WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 5, Issue 1, 125-134. Research Article ISSN 2277– 7105

EFFECT OF DENSITY GRADIENT TECHNIQUE ON CRYOPRESERVED SEMEN OF ASTHENOZOOSPERMIC MEN USING PENTOXIFYLLINE

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Article Received on
08 Nov 2015,

Revised on 29 Nov 2015,
Accepted on 20 Dec 2015

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ABSTRACT

Background: Pentoxifylline (PTX) as motility stimulant have shown positive effect on *in vitro* sperm activation and improved the forward movement. Indication for sperm cryopreservation is expanding results frozen sperm being as good as fresh sperm in fertilizing oocytes. **Objective:** This experiment has been designed to examine the certain sperm function parameters after *in vitro* preparation of cryopreserved semen by PTX using density gradient method. **Materials and methods:** forty five men were involved in this study that divided into two groups, namely; asthenozoospermic group(no=25), and normozoospermic group (no=20), depending on the results of semen analysis. Ejaculated semen were analyzed and *in vitro* activation technique after cryopreservation-thawing was adopted by using Ham's F-12 (control media) and PTX medium to evaluate the sperm functional characteristics before and after cryopreservation and after activation of cryopreserved human semen by density gradient technique. **Results**: *in vitro* activation of cryopreserved semen with PTX medium have shown a positive effect on sperm motility, grade activity of progressive forward movement and morphologically normal sperm (MNS) in control and treated group compared to post-thawing and other media. **Conclusion:** The study has shown that adding of PTX medium (1 ml) to the cryopreserved semen samples resulted in an improvement in certain sperm functional parameters. It is recommended for more studies in future to carrying out the effect of these stimulants on the DNA of cryopreserved semen.

KEYWORDS: Pentoxifylline, *In vitro* activation, cryopreservation, asthenozoospermia.

INTRODUCTION

The clinical definition of male infertility is the presence of abnormal semen parameters in the partner of couple who have been unable to conceive after 1 year of unprotected intercourse.[1] Half of infertility cases are due to male factors infertility which is due to low sperm production, abnormal sperm function or blockages that prevent the delivery of sperm.Illnesses, injuries, chronic health problems, lifestyle choices and other factors can play a role in causing male infertility.[2]

Male fertility requires normal sperm production and sperm transport, and adequate sexual performance, functions that requires normal levels of testosterone.[3] A semen analysis is the most important examination to be performed to approach the infertile couples.[4] Furthermore, sperm count, motility and percentage of normal sperm form are considered criteria for semen quality.[5]

The PTX is a methylxanthine that acts as a phosphodiesterase inhibitor and subsequently increases intracellular cyclic adenosine monophosphate (cAMP) levels.[6] In general, PTX has been reportedly effective in preserving sperm motility *in vitro*, by mechanism of Cyclic AMP, in turn, is believed to stimulate a cAMP-dependent kinase, which itself induces sperm tail protein phosphorylation with subsequent increase in sperm motility.[7,8]

Cryopreservation 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.[9] Freezing and thawing involve damage to the plasma membrane and acrosome of human spermatozoa as evidenced by significant ultra-structural changes demonstrated by electron microscopy.[10] This damage is exacerbated during the cryopreservation process by a decrease in the lipid content of the membranes, which contain a high proportion of polyunsaturated fatty acids; the decrease in lipid content indicates lipid peroxidation in the presence of reactive oxygen species.[11] Thus the goal of the present work is to found out the effect of PTX medium on the certain sperm function parameters before and after cryopreservation.

MATERIALS AND METHODS

1. Subjects

This study was carried out in the high Institute for Infertility diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. Through the period from November 2014 till April 2015. The clinical examination performed by a consultant urologist in the charge of male infertility Unit in the Institute for all the men attended in this study.

2. Semen samples

Eighty semen samples were taken from the patients who attended to the infertility clinic. Semen samples were collected by masturbation after 3–7 days of sexual abstinence into wide-mouth, clean, dry and sterile glass or plastic petri- dish at especially collection room for this purpose in the institute. Each sample was transported to the laboratory immediately and placed in an incubator at 37C^{o} till complete liquefaction, then semen samples were analyzed by a macroscopic and microscopic examination using standardization of WHO (1999). Then after, the men were divided into two groups depending on their seminal analysis results: Normozoospermic patients and Asthenozoospermic patients.

3. Preparation of Culture Media

3.1. Preparation of Phosphate Buffer Saline (PBS): One liter of PBS was prepared by dissolving the component (8gm of NaCl, 0.1444gm of KH_2PO_4 (BDH) and 0.795gm of Na_2HPO_4) in 990 ml deH_2O then adjust pH to 7.4 after that the volume was corrected to 1000 ml of deH_2O. This solution was filtered by using 0.45 µm Millipore filter, and it was sterilized by autoclaving at 121 ºC and 15 pound/inch for 15 minutes. Finally, it was stored at 4 ºC temperatures in sterile bottle.[12]

3.3.5. Preparation of pentoxifylline for *in vitro* sperm activation

This solution was prepared by dissolving 10 mg from PTX powder (Sigma, USA) in 10 ml of PBS (0.1%) then stirring until dissolve. These concentrations prepared daily under sterile condition using U/V light and Millipore filter (0.45µM).[12]

3. Preparation of Cryoprotectant: The cryoprotectant (Glycerol 15%); was prepared by adding 15 ml of Glycerol (BDH, England); to the 85 ml culture medium (Ham's F-12); then filtrated by (0.22 µM) filter Millipore. The freezing medium was exposed to the U/V light for sterilization.

4. Study Design: After macroscopic and microscopic examination using standardization of WHO (1999), each sample divided into two aliquots and cryopreserved. After one month of cryopreservation. The thawing processes of sample was done through rapidly transfer cryovial from liquid nitrogen to the water bath (Kotterman) 37 ºC until melting ice, for at least 5 minutes. as described by .Then *in vitro* activation was done using the following media :

-Medium (1): Control medium (Hams F 12 free- PTX) was used by adding Ham's F-12(0.5 ml) to (0.5 ml) of cryopreserved thawed sperm.

-Medium (2): Treated medium from PTX (0.1 mg/ml) was used by added 0.5ml of prepared PTX to 0.5ml of cryopreserved thawed sperm .

For both media, the suspension was remained in the incubator at 37ºC for 30 minutes. After that, 1 ml of density gradient solution (80% ,lower layer) and 1 ml of density gradient solution (40% upper layer) were prepared for each sample after thawing .Samples were put in the centrifuge at (4000 rpm) for 20 minutes, then certain sperm function parameters were examined.

- Statistical Analysis: The data of this study were expressed as mean ±SEM. Different statistical analysis were used depending on the nature of data. Student's t-test was used to compare values from experimental and control groups. Differences between values were considered significant at $P<0.05$. Analysis of variance (ANOVA) was used to compare the differences between the four prepared media.

RESULTS

1. Effect of *in vitro* activation by medium containing PTX on certain sperm function parameters of cryopreserved semen of Normozoospermic Group (control) using density gradient technique

In figure (1) showed the result of sperms activation with (Ham's F-12) medium, there was highly significant decrease ($P<0.001$) in sperm concentration compared to the result before activation, and a significant ($P<0.05$) difference in the mean of sperm concentration compared to result of sperm concentration after thawing.

There was a highly significant ($P<0.001$) increase in active sperm motility grade A, B and C activated with Ham's F-12 medium compared with before activation and after thawing. Activation of sperms *in vitro* cause a highly significant ($P<0.001$) increase in the percentage

of MNS in treated Ham's F-12 medium when compared to the results of before activation and after thawing.

The results of *In vitro* sperm activation with PTX medium showed a highly significant decrease (P<0.001) in the sperm concentration compared to the results before activation and a highly significant (P<0.05) difference in the mean of sperm concentration after thawing.

There was a highly significant (P<0.001) increase in active sperm motility grade A, B and C activated with PTX medium compared with before activation and after thawing. Activation of sperms *in vitro* cause a significant (P<0.005) difference in the percentage of morphologically normal sperm in treated PTX medium when compared to the results of before activation and a highly significant (P<0.001) increase in normal sperm morphology after thawing.

