopreservation, Pentoxifylline, Asthenozoospermia, Normozoospermia, oligoasthenozoospermia, oligozoospermia, azoospermia

INTRODUCTION

After at least a year of routine, unprotected sexual activity without conception, infertility is identified. About 187 million couples worldwide receive the diagnosis of infertility, which has emerged as a global health concern [1]. The study conduct found that male factors account for approximately half of cases of infertility, also showed patients with poor motility having the highest frequency of cases. Asthenozoospermia, defined as 32% progressive sperm motility [2]. Although, it is easily distinguished through semen analysis, the underlying causes remain unknown [3].

In a successful fertilization, impaired motility can lead to ineffective fertilization. Asthenozoospermia has been explained by Numerous studies have demonstrated various causes of asthenozoospermia, including proteomic [4], metabolic abnormalities [5, 6] and genetic [7, 8]. Authors indicated that altered in gene of androgen receptor as a cause of male infertility in Iraq in all groups of male patients with infertility (oligoasthenozoospermia, oligozoospermia and non-obstructive azoospermia), also showed the maximum percentage of loss in gene of androgen receptor was recorded in oligoasthenozoospermic patients [9]. One of the most practical and accepted ways to preserve a man's fertility potential is sperm cryopreservation, which is regarded as a standard technique in assisted reproductive technology (ART). When male fertility needs to be preserved before radiotherapy, cytotoxic chemotherapy, or specific surgical procedures, this approach becomes very crucial [10]. The negative effects of freezing and thawing on sperm are undeniable. After the frozen spermatozoa undergo the thawing process, not all of them are able to survive. In humans, the concentration and motility of sperm post-thaw are significantly lower, reaching up to 50% below the original pre-freeze levels. This decline can be attributed to various factors such as ice formation and osmotic stress, which adversely affect the sperm during freezing [11]. Pentoxifylline (P.X) belongs to the methylxantine group, inhibits phosphodiesterase. It prevents cyclic adenosine monophosphate (cAMP) from degrading. Furthermore, it is well-known that sperm motility is significantly influenced by intracellular cAMP concentration. For human IUI, PX was utilized to activate sperm. Cyclic AMP is thought to stimulate a cAMP-dependent kinase, which in turn causes sperm tail protein phosphorylation and an increase in sperm motility. In general, PX has been observed to be helpful in retaining sperm motility in vitro [12,13].

This study aimed to investigate the effect of adding pentoxifylline different concentrations to thawing solutions on percentage of progressive motility, immotile sperm %, viability % and DNA Fragmentation Index (DFI) % for Asthenozoospermia and normozoospermia.

MATERIALS AND METHODS

Semen Samples collection

The semen samples were taken in a clean, dry, and sterile disposable plastic wide mouth container in a chamber designed especially for this function in the institute after the subjects had abstained from sexual activity for three days. The samples were immediately sent to the facility for semen analysis, where they were maintained in an incubator for 30 minutes at 37 °C. After the semen was fully liquefied, the semen analysis was performed according to standard criteria [14,15]. Based on the results of the semen analysis, samples were subsequently divided into asthenozoospermia and normozoospermia.

Seminal Fluid Analysis

Every tests performed on semen sample used in the study before and after cryopreservation.

Measurement of Sperm Motility %

The sperm analyzer was used to measure sperm motility %. 10 µl of semen after complete liquefied were mounted onto slide and covered with a coverslip then placed on stage of the microscope which linked with sperm analyzer. Choice as a minimum 10 fields from the slide then sperm analyzer record their results and collects all 10 results in one report which including progressively (PR) motility % and immotile sperms (IM) % and other results

Measurement of Sperm viability

The percentage of Sperm viability was determined by Eosin Nigrosin stains method (16). The method for measuring sperm viability as following:

A- one drop of liquefied semen was added to two drops of eosin Y solution 1 %.

B– After 30 seconds, three drops of 10 % nigrosin solution were added and mixed.

