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A STIMULATORY APPROACH FOR ACTIVATION OF FROZEN AND THAWED SPERM MOTILITY BY USING THEOPHYLLINE IN ASTHENOZOOSPERMIC MEN

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ABSTRACT

Background: From each six couples, one is affected by infertility and half of these cases traced back to male. Sperm cryopreservation is a useful domain of Assisted Reproductive Technologies (ART's) used to preserve men fertility but unfortunately may be consequence of cryoinjury. Theophylline is an activating substance using to enhance and prolong fresh and thawed sperm motion by increasing a source of energy. **Objective:** This study was designed to evaluate whether in *vitro* sperm activation by Theophylline solution before cryopreservation improves percentages of sperm parameters post freezing-thawing process as compared with activation if done by the same substance but after cryopreservation. **Subjects, Materials and Methods**: Sixty male were participated in this study, twenty normozoospermic male and forty asthenozoospermic male. Semen samples were collected and seminal fluid analysis was done according to (WHO 1999, 2010). Each sample was divided into two aliquots, one of which was equally divided into two groups, the centrifugation was done to remove seminal plasma and then Swim-up technique was dependent for *in vitro* sperm activation by using FertiCult medium and Theophylline solution respectively prior to cryopreservation and one of which was also divided and activated by the same materials but post cryopreservation. Sperm parameters were measured in each activated group according to (WHO 1999). **Results:** using of FertiCult Flushing medium and Theophylline solution to activate fresh semen resulted in significant reduction in sperm concentration and also resulted in highly significant improvement (P<0.001) in percentage of sperm parameters in comparison with non-activated ones. However, Theophylline solution

resulted in least reduction in sperm concentration and better results of progressive and non-progressive sperm motility also the percent of morphologically normal sperm (MNS). Pre-freeze activation of healthy control subjects and asthenozoospermic male patients did not improve post-thaw sperm parameters as compared when used these activation media post-freeze to activate post thawed sperm (non-activated), the later activation gave it significant increase ($P<0.05$) in percent of sperm motility and MNS. **Conclusion*:*** From the comparisons among both groups before cryopreservation and after cryopreservation, whatever treatment of semen is, the concentration of sperm will be reduced and sperm motility and MNS will be improved after activation with FertiCult Flushing medium and Theophylline solution. In both procedures, fresh semen and thawed semen, using of Theophylline resulted in the best increases in sperm motility and MNS percent.

KEYWORDS: Asthenozoospermic, *In vitro* activation, Theophylline, Cryopreservation.

INTRODUCTION

The World health Organization reports infertility as a failure of reproductive tract to achieve pregnancy after one year or more of regular unprotected sexual intercourse.[1] Infertility affecting about 15% of couples at reproductive age and male cause is responsible for about half of these cases. Etiology for male factor infertility is multifactorial.[2] The basic diagnosis can offer insight into the cause of male infertility may be done by the conventional semen analysis. Asthenozoospermia is one of most important causes of male infertility and can influence the successful of pregnancy rates next ARTs.[3] The culture media (CM) deliver spermatozoa with needs that maintain optimal function in order to give a good results during preparation, methylxanthines supplementation to CM are commonly used in order to improve sperm characteristics, resulted in better motility in fresh and cryopreserved spermatozoa and among methylxanthine derivatives, Theophylline has been used for enhanced motility in human semen by increasing of intracellular concentrations of cyclic adenosine mono phosphate (cAMP), a molecule involved in generation of sperm energy which is a result of its inhibitory properties on phosphodiesterase (PDE) function.[4,5] Cryopreservation of human sperm is an important routine process nowadays used for numerous purposes such as protection of sperm for patients of chemotherapy, but unfortunately, the negative effects of cryopreservation on viability, motility, morphology and the Deoxyribonucleic acid (DNA) integrity of spermatozoa and fertilization ability have been proved.

SUBJECTS, MATERIALS AND METHODS

Subjects

Sixty male were involved in this research was carried out in the high Institute for Infertility diagnosis and Assisted Reproductive Technologies at Al-Nahrain University and Kamal Al-Samarrai Hospital Fertility Center Infertility Treatment and IVF. During the period from November 2016 till April 2017, twenty normozoospermic and forty asthenozoospermic male. The clinical examination performed by a consultant urologist in charge of male infertility Unit.

