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Comparing the Activation Role of Centrifugation Swim-up and the Density Gradient Combined with ALLGrad Sperm Preparation Techniques

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Abstract

In about 40% of infertile couples, the husband was found to be either the single or a contributing cause of infertility and the systemic management ready for those couples with male infertility are restricted. 135 couples with infertility shared herein; the husbands required *in vitro* activation for their sperm samples, their wives however; had no fertility problem and were submitted ovarian induction protocol. However; Sperm preparation was done using both, the centrifugation indirect swim-up technique with Global medium and density gradient with ALLGrad solution. While ALLGrad 45% is a ready-to-be-used upper layer. There was an acceleration in seminal parameters after preparation using both various techniques. So, a highly significant increment ($P \leq 0.001$) in the percentages of progressive forward motility was in grades (A and B) and grade (A) progressive sperm motility, also a significant enhancement ($P \leq 0.05$) in percentage of morphologically normal sperms when applying density gradient mechanism in relation to centrifugation swim-up technique. This study concludes that the use of density gradient mechanism with ALLGrad solution and Global culture medium are more efficient in achieving better percentages of progressive forward motility grades (A and B), grade (A) progressive motility and morphologically normal values of spermatozoa than centrifugation indirect swim-up technique by using Global culture medium. However, it was found there was a non-significant difference when we use both techniques concerning other sperm parameters.

Keywords: Seminal fluid analysis, Sperm activation techniques, Centrifugation swim-up techniques, Density gradient technique, ALLGrad solution

1. Introduction

Infertility is stated by the American Society for Reproductive Medicine (ASRM) as being a disorder of the reproductive system that inhibits the ability to do the main function of reproduction in the body, so it typically means the inability to have a pregnancy after one year of unprotected intercourse. A severe disorder of sperm production is, however; including decreased sperm number, diminished motility of sperms and more values of sperm with abnormal morphology, which will also be relevant. However, mild disorders may just become apparent as both the age of the female partner and the duration of infertility increases (Templeton, A. [1]), male infertility is as a result of the absolute absence of sperm in the ejaculate and is relatively unusual so male subfertility can be due to decreased counts of sperm and a decreased percentage of sperm with progressive motility or abnormalities in the sperm's capacity to fertilize an egg (Francavilla, et al. [2]). Furthermore, it was found that there are many usual causes of infertility in males and these include hormonal as well as gonadal abnormalities, also medical problems such as cystic fibrosis and sickle cell anemia, further renal disorders, urological diseases and problems such as cryptorchidism, varicoceles, cancer of testes, and genital tract trauma and/or obstruction are also included in the causes of male infertility (Smith, et al. [3]). However; these problems can either temporarily or constantly cause spermatogenesis impairment, decreased sperm transport in the male and also in the female genital tracts due to impaired motility can result in decreased morphology and affect sperm function (acrosome reaction and sperm oocyte fusion), all of which may inhibit pregnancy (Agarwal, et al. [4]). Furthermore; many of these problems can enhance the production of reactive oxygen/nitrogen species that will cause oxidative stress, which results in both spermatozoal dysfunction and infertility (Agarwal, et al. [5]). For couples with age

over 35, it is usually recommended that infertility investigations better to begin beyond 6 months of unsuccessful trials to conception (Williams and Elam [6], American Society for Reproductive Medicine (ASRM) [7]). In some cases, testing may, however, be done before one year if there is a possible known infertility risk factor in the male such as bilateral cryptorchidism or also known female risk factor (American Urological Association [8]). According to the (World Health Organization [9]), infertility as a problem is a worldwide problem affecting more than 80 million people all over the world. Indeed, most of those males with infertility were found to live in the developing countries where infertility treatments in general and especially Assisted Reproductive Technologies (ART's) are not much available (American Society of Reproductive Medicine [10]). 40% of cases of infertility however; have been caused by male factors where the systemic treatments present for those couples with male infertility are not much available (Comhaire and World Health Organization [11]). In fact, assisted reproduction has been transformed from the only gynecological indications to both gynecological and also andrological indications during the past years (Dokras and Olive [12]). Furthermore; This has urged andrological scientists and workers to comprehend the physiology of male germ cells in a better way and to develop furthermore difficult mechanisms to separate active motile spermatozoa from those with no motility, have poor morphological features or are not having the ability to fertilize oocytes (Hansen, et al. [13]). To start, beginning from just simple sperm wash, several activation techniques that depend on various principles like migration, filtration or density gradient centrifugation have however been developed (Henkel and Schill [14]). Sperm separation techniques have increased with the increasing knowledge of sperm physiology and their genetic contribution to the embryo (Shekarriz, et al. [15]). Because of this, there has been increasing interest in the safety of

any sperm activation techniques concern about the safety of any sperm separation technique with respect to the viability of the sperm and also the long-dated effects on any resulting pregnancy (Khorram, et al. [16]). Currently, density gradient centrifugation, swim-up, and one-step wash are the most relevantly used techniques for sperm activation (Agarwal, et al. [17]).

2. Materials and Methods

A hundred and thirty-five couples with infertility shared in the present study where we have the males needed sperm preparation and activation in vitro, however; their wives were having no problems and they had ovarian induction during their coming to the Infertility Clinic at Institute of Infertility Diagnosis and Assisted Reproductive Technologies; Al-Nahrain University.

a. Instruments and equipment

Centrifuge (D-78532), Disposable Petri-dish (D-810), Air incubator (B-6060), Air incubator (B-6060), Glass Pasteur Pipette (150 mm), Eppendorff pipette, Light microscope (N-200M), Vaginal ultrasound, intra-uterine catheter, