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RESEARCH ARTICLE

THE NEW BENEFICIAL APPLICATION OF CAFFEINE AS A PROMOTING FACTOR OF SPERM MOTILITY COMBINED WITH DENSITY GRADIENT CENTRIFUGATION TECHNIQUE FOR BEST YIELD FOR ASTHENOZOOSPERMIC AND OLIGOZOOSPERMIC MEN.

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Key words:-

Asthenozoospermia, Oligozoospermia,
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 ,Caffeine.

Abstract

Background: Seminal fluid of males is varied in quality, not only between males but also within a single sample. A verity of *in vitro* sperm preparation techniques were developed to separate normal and motile spermatozoa from other constituents of sample to provide successful assisted reproductive techniques.

Objectives: The goal of this study was to compare the effects of the four *in vitro* sperm activation techniques on sperm function parameters specially sperm motility of asthenozoospermic and oligozoospermic semen samples.

Subjects, Materials and Methods: Thirty-five asthenozoospermic and fifteen oligozoospermic men were participated in this study during their attendance to the Infertility Clinic at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies; Al- Nahrain University. Collection of semen samples and seminal fluid analysis was done according to WHO (1999) and WHO (2010). Each semen sample was divided into four parts. The first one prepared as *in vitro* sperm activation using the direct swim-up technique, the second using indirect swim-up technique, the third using density gradient centrifugation technique, while the last part prepared using density gradient centrifugation and Caffeine (combined techniques).

Results: A significant increase was observed in the sperm function parameters including sperm motility and morphologically normal sperm for asthenozoospermic and oligozoospermic samples when using density gradient centrifugation and Caffeine as compared to density gradient centrifugation alone. Also, there was a significant increase was observed in the same sperm function parameters when using density gradient centrifugation as compared to direct swim-up technique and indirect swim-up technique.

Conclusion: In this study, the outcome of sperm function parameters using combined density gradient technique with Caffeine were higher than other techniques, especially when using a low quality of semen samples such as decreased sperm motility, therefore it was fit in cases of asthenozoospermia and oligozoospermia which are taken as examples of infertile group in this study.

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Introduction:-

Assisted reproductive technologies (ART's) were advanced through the earlier decades to produce high-yielding numbers of embryos, and have been revealed the need for suitable and effective techniques of sperm treatment in the laboratory⁽¹⁾. Many of sperm separation or isolation methods exist to select spermatozoa. These methods include swim-up methods, density gradient centrifugation, Pentoxifylline wash, and others. A number of techniques have been developed to separate viable sperm from the seminal fluid for use in ART's⁽²⁾.

The density gradient centrifugation technique can be 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⁽³⁾.

Spermatozoa of mammals do not have the ability to fertilize the ova immediately after ejaculation process; however, the sperm gain this ability after capacitation and hyperactivation process^(4,5). One of the continuous physiological processes occurring for spermatozoa to obtain the fertilizing efficiency is hyperactivity. Motility of sperm is very important to penetrate the cumulus cells and zona pellucida surrounding the oocyte^(6,7). 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^(8,9).

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⁽¹⁰⁾.

Caffeine is a cyclic nucleotide phosphodiesterase inhibitor, causing an increase in the intracellular cyclic adenosine monophosphate (cAMP), stimulating capacitation and the spermatozoal reaction⁽¹¹⁾, and increasing of sperm motility⁽¹²⁾. Depending on the concentration of calcium ions, Caffeine may have a direct effect on metabolism of cells⁽¹³⁾.

Subjects, Materials and Methods:-

The study was done for thirty-five asthenozoospermic and fifteen oligozoospermic infertile men of different age, during their attendance to the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies (ART's) at Al-Nahrain University. The samples of semen were collected and analysis was done according to WHO (1999, 2010). 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 Caffeine, the third one using the centrifugation swim-up migration technique and the last one using direct swim-up technique, then sperm parameters were assessed for these four techniques and the results were statistically analyzed.

Discontinuous Density Gradients Technique with Caffeine:-

This sperm preparation method was prepared as same as discontinuous density gradients technique with adding 0.5mL of Caffeine stock 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 slide and covered with cover slip.

Statistical Analysis:- The Statistical Analysis System- SAS (2016) program was used to effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means in this study⁽¹⁴⁾.

Results:-

After *in vitro* sperm activation for asthenozoospermic and oligozoospermic samples using these four techniques, a significant increase was observed in the sperm function parameters including sperm motility and morphologically normal sperm as compared to pre-activation. Besides, it was observed a significant or 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, progressive sperm motility, and morphologically normal sperm for all types of samples using combined techniques (density gradient centrifugation and Caffeine) as compared to the three other techniques (table 1,2). In all types of samples, 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(table 1,2).

Table 1:- Sperm parameters for asthenozoospermic male group pre-and post *in vitro* sperm activation.