C– A thin smear of the semen–eosin–nigrosin mixture was made within the 30 seconds of adding nigrosin.

D– Let to air dry, then inspected under a microscope (100 ×).

Eosin stained dead sperm, whereas live sperm maintained the integrity of their cell membrane, preventing the stain from entering cells. Background stains were created with nigrosin. 200 sperm in total were examined using a light microscope and a (100X) objective lens while submerged in oil. Spermatozoa that appeared white and were unstained were categorized as "live," whereas those that displayed any pink or red hue were categorized as "dead."

According to the following equation, the amount of viable sperm was determined by dividing the total number of spermatozoa by 100.

Sperm vitality = number of viable sperm / total number of spermatozoa × 100.

Assessment of DNA Fragmentation Index (DFI)

DNA Fragmentation Index (DFI) was measured by the fluorescent dye Acridine Orange (AO). According to (14), The DFI was assessed using a fluorescence microscope with spermatozoa counted in as a minimum 5 fields and excitation at 450-490 nm. defective spermatozoa with DNA fragments fluoresced in yellow, orange, or red, typical spermatozoa with double-stranded DNA intact stained green. The DFI was calculated by the following equation [17].

DFI= (yellow + orange + red)/ total x 100

Procedure of rapid Cryopreservation and thawing semen sample from Asthenozoospermia and Normozoospermia after performing all the previous tests, 0.8 ml of the sample with 1mL cryo-solution (SMART cryopreservation medium and Dimethyl sulf-oxide DMSO cryo-protectant substance) placed in cryo-vials, Each cryo-vial was labeled with code number and date of cryopreservation. then the cryo-vials exposure to liquid nitrogen vapor (-80 °C) for 15 minutes and 10cm above the surface of liquid nitrogen [18]. Cryo-vial were plunged inside liquid nitrogen and they were stored at -196 °C for two months using a cryopreservation liquid nitrogen tank (40L).

Preparation pentoxifylline (PX) solution.

Two different concentrations of pentoxifylline solution were prepared.

High concentration of pentoxifylline (10 mg/mL)

High concentration of pentoxifylline was prepared in two steps, the first step dissolving 1000 mg from pentoxifylline (PX) in 10 ml of normal saline then stirring until dissolve. These called stock solution prepared daily under sterile condition using U/V light then filtered by Millipore filter (0.45µM) then filtered by Millipore filter (0.22 µM). The second step to add(0.1 mL) from stock solution to 9.90ml from SMART medium. A solution was obtained in which the concentration of pentoxifylline is (10 mg/mL).

Low concentration of pentoxifylline (5mg/mL).

Low concentration of pentoxifylline was prepared in two steps, the first step dissolving 500 mg from pentoxifylline (PX) in 10 ml of normal saline then stirring until dissolve. These called stock solution prepared daily under sterile condition using U/V light then filtered by Millipore filter (0.45µM) then filtered by Millipore filter (0.22 µM). The second step to add (0.1 mL) from stock solution to 9.90 ml from SMART medium. A solution was obtained in which the concentration of pentoxifylline is (5mg/mL).

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Thawing Technique

The semen samples were thawed. Both Asthenozoospermia and Normozoospermia were divided depend on adding pentoxifylline in certain concentrations to the thawing solutions, into three groups:. G1: without pentoxifylline, G2: contain low concentration of pentoxifylline (5mg/mL) and G3: contain high concentration of pentoxifylline (10 mg/mL).