Semen Specimen Collection

Semen samples were collected by masturbation into wide-mouth containers, collected in a private room near laboratory, after a 3 -7 days abstinence period, sample directly placed in an incubator at 37C° for 30-60 minutes for liquefaction completing and analyzed macroscopically and microscopically according to standardization of WHO 1999, 2010.[6]

Preparation of Theophylline Solution for in *vitro* sperm activation

Theophylline solution was prepared by dissolving (0.036 mg) from Theophylline powder (TRANSHUMAN TECHNOLOGIES LTD-Uinted Kingdom)in (10 ml) of FertiCult Flushing medium.[4] The solution was prepared everyday under sterilized conditions by U/V light and Millipore filter (0.45μM).

In vitro sperm activation and cryopreservation technique

After liquefaction, each semen sample was divided into two equal portions, first one (non-activated, then later after cryopreservation will be divided into two equal portions) is washing by using FertiCult Flushing Medium and centrifuged 1500 rpm for 10 minute to remove seminal plasma and then freeze; Sperm Freeze medium added slowly with swirling for 10 minute at a room temperature (0.7 ml of sperm Freeze medium to every 1 ml semen). Sample was exposed to static vapor phase cooling for 15 minute before it was plunged into liquid nitrogen (LN_2) tank for one month. The other half of semen sample was divided into two equal portions, processing also was done by centrifugation of semen with FertiCult medium to wash it and pellet forming from these portions were resuspended by FertiCult medium and Theophylline solution respectively to complete sample volume to the original volume which centrifuged for each portion and then they were incubated for 30 minute. Certain sperm function parameters were assessed microscopically according to (WHO1999) guidelines[7], and then sperm Freeze medium was added and same steps of freezing done.

After one month of freezing, thawing process of all samples was done via a rapid transfer of cryovials from LN_2 to water bath at 37ºC until melting ice. Then transfer contents of each one into conical – bottom tube, the content of the first thawed cryovial was non-activated thawed sample, divided into two equal portions each one were put in a tube, washing medium was added slowly to all four tubes; (the first two tubes were contained thawed semen were non-activated before cryopreservation and the last two tubes contained thawed semen were pre-activated before cryopreservation); with centrifugation in order to remove freeze medium out from four samples, supernatant was discharged and 0.5ml of FertiCult Flushing medium was added to pellets of the last two groups. One drop of each sample was examined microscopically to assess sperm parameters after thawing.While pellets of the first two groups were activated by incubation with FertiCult Flushing medium and Theophylline solution and also each sample was tested microscopically to assess sperm parameters.

Statistical Analysis

Data were collected, summarized, analyzed and presented using three statistical software programs: the statistical package for social science (SPSS version 22), Microsoft Office Excel 2013 and MedCalc 2014. Categorical variables were presented as number and percentage whereas numeric variables were presented as mean and standard deviation (SD). Comparison of mean values between any two groups was carried out using independent samples-t test; comparison of mean values among more than two groups was carried out using one way ANOVA followed by post hoc LSD test. P-value was considered significant when it was equal to or less than 0.05.[8]

RESULTS

***In vitro* sperm activation before cryopreservation**

Results of most sperm parameters in healthy control subjects and asthenozoospermic male patients before cryopreservation were appeared highly significant ($P<0.01$) after activation by using of FertiCult Flushing medium and Theophylline solution as a comparison between them within the same group (control or asthenozoospermic male group) and without activation. In the healthy control subjects, after being activated with FertiCult Flushing medium resulted in: significant reduction in sperm concentration, significant increase in sperm motility grade A %, insignificant change in sperm motility grade B %, significant increase in combined grade A and B %, insignificant change in sperm motility grade C %, significant reduction in sperm motility grade D % and significant increase in MNS % as

compared with non-activated semen as shown in (Table1). While in the asthenozoospermic male patients, a comparison between non - activated semen and after being activated with FertiCult Flushing medium; resulted in significant reduction in sperm concentration, significant increase in sperm motility grade A%, B%, combined grade A and B%, C% and MNS% with significant reduction in sperm motility grade D%.

Table 1: Sperm characteristics in both groups after *in vitro* activation by FertiCult Medium.