Certain Sperm Function Parameters	M ± SE					
	Sperm Concentration Value (m/ml)	Progressive Sperm Motility (%)		Non-Progressive Sperm Motility (%)	Immotile Sperm (%)	Morphologically Normal Sperm (%)
		Grade A	Grade B	Grade C	Grade D	
Before activation	49.23 ± 3.54 a	0.742 ± 0.37 d	28.23 ± 1.63 d	37.46 ± 1.34 a	34.08 ± 1.75 a	34.00 ± 1.77 d
After <i>in vitro</i> activation						
Direct technique	19.23 ± 2.15 d	1.94 ± 0.62 d	40.48 ± 1.82 c	39.23 ± 1.56 a	18.29 ± 1.13 b	49.11 ± 1.57 c
Indirect technique	25.28 ± 2.43 c	4.08 ± 0.97 c	47.37 ± 2.08 b	36.40 ± 2.54 a	12.14 ± 1.16 c	57.20 ± 1.75bc
Density gradient	30.70 ± 2.73bc	7.45 ± 1.44 b	53.48 ± 1.60 b	30.94 ± 2.26 b	8.11 ± 1.21 cd	65.97 ± 1.63 ab
Density gradient + Caffeine	36.80 ± 2.73 b	11.88 ± 1.92 a	59.80 ± 1.48 a	24.08 ± 2.15 c	4.23 ± 0.75 d	75.00 ± 1.57 a
LSD value	7.215 *	2.679 *	6.463 *	5.225 *	5.507 *	11.726 *
* (P<0.05). Means having with the different letters in same column differed significantly. M ± SE: Mean ± Standard error, N=35						

Table 2:- Sperm parameters for oligozoospermic male group pre-and post *in vitro* sperm activation.

Certain Sperm Function Parameters	Mean ± SE					
	Sperm Concentration value (m/ml)	Progressive sperm motility (%)		Non-progressive sperm motility (%)	Immotile sperm (%)	Morphologically Normal sperm (%)
		Grade A	Grade B	Grade C	Grade D	
Before activation	11.13 ± 0.96 a	0.60 ± 0.10 c	25.06 ± 2.86 d	33.26 ± 2.10 b	14.90 ± 3.85 cd	15.11 ± 3.90 d
After <i>in vitro</i> activation						
Direct technique	3.38 ± 0.49 c	0.876 ± 0.54 c	34.13 ± 3.12 c	39.20 ± 2.84 a	25.86 ± 2.85 a	42.67 ± 1.99 c
Indirect technique	4.15 ± 0.66bc	2.07 ± 1.08bc	39.00 ± 3.52bc	36.80 ± 2.89 ab	21.46 ± 2.90 b	49.00 ± 2.59bc
Density gradient	5.58 ± 0.78bc	3.60 ± 1.47 b	43.00 ± 3.21 b	35.53 ± 3.10 ab	17.86 ± 4.29bc	56.46 ± 3.12 ab
Density gradient + Caffeine	7.04 ± 0.87 b	6.33 ± 2.12 a	50.46 ± 2.93 a	33.26 ± 2.97 b	10.93 ± 3.42 d	65.93 ± 3.39 a
LSD value	3.530 *	2.094 *	6.283 *	5.471 *	4.392 *	10.613 *
* (P<0.05). Means having with the different letters in same column differed significantly. M ± SE: Mean ± Standard error, N=15						

Discussion:-

The importance of sperm preparation techniques in ART's, is to obtain the highest sperm recovery and *in vitro* treatment of spermatozoa to improve their function like, motility and improve or maintain the functional capacity for successful fertilization through supplementation of protective media ⁽¹⁵⁾.

The results of sperm concentrations parameter for asthenozoospermic and oligozoospermic semen samples showed a significant ($P < 0.05$) decrease in the concentration of sperm in post- *in vitro* sperm activation techniques; direct swim-up, in direct swim-up, density gradient and density gradient combined with Caffeine as compared with pre-activation sperm concentration. This reduction occurs as a result of abnormal and dead sperms exclusion during sperm preparation techniques.

Density gradient centrifugation technique with and without Caffeine sperm migrate through density of media and then from pellet to upper media, the data of this study showed the high concentration rate of sperm was in these two techniques. These results were in contract with other studies that activate and isolate spermatozoa *in vitro* sperm preparation using density gradient centrifugation techniques compared with other techniques, as reported by Sakkas, *et al*⁽¹⁶⁾.

In this study there is a significant ($P < 0.05$) result for the four techniques of increase sperm motility noticed with progressive motile sperm (grade A and grade B) as grade system which used. 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⁽¹⁷⁾.

These results were similar to other 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^(17,18, 19, 20).

In general, density gradient centrifugation technique showed result high than other techniques as a result of silane coated silica that used for sperm preparation, and these result agreements with the findings of other studies that reported by the main improving role of this technique on the recovery of motile spermatozoa^(21,22,23,24).

The high result showed with density gradient centrifugation technique with and without Caffeine, the reason of these result 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⁽²⁵⁾; on other hand, the separation of sperm based on their motility and morphology with used of activated medium which a cause of normal morphologically and motile sperm separation from the total sperm population⁽²⁶⁾.

In this study, the best choice to prepare or select functional sperm depending on the feature of the semen samples for asthenozoospermic, oligozoospermic samples is density gradient centrifugation technique specially when combined with Caffeine, in which yielded high result for sperm motility and morphology more than other techniques which used in this study.^(27,28, 29)

The Active progressive sperm motility grades A and B are increased following activation for 30 minutes and one hour compared with before activation⁽³⁰⁾, because activation techniques of sperm lead to excellent yields in motile spermatozoa especially in density gradient technique combined with Caffeine⁽³¹⁾, in addition to that the components of liquid artificial medium may improve the percentage of morphologically normal sperm and progressive motility because it diluted and reduced the semen viscosity⁽²⁰⁾. Therefore, these good sperm preparation techniques can improve sperm quality by selecting high percentage of motile and morphologically normal spermatozoa⁽³²⁾.

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