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Comparison of Best Yield of *in vitro* Sperm activation Techniques with New technique of Caffeine Combined with Density Gradient Centrifugation in Iraqi Patients

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Abstract

Background:

Many of sperm preparation techniques exist to select spermatozoa with good quality, as the classical parameters of sperm number, motility and morphology and other sperm parameters to provide successful assisted reproductive techniques.Many studies have been conducted on Caffeine as sperm motility stimulants which have shown positive effect on the *in vitro* activation of sperm and improved the progressive motility.

Objective:

The aim of this study was to assess the beneficial role of Caffeine combined with Density Gradient Centrifugation technique for activation of human sperm of asthenozoospermic,oligozoospermic and normozoospermic subjects compared with the other *in vitro* activation techniques.

Patients, Materials and Methods:

Sixty-five males were involved in this study, divided into three groups, (Thirty-five asthenozoospermic, fifteen oligozoospermic and fifteen normozoospermic subjects)during their attendance to the Infertility Clinic at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies; Al- Nahrain University. Semen samples were obtained, and seminal fluid analysis was assessed. Each semen sample was divided into four parts. The first part prepared as *in vitro* sperm activation using the direct swim-up technique, the second part usingin direct swim-up technique,the third part using density gradient centrifugation technique, while the last part prepared using density gradient centrifugation combined with Caffeine.

Results:

After *in vitro* sperm activation for asthenozoospermic, oligozoospermic and normozoospermic samples a significant increase was observed in the sperm function parameters including sperm motility and morphologically normal sperm percentage as compared to pre-activation using combined techniques (density gradient centrifugation and Caffeine) as compared to the three other techniques.

Conclusions:

Density gradient centrifugation techniquealone and combined with Caffeine was found a higher significant result on sperm function parameters (sperm motility and morphology) when using a low quality of semen samples such as decreased sperm motility as compared with the two other technique (direct and indirect swim-up techniques).

Key words: Density gradient centrifugation, Caffeine,Asthenozoospermia and Oligozoospermia.

INTRODUCTION:

Many of sperm preparation techniques exist to separate viable sperm from the seminal fluid for use in assisted reproductive technologies (ART's) [1]. Motility of sperm is very important to penetrate the cumulus cells and zona pellucida surrounding the oocyte [2,3] .Moreover, a change in the sperm motility can be indicated by hyperactivation , the change of motility pattern is from steady and symmetrical flagellate bends to high amplitude and asymmetrical flagellate bends [4,5] .Density gradient centrifugation technique modified to treat the issues of each individual specimen, and it is the method of choice for preparation of the sperm in the majority of ART's and andrology laboratories[6].

In ART's and andrology laboratories, to study the hyperactivation of sperm there are many ingredients were used as an attempt to improve the semen quality by using Caffeine as an example of sperm motility stimulating factors[7]. Caffeine is a cyclic nucleotide phosphodiesterase inhibitor, causing in an increase in the intracellular cyclic adenosine monophosphate (cAMP), stimulating of capacitation and the sperm acrosomal reaction [8], and increasing of sperm motility [9] . Depending on the concentration of calcium ions, Caffeine may have a direct effect on metabolism of cells [10].

PATIENTS, MATERIALS AND METHODS:

In this study,samples were collected for thirty-five (35) asthenozoospermic, fifteen (15) oligozoospermic infertile men and fifteen (15, as control) fertile men during their attendance to the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies (ART's) at Al- Nahrain University,during the period was from September 2016 to March 2017.

Examination of the samples was done before and after sperm preparation techniques according to WHO (1999, 2010). The effect of these four techniques on sperm motility parameters was examined to select the most effective techniques. Every sample was divided into four parts. The first one using the density gradient centrifugation technique, the second one using the density gradient centrifugation technique with 3mM/mL of Caffeine, the third one using the centrifugation swim-up migration technique and the last one using direct swim-up technique. The effect of activation by these methods on sperm concentration, sperm motility, grade activity and morphologically normal sperm percentage were examined and statistically analyzed**.**

1-Direct Swim-Up Technique

The technique was performed by adding (1mL) of semen to tube containing (1mL) of FertiCult^{TM}–Flushing medium (semen layered beneath a culture medium), then incubate at 37degree for (30-60) minutes in air incubator. After incubation the supernatant was collected, which contain the progressive motile spermatozoa that's migrate from the semen layer into the culture medium. Then, assessment of the sperm parameters done by a drop of 10µL was aspirated, put on a slide with cover slip and examined under the microscope at 400X objective [11].