Groups	Sperm characteristics	No activation	FertiCult media	P-value
		Mean ± SD	Mean ± SD	
Control group n = 20	Concentration(millions/mL)	47.00 ±10.66	30.55 ±6.50	<0.001
	Grade A %	3.60 ±4.50	9.10 ±5.42	<0.001
	Grade B %	36.30 ±7.99	39.25 ±6.73	0.074
	Grade A+B %	39.90 ±5.19	48.35 ±8.15	0.001
	Grade C %	29.95 ±6.82	27.60 ±7.34	0.189
	Grade D %	30.15 ±7.29	24.05 ±5.84	<0.001
	MNS %	40.25 ±4.72	41.90 ±4.19	<0.001
Study group n = 40	Concentration(millions/mL)	33.40 ±8.70	17.98 ±4.67	<0.001
	Grade A %	2.55 ±3.18	10.25 ±5.45	<0.001
	Grade B %	22.05 ±4.15	32.38 ±5.55	<0.001
	Grade A+B %	24.60 ±4.20	42.63 ±8.68	<0.001
	Grade C %	24.53 ±6.61	28.10 ±6.86	0.001
	Grade D %	50.88 ±7.17	29.28 ±8.22	<0.001
	MNS %	35.38 ±3.08	36.50 ±3.08	0.031

As shown in Table (2) it showed a comparison between sperm functional parameters of non-activated semen and after being activated with Theophylline solution. In healthy control group Theophylline solution resulted in: significant reduction in sperm concentration, significant increase in percentage of sperm motility grade A, B, combined A and B, significant reduction in percent of sperm motility grade D and significant increase in percentage of MNS. While in asthenozoospermic male group, Theophylline solution resulted in the following: significant reduction in sperm concentration, significant increase in percent of sperm motility grade A, B sperm, combined A and B, C and significant reduction in grade D with significant increase in percent of MNS as shown in table (2).

Table 2: Sperm characteristics in both groups after *in vitro* activation by Theophylline.

Groups	Sperm characteristics	No activation	Theophylline solution	P-value
		Mean ± SD	Mean ± SD	
Control group n = 20	Concentration (millions/mL)	47.00 ±10.66	31.20 ±7.46	<0.001
	Grade A %	3.60 ±4.50	10.70 ±5.86	<0.001
	Grade B %	36.30 ±7.99	39.95 ±8.44	0.040
	Grade A+B %	39.90 ±5.19	50.65 ±8.31	<0.001
	Grade C %	29.95 ±6.82	28.10 ±7.87	0.302
	Grade D %	30.15 ±7.29	21.25 ±6.78	<0.001
	MNS %	40.25 ±4.72	41.65 ±3.80	0.122
Study group n = 40	Concentration (millions/mL)	33.40 ±8.70	18.28 ±5.14	<0.001
	Grade A %	2.55 ±3.18	9.25 ±5.28	<0.001
	Grade B %	22.05 ±4.15	35.28 ±7.36	<0.001
	Grade A+B %	24.60 ±4.20	44.53 ±8.94	<0.001
	Grade C %	24.53 ±6.61	29.23 ±6.45	<0.001
	Grade D %	50.88 ±7.17	26.23 ±7.09	<0.001
	MNS %	35.38 ±3.08	37.20 ±3.28	<0.001

***In vitro* sperm activation after thawing and comparison with the result of thawed sperm has been activated prior freezing.**

Post thawing and for purpose of comparison of sperm functional characteristics in healthy control subjects and asthenozoospermic patients, four groups in control category and four groups in asthenozoospermic category, the first and second groups in which post-thawed semen were activated with FerticCult Flushing medium and Theophylline respectively, while third and fourth groups were preserved with FertiCult medium and Theophylline solution respectively then they were thawed.

The results in which first group (FertiCult Flushing medium) as compared with last two groups (pre-activated groups) regarding healthy control subjects and asthenozoospermic male patients was the best as shown in table (3), regarding sperm concentration, percentage of sperm motility grade A,B, combined A and B, C and MNS. Also showed in the same table results in which second group (activated cryopreserved- thawed semen with Theophylline solution after being thawed) was compared with last two pre-activated groups for both of healthy control subjects and asthenozoospermic male patients; Theophylline resulted in better results regarding sperm functional parameters.

Table 3: Sperm characteristics in healthy control subjects and asthenozoospermic patients after *in vitro* activation by FertiCult medium and Theophylline solution.