Procedure of Thawing

Thawing was done for each semen sample after two months of cryopreservation in liquid nitrogen (19) .the Procedure of thawing as following :

a. The Cryo-vials were taken out of the liquid nitrogen.

b. Cryo-vials were submerged in water bath at 37 °C for 5 minutes.

c-Each semen sample in cryo-vial divided to three groups (G1,G2 and G3) and placed in three Eppendorf tubes; then centrifugation was done for all samples by centrifuge at 1500 rpm for 5 minutes, supernatant was removed and remind the pellet in Eppendorf tubes.

d- Adding 1mL from thawing solution contains either only SMART medium or SMART medium and pentoxifylline (high or low concentration) for all eppendorf tubes and put in the incubator for 30minute.

e- Post- thawing process, all samples were divided into three sub groups: G1: freezing semen sample with SMART medium without pentoxifylline, G2: freezing semen sample with SMART medium containing 5μm pentoxifylline (low concentration), G3: freezing semen sample with SMART medium containing 10μm pentoxifylline (high concentration).

All semen analysis that were performed on semen before cryopreservation in this study are repeated in the same way after thawing. These analysis include (progressive motility %, immotile Sperm %,Sperm Viability % and DFI %).

Statistical analysis

The mean ± SEM is used to express data. Version 23 of SPSS was used for statistical analysis. A one-way ANOVA and student t-test were used to compare the groups, and LSD was then applied. A significance level of $p < 0.05$ was considered.

RESULTS

Effect of adding different Concentrations of P.X on Percentage of Progressive Motility Post-thawing in Normozoospermia and Asthenozoospermia

The results of this study showed that the percentage of progressive motility was decreased significantly ($p<0.05$) after thawing in all subgroups (G1,G2 and G3) comparing with pre cryopreservation, also noted the percentage of progressive motility decrease significantly($p< 0.05$) in G1 than other groups (G2 andG3) in both normozoospermia and asthenozoospermia, the results indicated a significant increase($p<0.05$) in G3 than G2 in asthenozoospermia, while in normozoospermia showed non-significant difference($p<0.05$) when compared between G3 and G2, as in table (1).

Groups	Pre cryopreservation	Groups			LSD
		G1 (No.P.X)	G2(5 mg/mL P.X)	G3(10 mg/mL P.X)	
Normozoospermia	**51.04±8.15A**	**23.27±4.3B**	**30.59±4.9C**	**30.82±4.86C**	**2.724**
Asthenozoospermia	**24.61±5.51A**	**5.33±1.44B**	**7.79±2.16C**	**9.58±2.55D**	**1.132**

Table 1: Percentage of progressive motility pre-cryopreservation and post-thawing for using different concentrations of P.X in normozoospermia and asthenozoospermia.

Means with different capital letters in the same row are significantly different

Effect of adding different Concentrations of P.X on Percentage of Immotile Sperm Post-thawing in Normozoospermia and Asthenozoospermia

The current results investigated that the percentage of Immotile sperm was increased significantly ($p< 0.05$) after thawing in all groups (G1, G2 and G3) comparing with pre-cryo-preservation in both normozoospermia and asthenozoospermia, furthermore, a significant increase ($p<0.05$) in G1 of both normozoospermia and asthenozoospermia after thawing than others groups G2 andG3, the results indicated non-significant difference ($p<0.05$) when compared between G3 and G2 in normozoospermia, while the results showed a significant decrease ($p< 0.05$) after thawing in G3 comparing with G2 in asthenozoospermia, as in table (2).

Table 2: Percentage of immotile sperm pre-cryopreservation and post-thawing using different concentrations of P.X in normozoospermia and Asthenozoospermia

Groups	Pre cryopreservation	Groups			LSD
		G1(No.P.X)	G2(5 mg/mL P.X))	G3(10mg/mL P.X)	
Normozoospermia	27.73±7.51A	59.07±4.59B	48.63±4.33C	48.47±4.5C	2.553
Asthenozoospermia	64.12±7.79A	86.09±3.63B	82.52±3.69C	80.67±3.63D	1.518

Means with different capital letters in the same row are significantly different

Effect of adding different Concentrations of P.X on Percentage of Sperm Viability Post-thawing in Normozoospermia and Asthenozoospermia

The percentage of sperm viability was decreased significantly ($p<0.05$) after thawing in all groups (G1, G2 andG3) comparing with pre cryopreservation, also showed a significant decrease ($p < 0.05$) in G1 comparing with G2 andG3 in both normozoospermia and asthenozoospermia, in addition non-significant difference ($p<0.05$) was noted between G2 and G3 of normozoospermia, while in asthenozoospermia showed a significant increase ($p<0.05$) in G3 comparing with G2, as in table (3).