2-Direct Sperm Activation Method (Centrifugation Swim-Up Migration Technique)

The technique was performed by washing the seminal fluid with culture media (FertiCult™–Flushing medium), and centrifuged for 5 minutes at 2500 R.P.M. The supernatant was discarded and 0.5 mL of FertiCult™ -Flushing medium was added to the sperm pellet slowly. The pellet with culture media was incubated for 30 and 60 minutes in air incubator. Methodology of this method is based on the spermatozoa active movement from the prewashed cell pellet into a culture medium. Then assessment of the sperm parameters done by a drop of 10µL was aspirated, put on a slide with cover slip and examined under the microscope at 400X objective [12].

3-Discontinuous Density Gradients Technique

Separation of the sperm by density gradient technique, has become a standard technique in many ART's laboratories because it can be done easily and quickly resulting a high motile sperm recovery [13]. The technique was performed by adding 1mL of 80% Sil-Select Plus gradient as the lower layer, then 1mL of 40% Sil-Select Plus gradient as the upper layer of solution and liquefied semen is layered over density-gradients system (colloidal silica coated with silane) in tube. Centrifugation of the prepared tube, motile spermatozoa swim actively through the gradient material which separates cells by their density to form a soft pellet at the bottom of the tube. Discard of the supernatant and adding 0.5mL of FertiCult™–Flushing medium in the tube to the pellet. Incubate the tube at 37degree in air incubator for 30-60 minute. Sperm parameters were examined by aspiration of 10µL with micropipette which put on a slid and covered with cover slip [13].

4- Discontinuous Density Gradients Technique with Caffeine

This method as same asdiscontinuous density gradients technique without Caffeine but, 0.5mL of Caffeine stock was added in the tube to the pellet when the supernatant was discarded and sperm parameters were examined by aspiration of 10µL with micropipette which put on a slid and covered with cover slip.

RESULTS:

Result of *in vitro* sperm activation for asthenozoospermic and oligozoospermic and normozoospermic samples using these four techniques, a significant increase was observed in the sperm function parameters including progressive sperm motility and morphologically normal sperm as compared to pre-activation. Besides, it was observed a significant reduction in the other sperm function parameters (concentration, agglutination and round cells count). Furthermore, the present study showed a significant increase in certain sperm function parameters such as sperm motility stimulation and morphologically normal sperm for all types of samples using combined techniques (density gradient centrifugation and Caffeine)as compared to the three other techniques. It was appeared that density gradient centrifugation combined with Caffeine resulted in significantly superior results and shown a positive effect for the concentration and total number of progressive motile of the sperm rather than direct swim-up technique,indirect swim-up technique, and density gradient centrifugation technique in all types of samples.

Table (1):Pre-and post *in vitro* sperm activation comparison in sperm progressive motility grade A between groups.

Activation Technique	Mean ± SE of A Progressive			LSD value
	Asthenozoospermia	Normozoospermia	Oligozoospermia	
Before activation	0.742 ± 0.37 D b	8.33 ± 1.12D a	0.60 ± 0.10C b	3.742 *
Direct technique	1.94 ± 0.62 D b	12.73 ± 1.47CD a	0.876 ± 0.54C b	4.167 *
Indirect technique	4.08 ± 0.97 C b	16.73 ± 1.56C a	2.07 ± 1.08BC b	4.092 *
Density gradient	7.45 ± 1.44 B b	23.93 ± 1.80B a	3.60 ± 1.47B b	4.175 *
Density gradient + Caffeine	11.88 ± 1.92 A b	31.06 ± 2.34A a	6.33 ± 2.12A c	3.996 *
LSD value	2.679 *	5.802 *	2.094 *	---

* (P<0.05) Means having with the different big letters in same column and small letters in same row differed significantly.

Table (2):Pre-and post *in vitro* sperm activation comparison in sperm progressive motility grade B between groups.

Activation Technique	Mean ± SE of B Progressive			LSD value
	Asthenozoospermia	Normozoospermia	Oligozoospermia	
Before activation	28.23 ± 1.63 D b	44.53 ± 0.81B a	25.06 ± 2.86D b	6.027 *
Direct technique	40.48 ± 1.82 C b	47.80 ± 1.75AB a	34.13 ± 3.12C b	7.084 *
Indirect technique	47.37 ± 2.08 B a	49.73 ± 2.05A a	39.00 ± 3.52BC b	6.463 *
Density gradient	53.48 ± 1.60 B a	51.73 ± 1.78A a	43.00 ± 3.21B b	6.902 *
Density gradient + Caffeine	59.80 ± 1.48 A a	50.20 ± 2.09A b	50.46 ± 2.93A b	6.703 *
LSD value	6.463 *	5.275 *	6.283 *	---

* (P<0.05). Means having with the different big letters in same column and small letters in same row differed significantly.