Groups	Sperm characteristic	FertiCult medium	Theophylline solution	Pre-Activated FertiCult medium	Pre-Activated Theophylline solution
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control group n = 20	Concentration (millions/mL)	15.25 ±3.34 a	15.55 ±3.78 a	14.05 ±3.05 b,b	13.65 ±3.03 c,c
	Grade A %	3.35 ±2.89 b	3.85 ±3.53 b	3.00 ±3.08 c,c	1.20 ±1.79 d,d
	Grade B %	21.25 ±5.53 a	23.35 ±5.74 a	19.90 ±3.81 b,b	17.80 ±4.02 c,c
	Grade A+B %	24.60 ±6.18 a	27.20 ±5.51 a	22.90 ±4.87 b,b	19.00 ±4.04 d,d
	Grade C %	26.55 ±5.24 a	25.05 ±5.39 b	26.00 ±3.24 a,a	24.25 ±3.63 b,c
	Grade D %	48.85 ±3.30 d	47.75 ±4.09 d	51.10 ±4.81 c,c	56.75 ±4.04 b,b
	MNS %	33.55 ±3.32 a	34.55 ±2.76 a	29.75 ±2.40 b,b	27.20 ±2.17 c,c
Study group n = 40	Concentration (millions/mL)	12.10 ±1.95 a	12.33 ±2.03 a	10.40 ±1.77 b,b	9.75 ±1.79 c,c
	Grade A %	2.48 ±3.03 c	4.70 ±4.72 a	2.88 ±3.57 b,b	1.03 ±2.40 d,c
	Grade B %	20.20 ±5.44 a	20.83 ±5.76 a	17.93 ±4.71 b,b	16.55 ±5.80 c,c
	Grade A+B %	22.68 ±7.24 a	25.53 ±6.39 a	20.80 ±6.28 b,b	17.58 ±6.22 d,d
	Grade C %	24.33 ±5.96 a	26.58 ±7.17 a	23.05 ±4.38 b,b	23.08 ±5.23 b,b
	Grade D %	53.00 ±7.47 d	48.88 ±6.13 d	55.90 ±5.92 c,c	59.20 ±5.38 b,b
	MNS %	28.93 ±2.77 a	33.68 ±2.76 a	28.08 ±2.96 a,b	26.90 ±2.73 b,c

Values are expressed as Mean±SD.

Different small letters mean significant difference at $P<0.05$ between activated groups.

DISCUSSION

Before cryopreservation, *in vitro* sperm activation of healthy control subjects and asthenozoospermic male patients by using of FertiCult Flushing medium and Theophylline solution result in, significant reduction in sperm concentration as comparison with non-activated semen samples, this finding may be related to effects of semen preparation used in the present work by centrifugation, swim-up technique to eliminate a low motility sperm,

immotile sperm and semen debris and just good quality sperm could swim up to upper and dead sperm remain down.[9,10] The using of FertiCult Flushing medium as a culture medium led to improve of progressive sperm motility and MNS as compared with non- activated fresh semen as shown in table (1), that because of their aqueous nature with lower viscosity than of seminal plasma resulted in making movement of sperm more freely.[11] FertiCult medium contains many components that are essential in physiology and metabolism of sperm such as serum albumin which plays a protective role in spermatozoa integrity, it (contains cysteine-34(34Cys) which may capture radicals that is critical for maintaining MNS.[12] As shown in table (2) there was a highly significant increase in progressive sperm motility and MNS after activation by Theophylline at concentration (20mM), this result was in agreement with Loughlin KR.[4], Theophylline offering potential to augment sperm functional parameters especially motility and this due to Theophylline ability on inhibition of PDE activity and thus increasing intracellular cAMP level which play a major role in glycolytic pathways of sperm and it can influence the energy generation which required for sperm motility.[4,13] Motility is a critical function of sperm in that it is required to complete its function of reaching the oocyte and ultimately for fertilization to occur (WHO, 2010). In present study the quantitative motility for asthenozoospermic male group was 48% improved to became 73% after activation by Theophylline, about 25% percent more than of original sample, this result was in agreement with Hong *et al*.[14] who found the stimulating effect of dimethylxanthine can induce amplitude sperm motility by more than 50% of control.

Also showed in table (2) a highly significant increase in progressive sperm motility grade (A) for both groups after activation by Theophylline solution; the increasing were about (3 and 4) fold of original samples this result was near to those reported in Jaiswal**[15]** who noticed three fold increments when Theophylline used at (30mM) concentration, the possible explanation was that Theophylline activation role may increase intra sperm cAMP content to level greater than the threshold level, thereby causing induction of forward motility. Regarding increase in MNS percentage, after activation by Theophylline this may be due to effect both of the sperm preparation technique and components of liquid artificial medium that used in this study to enhance MNS, Theophylline has a protective effect on sperm membranes as it being oxygen radical scavengers, neutralizing ROS and reducing lipid peroxidation that prevents oxidative stress-induced DNA damage.[16]