Table 3: Percentage of sperm viability pre-cryopreservation and post-thawing using different concentrations of P.X in normozoospermia and Asthenozoospermia

Groups	Pre cryopreservation	Groups			LSD
		G1(No.P.X)	G2 (5 mg/mL P.X))	G3 (10mg/mL P.X))	
Normozoospermia	81.35±6.18A	30.47±3.87B	36.51±4.07C	36.65±3.98C	1.227
Asthenozoospermia	54.18±3.92A	21.45±2.94B	24.31±3.01C	26.28±2.08D	1.185

Means with different capital letters in the same row are significantly different

Effect of adding different Concentrations of P.X on Percentage of DNA Fragmentation Index (DIF) of Sperm Post-thawing in Normozoospermia and Asthenozoospermia

The significant increase ($p<0.05$) in the percentage of DNA Fragmentation Index (DIF) of Sperm after thawing in all groups (G1, G2 and G3) compared with pre cryopreservation, also the results

indicated a significant increase ($p<0.05$) in G1 than G2 and G3 in both normozoospermia and asthenozoospermia, in addition showed non-significant difference ($p<0.05$) between G3 and G2 in normozoospermia, while in asthenozoospermia showed a significant decrease ($p < 0.05$) in G3 compared with G2 as in table (4).

Table 4: Percentage of DNA fragmentation Index(DIF)of sperm pre-cryopreservation and post-thawing using different concentrations of P.X in normozoospermia and Asthenozoospermia.

Groups	Pre cryopreservation	Groups			LSD
		G1(No.P.X)	G2 (5 mg/mL P.X)	G3(10mg/mL P.X)	
Normozoospermia	13.75±2.67A	35.67±2.08B	30.45±1.15C	30.72±2.3C	2.23
Asthenozoospermia	43.77±6.23A	71.48±6.91B	66.71±6.57C	63.79±6.58D	2.47

Means with different capital letters in the same row are significantly different

Discussion

Cryopreservation is usually performed to preserve the sperm of patients undergoing assisted reproductive technology In patients who need to preserve their sperm before undergoing treatments that may lead to infertility for example radiotherapy, chemotherapy, and orchiectomy or vasectomy [19].

In the current results of sperm cryopreservation were a significantly reduced in sperms function factors(, progressive Motility% and Viability %) in all groups post-thawing compared with pre-cryopreservation in both normozoospermia and asthenozoospermia, these results agreement with study performed and indicated a pointedly decreased in Sperm progressive motility and vitality when compared between before cryopreservation and after thawing [20].

Despite many advances in sperm freezing and the widespread clinical use of cryopreserved sperm, not all sperm survive after freezing and thawing process, After freezing and thawing, sperm concentration and motility% decrease about 50% compared to before freezing [13].

Also the results revealed a major rise in DNA Fragmentation Index (DIF) in all groups after thawing comparing with pre cryopreservation in both normozoospermia and asthenospermia. These result is agreement with [21] reported a major rise in DIF post thawing than pre-cryopreservation.

DFI provides additional information about men's reproductive potential in addition to the standard sperm parameters. It has been shown that spermatozoa with broken DNA play a role in infertility and miscarriage [22]. Cryopreservation has been shown in multiple studies to enhance sperm DNA fragmentation after thawing [23,24].

Other showed that cryopreservation has negatively effect on sperm DNA [25]. In contrast with [26] indicated that cryopreservation does not increase sperm DNA destruction. Also, other noted that the freezing and thawing process did not effect on sperm chromatin integrity [27]. The study of [28] reported that spermatozoa from fertile men are found to be more resistant to damage than those from infertile men.