Table (3):Pre-and post *in vitro* sperm activation comparison in Morphologically Normal Sperm between groups.

Activation Technique	Mean ± SE of MNS			LSD value
	Asthenozoospermia	Normozoospermia	Oligozoospermia	
Before activation	34.00 ± 1.77 D b	45.33 ± 1.35D a	15.11 ± 3.90D c	7.935 *
Direct technique	49.11 ± 1.57 C b	57.00 ± 1.75CD a	42.67 ± 1.99C c	6.003 *
Indirect technique	57.20 ± 1.75 BC a	63.40 ± 1.54BC a	49.00 ± 2.59BC b	6.931 *
Density gradient	65.97 ± 1.63 AB b	73.20 ± 1.27B a	56.46 ± 3.12AB c	6.402 *
Density gradient + Caffeine	75.00 ± 1.57 A b	85.80 ± 1.89A a	65.93 ± 3.39A c	7.259 *
LSD value	11.726 *	10.464 *	10.613 *	---

* (P<0.05).Means having with the different big letters in same column and small letters in same row differed significantly.

DISCUSSION:

Semen analysis is an important diagnostic step to evaluate male fertility for assisted reproductive technologies (ART's), because of spermatozoa nature as morphology, vitality, motility and seminal fluid composition are important for sperm function [14] .

Morphologically normal sperm besides the concentration and motility of sperm are good indicators of semen quality, as ability to fertilize the oocyte[15] .The aim of sperm preparation techniques is to separate the motile sperm cells from the other contents of semen as immotile sperm cells, leukocytes, epithelial cells, debris, any other content that produce reactive oxygen species and eliminate agglutination [16] , which may be resulted from the anti-sperm antibodies presence and cause sperm sticking to each other in a variable degree which result in limitation of sperm motility, to provide better quality of sperm regarding concentration and motility, which may lead to advanced success rates for assisted reproduction [17].

In this study, there is a significant(P<0.05)result for the four techniques of increasing sperm motility noticed with progressive motile sperm (grade A and grade B). This result due to activation of sperm with culture media, so sperm of grade B become grade A and the same for other grades. But, the data of this study showed the high percentage of motility was found with density gradient combined with Caffeine. Since cyclic AMP is known to rise the sperm motility, high concentrations of Caffeine can cause an inhibition of cyclic adenosine 3',5'-monophosphate (AMP) phosphodiesterase when added to semen thus in turn increase sperm motility, while Caffeine in low concentrations produced a dose-dependent increase in motility of sperm, which suggest that Caffeine can stimulate motility of sperm by a mechanism other than inhibition of phosphodiesterase [18].These results were similar toother studies that reported a significant stimulatory result of Caffeine on motility of sperm was observed, when compared with other chemicals used to stimulate motility and improve the effect of Caffeine activated the non-motile live spermatozoa[18,19, 20,21].

Density gradient centrifugation technique showed results higher than other techniques as a result of silane coated silica that used for sperm preparation, and these results were in an agreement with the findings of other studies that reported by the main improving role of this technique on the recovery of motile spermatozoa[12,22,23].The high results showed with density gradient centrifugation technique with and without Caffeine, the results of this technique is directly linked to the gradient material which used to prepare sperm cells separation in the presence of round cells in large number, antisperm antibodies and low recovery motile spermatozoa in samples [24] . On other hand, the separation of sperm based on their motility and morphology with used of activated medium which a cause of morphologically normal and motile sperm separation from the total sperm population[25] .

As semen centrifugation is known to induce sperm dysfunction mediated through reactive oxygen species production by spermatozoa and leucocytes. Therefore, density gradient centrifugation and swim-up are more gentle techniques and widely applied in clinical practice. In assisted reproductive techniques the success rates of sperm selection are currently based on standard criteria such as motility, viability and morphology[26].

In this study, the best choice to prepare or select functional sperm depending on the feature of the semen samples for all the three groups of samples asthenozoospermia,oligozoospermic and normozoospermic samples is density gradient centrifugation technique especially when combined with Caffeine, in which yielded high result for sperm motility and morphology more than other techniques which used in this study.

CONCLUSIONS:

Density gradient centrifugation technique alone and combined with Caffeine was found a higher significant result on sperm function parameters (sperm motility and morphology) when using a low quality of semen samples such as decreased sperm motility as compared with the two other technique (direct and indirect swim-up techniques).

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