After cryopreservation, there were significant decreased in sperm concentration, percentage of progressive and non - progressive sperm motility and MNS for all groups after freezing-Thawing as compared with pre –freezing samples these indicated to deleterious changes in cryopreserved sperm which can be attributed to physical and chemical stress occurring during freezing and thawing process that due to thermal shock with formation of intracellular and extracellular ice crystals, dehydration of cell, increased salts concentration, oxidative stress and osmotic shock that can modify structure of sperm plasma membrane after cryopreservation.[17,18] Regarding healthy control subjects and asthenozoospermic patients, results of first group(activating with FertiCult Flushing medium after being thawed) was compared with the last two groups (pre-activated groups), was the better results regarding sperm concentration, percentage of sperm motility grade A, B, combined A and B,C and MNS as shown in table (3), This may be due to good preparation of post-thawed semen (first group) by washing and swim up technique with FertiCult Flushing medium was performed to discard cryoprotectant medium and decrease of abnormal spermatozoa that resulted in cryopreservation and increase of motile sperm with good yield[19] and the first group result was better than pre-activated groups may be from the production of ROS with prolonged incubation time for the last two group; as compared with first group incubation was just after cryopreservation and may be as a result of higher concentration of spermatozoa and higher of dead sperm cells of pre-activated groups before cryopreservation resulted in greater amounts of ROS level production that induce peroxidative damage and a loss of sperm function additional to that occurred or induce during freezing and that is possibly explains the diminished survival of a much yield of sperm obtained from pre-treatment with activation media after thawing.[20] Theophylline can enhance fresh and thawed sperm functions especially motility by its inhibitory effect on PDE, which in turn helps in maintaining a high cAMP level in spermatozoa.[4,21] In this study, there was significant increase in sperm functional parameters in the first group as compared with Theophylline (pre-activated) groups, the negative effects resulted from adding Theophylline prior to freezing on thawed human spermatozoa could be result of a longer exposure of sperm to this compound through activation, freezing and thawing.[22] Deterioration in plasma membrane integrity rise after each cryopreservation step and sperm perhaps to be sensitized and need a different concentrations of this compound, some studies referred to the concentration-dependent toxic effect also may be occur when the substance is added to semen before freezing.[23] The quality and percent of sperm before freezing is related to their cryo behavior and survival of cryopreservation.[24]

There was significant increase in MNS% for first groups as compared with last two groups (pre-activated groups), this due to the role of sperm preparation techniques and FertiCult medium component as mentioned earlier, while results of MNS in pre-activated groups were significant decreased, that could be due to osmolality change in the media which may be responsible for change in the membrane permeable to solutes concentration, thereby increasing of solutes and osmotic pressure inside cell and causing changes in sperm volume due to dehydration and tail coiling.[25]

In the healthy control subjects and asthenozoospermic patients, second group activating with Theophylline solution (after being thawed) resulted in best results regarding sperm functional parameters as compared with last two groups (pre-activated groups). Methylxanthine enhance fresh and cryopreserved spermatozoa functions especially motility and these effects has been attributed to their potent of PDE inhibitory action and elevation of cAMP levels.**[21,26]** On the other hand, Theophylline acted as oxygen radical scavengers used to stop the auto-oxidation which occur during thawing process that causes a chain of reaction in the unsaturated fatty acids in lipid and that help in slowing down the oxidation of lipid oxygen reacts preferentially with antioxidants rather than oxidizing lipid, thereby protecting them from hurt. The interaction of Theophylline with polyunsaturated fatty acid will have a positive effect; it decreases lipid peroxidation and increase in DNA normality after activation of cryopreserved samples.**[27,28]** Numerous studies have claimed that a high quality of motile and MNS also is obtained via the swim-up procedure, that due to the size and shape of normal mammalian sperm are separated under hydrodynamic selection for optimal swimming efficiency.[29]

In current study there was significant improvement in progressive sperm motility after activation with Theophylline and was more obvious in asthenozoospermic male patients, this was in agreement with results of previous studies which demonstrated that methylxanthine was useful for improving the motility of spermatozoa in the suboptimal human semen sample.**[30,28]** The concentration of the ROS is unusually high in subfertile men. Moreover, It is also recognized that cryopreservation can induce sperm cells to lose their antioxidant defense and shift in the levels of ROS towards pro-oxidants in semen can induce an oxidative stress on spermatozoa.[31] Addition of Theophylline solution to post-thawed sperm was necessary to neutralize excessive of ROS and that increase intracellular cAMP concentrations often cause an increase in sperm motility and kinematics.[32,33]

CONCLUSION

All activation media which used in this study before and after cryopreservation resulted in significant reduction in sperm concentration(best yield), Theophylline solution resulted in least reduction in sperm concentration and better result of percentage of sperm motility grade A, B, combined grade A and B, C and morphologically normal sperm.

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