Furthermore, Lusignan et al, [29] concluded that the sperm DFI was not affected during cryopreservation. Cryopreservation may cause to harmful alterations of sperm construction and function [30]. Many damage processes, including thermal shock with the formation of intracellular and extracellular ice crystals, cellular dehydration, increased production of free radicals and reactive oxygen species (ROS) leading to decreased membrane fluidity, increased DNA fragmentation, morphological changes, and decreased spermatozoa's ability to fertilize, have been widely reported to occur during the freezing and thawing of human spermatozoa [31,32].

Cryopreservation may alter the structure of spermatozoon plasma membrane and mitochondrial membranes, which could have a detrimental effect on acrosomal reaction and mitochondrial functions. Because the cryopreserved spermatozoa have a lower antioxidant status, the cryopreservation process is known to produce more reactive oxygen species (ROS), which in turn causes oxidative stress [33], oxidative stress is linked to lipid peroxidation of the sperm outer membrane, which results in sperm motility loss. It also increases damage to the chromatin and plasma membrane and promotes apoptosis and cell death [34]. Other researchers have reported that ROS by interruption of adenosine triphosphate (ATP) production can damage sperms motility [35].

Human spermatozoa that have been cryopreserved may undergo apoptosis due to a number of causes. Elevated levels of reactive oxygen species (ROS) have been found to be positively linked with the triggering of programmed cell death. When ROS concentrations increase or antioxidant enzyme concentrations decrease, the ROS effect overwhelms the cell and causes apoptosis. Under these circumstances, apoptosis is mediated by activation of the BCL-2 family of proteins, which recognize apoptotic stimuli and cause the outer mitochondrial membrane to permeabilize, allowing cytochrome c to be released along with it. The apoptosis cascade is then started when caspase-9 is triggered to generate an apoptosome [36,37]. DNA fragmentation results from the mitochondria's release of

apoptosis-inducing proteins [38]. Cryopreservation may also cause increased sperm DNA fragmentation and change the structure of the spermatozoon's nucleus. and changes to the chromatin structure [39]. During the freeze-thaw process, ROS generation is linked to damage of sperm DNA, chromatin structure, progressive motility, and viability [40]. Cryopreservation process causes changes in osmotic pressure that effect on cell survival during low-temperature storage [41]. Ice crystal formation is one of the primary reasons for cell death during the cryopreservation process. The presence of any solid material within the cell will induce the formation of intracellular ice crystals [42].

From this study, we founded that the addition of pentoxifylline in both concentration (5 mg/mL and 10 mg/mL) to the thawing solutions of some groups improve most sperm parameters and reduced DNA Fragmentation Index post thawing when compared with the groups without the addition of px in both normozoospermia and asthenozoospermia, During our results regarding the positive effect of pentoxifylline when added to thawing solutions, we agree with a study conducted that showed that the cryopreservation and thawing process decreased viability, motility and fertilization potential of human sperm [43]. Also showed use of 3 µ Mpentoxifylline in some group improved the functional parameters of sperm when compared with group without PX post thawing. Also, showed that PX can boost human sperm's resistance to stresses of freeze-thaw cycle and enhance sperm motility in asthenozoospermia without compromising the integrity of the sperm DNA during the vitrification process [44].

Accordingly, the incubating semen samples with 3.6 mmol/l P X for 30 minutes after cryopreservation accelerated the rate of progressive motility [44]. Furthermore, PX addition did not considerably compromise the integrity of the sperm's DNA in asthenozoospermia. The results of current study disagreed with that the addition of pentoxifylline to semen post-thawing did not improve percentage of viability and motility of sperm than groups without PX [45].