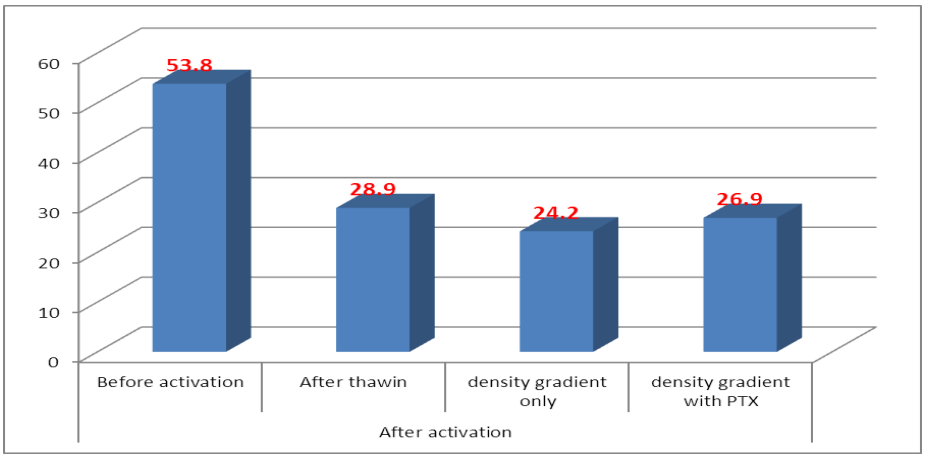

Figure (1): mean of sperm concentration of normozoospermic group after cryopreservation and activation with PTX using density gradient technique.

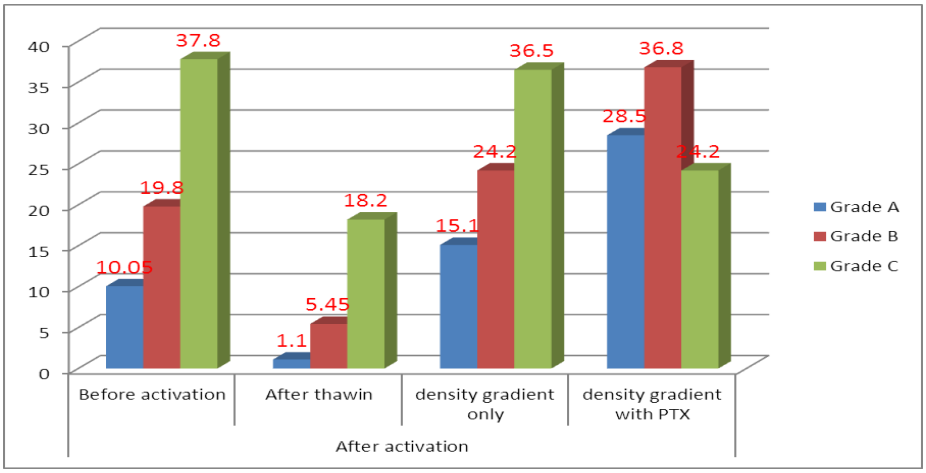

Figure (2): mean of sperm motility of normozoospermic group after cryopreservation and activation with PTX using density gradient technique.

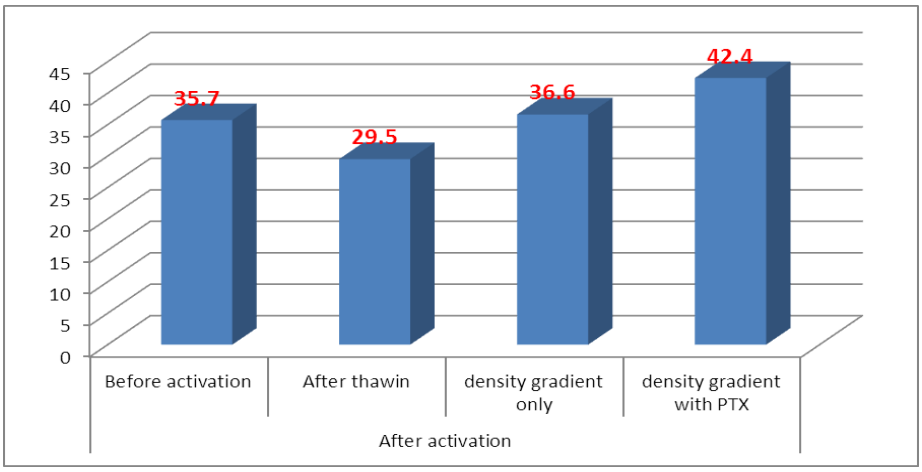

Figure (3): mean of morphologically normal sperm of normozoospermic group after cryopreservation and activation with PTX using density gradient technique

2. Effect of *in vitro* activation by medium containing PTX on certain sperm function parameters of cryopreserved semen of Asthenospermic Group using density gradient technique.

In figure (4), there was highly significant (P<0.001) decrease in sperm concentration compared to the result before activation when Ham's F -12 medium used and a significant (P<0.05) difference in the mean of sperm concentration compared to result of sperm concentration after thawing. The results of *In vitro* sperm activation with PTX medium showed a highly significant (P<0.001) decrease in the sperm concentration compared to the results before activation and a significant (P<0.005) difference in the mean of sperm concentration after thawing.