Authors explained that adding pentoxifylline to cryoprotectant of some groups leads to an increase in sperm motility before and after freezing compared to groups without the addition of pentoxifylline, also showed the Sperm viability was unchanged between all groups before and .after freezing [46]. Accordingly the pentoxifylline has a positive effect on sheep sperm viability and spermatic membrane preservation, but it has no discernible effect on sperm motility [47].

Pentoxifylline (PTX) is one of Phosphodiesterase inhibitors, PX used pharmaceutically to increase sperm motility in assisted reproductive technologies. It is primarily used to help select viable sperm in asthenozoospermic patients before intracytoplasmic sperm injection (ICSI) [13].

The positive affect of PX on spermatozoa function may be due to Pentoxifylline is an inhibitor of the enzyme cyclic adenosine monophosphate (cAMP) phosphodiesterase, It enhances cAMP levels by inhibiting phosphodiesterase enzyme, Thus, it prevents cAMP breakage cAMP play a crucial role in the glycolysis pathway of the sperm and lead to increases ATP production required for sperm motility [48,49]. The antioxidant capacity of pentoxifylline could be associated to ability to reduce H2O2 and improve sperm function parameters under stress conditions. Pentoxifylline has antioxidant capacity to neutralize H2O2 produced by the spermatozoa of small ruminants [50]. .

Additionally, pentoxifylline inhibits tumor-necrosis factor-alpha (TNF-alpha), which is involved in DNA destruction and programmed cell death (apoptosis) [51,52].

PX interacted with the phospholipid content of the sperm cell membrane, which contains of polyunsaturated fatty acids in great percentage. This interaction of PX with polyunsaturated fatty acid will effect positively to decrease the production of lipid peroxidation which is the source of ROS, These reasons play an important role in reducing DNA damage caused by freezing and thawing [53]. Pentoxifylline safe even at the level of sperms chromatin structure and DNA integrity and preserve ultrastructure of sperms after cryopreservation in asthenozoospermic patients [44].

From the current study, we found the best results of sperm parameters after thawing in the groups with high concentration of pentoxifylline (10mg/mL) than the groups with a low concentration(5 mg/mL) and the groups left without addition of pentoxifylline. Consequently, determining the ideal pentoxifylline concentration is crucial to achieving the best outcome by minimizing the impact of thawing on the effectiveness of frozen sperm.

The results of this study regarding the optimal concentration of pentoxifylline are disagreed with previous study showed that a lesser concentration of pentoxifylline (1 mM) was optimum concentration for cryopreservation purposes. However, showed that 1 mM of pentoxifylline improve percentage of progressive motility post-thawing from 15.3 ± 2.4 to 23.1 ± 3.8%, and percentage of total motility from 27.4 ± 3.3 to 38.2 ± 3.9% ($P < 0.05$).while Sperm motility was found to be unaffected by pentoxifylline at high concentrations of 10 mM [54].

The addition of 3 and 6 mM pentoxifylline were capable to improve percentage of motility and decrease the destruction of goat sperm which caused by cryopreservation and thawing processes [43]. While other showed that 3.5 mM concentration of pentoxifylline has not had any effect on motility of horse sperms but has significantly increased in sperm viability [55]. Additionally, another study revealed that pentoxifylline had a positive effect on sperm motility at low doses (0.01 mM) but a

negative effect at(10 mM) higher doses [56]. Accordingly, the Pentoxifylline at 200 µg/ml concentration lead to significantly increased of sperm viability % but did not significantly affect sperm motility in infertile men with oligoasthenozoospermia [56-59].

Conclusion

The rapid cryopreservation has numerous detrimental effects on sperm, including decreased progressive motility % and sperm viability %, increased immotile sperm %, and increased DFI %. Additionally, we indicate that adding pentoxifylline at certain concentration to the thawing solution that reduces immotile sperm % and DFI % for Asthenozoospermia and normozoospermia. The best results we succeeded with highest concentration of pentoxifylline (10 mg/mL) than low concentration (5 mg/mL).

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