A highly significant (P<0.001) increase was observed in active sperm motility grade A, B and C that activated with Ham's F-12 medium compared with before activation and after thawing. There was a highly significant (P<0.001) increase in active sperm motility grade A and B compared with before activation and after thawing and a highly significant (P<0.001) decrease was observed in active sperm motility grade C before activation and a highly significant (P<0.001) increase after thawing (figue-5).

Activation of sperms *in vitro* cause a significant (P<0.005) improvement in the percentage MNS in treated Ham's F-12 medium, while there was a highly significant (P<0.001) increase in sperm morphology that activated with Ham's F-12 medium compared with after thawing. Activation of sperms *in vitro* cause a highly significant (P<0.005) improvement in the percentage of MNS in treated PTX medium when compared to the results of before

activation, and a highly significant (P<0.001) increase in compared with after thawing as shown in (figure-6).

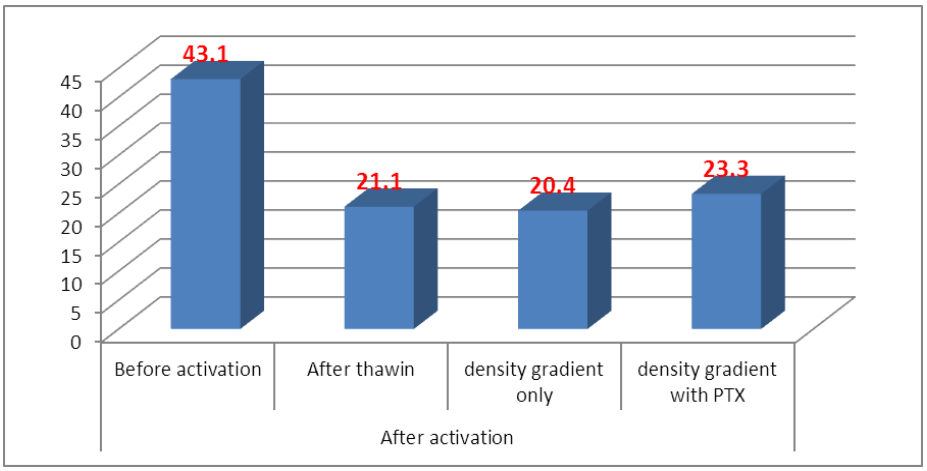

Figure (4): mean of sperm concentration of asthenozoospermic group after cryopreservation and activation with PTX using density gradient technique.

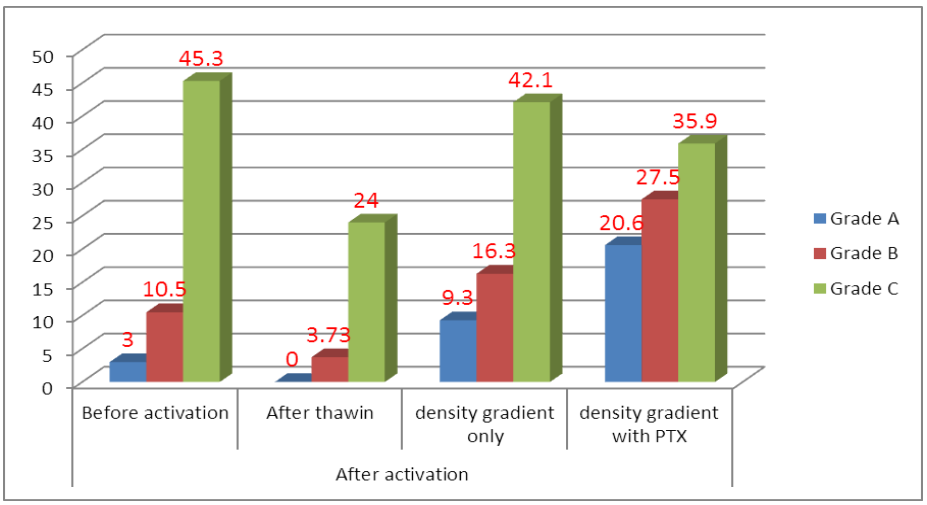

Figure (5): mean of sperm motility of asthenozoospermic group after cryopreservation and activation with PTX using density gradient technique

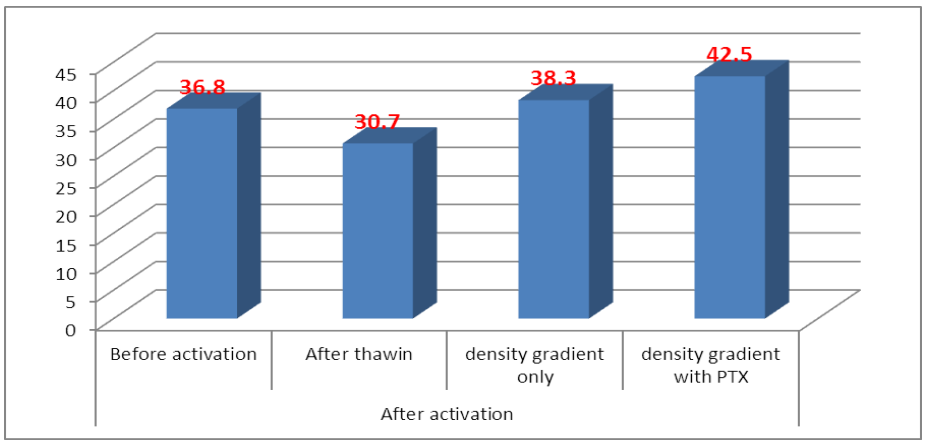

Figure (6): mean of morphologically normal sperm of asthenozoospermic group after cryopreservation and activation with PTX using density gradient technique

DISCUSSION

1. Effect of cryopreservation on certain sperm function parameters for study groups

The result of this study showed a highly significant decrease in sperm concentration, progressive motility and MNS after cryopreservation and thawing. These result compatible with the studies of AL-Dujaily *et al.*[13] The certain sperm parameters decreased as a result of process of freezing and thawing which may inflict irreversible injury on a proportion of human spermatozoa.[14]

2- Effect of *in vitro* activation by Pentoxifylline medium on cryopreserved human semen of study groups using density gradient technique

The presented study has shown that there is a positive effect of PTX medium on certain sperms function parameters (motility and MNS). However, the reason of significantly reduction of sperm concentration following *in vitro* activation is resulted from the inability of the dead and abnormal spermatozoa to swim up and migrate from sperm pellet to the upper layer of culture medium. All the immotile, dead and sluggish sperms remain in the down layer of the medium.[15] There are several external factors that may lead to reduction of sperm concentration of cryopreserved semen samples such as the addition of the diluents to the samples during flushing, activation, adding and removing cryoprotectant and dividing the sample into two groups.

Further significant increment has been shown in active progressive motility following *in vitro* activation by a medium which contains PTX. These results are similar to Tash.[16] This significant increment in forward progressive motility may be due to the effect of PTX on energy supplementation by cAMP. PTX is considered a phosphodiesterase inhibitor of the methylxantine group.[17] It is plays an important role in an inhibition of cAMP breakdown and also known that intracellular cAMP concentration plays great role in supplying the cell with energy and Ca^{+2} which in turn sustains sperm motility. Also increasing in cAMP leads to an increase in progressive sperm motility. Furthermore the cAMP play a central role in the glycolytic pathway of the sperm and, through its effect on glycolysis, it can effect on the energy generation required for sperm motion.[18]

It has been detected that cAMP activates cAMP-dependent protein Kinase.[18] This in turn is believed to induce enhancement of protein phosphorylation of a defined number of specific proteins.[19] Certain effects like Ca^{+2} may be linked to a stimulation of adenylate cyclase, resulting in an increase in intracellular cAMP production. In addition, PTX has protective

effect on sperm membranes, it may play a role in scavenging the reactive oxygen radicals and reduces lipid peroxidation.[19] PTX has been shown to scavenge hydroxyl as well as superoxide radicals and inhibit their release.[17] Small doses of PTX have proven to be effective in their ability to scavenge ROS in cryopreserved sperm. The PTX also demonstrates a beneficial effect in reducing ROS.[19]

The data also show a significant increase in morphologically normal sperm, these result are compatible with.[17] This finding may due to the fast movement of normal spermatozoa from seminal plasma into the upper layer of the culture medium, and consequently elicited from impact of some seminal plasma components like leukocytes, round cell and others leading to keep the sperm out of stress factor and ROS production responsible for DNA damage.[19] It was concluded that PTX medium can be utilized for *in vitro* activation to maintain the sperms from freezing and post thawing damage of asthenospermic men.

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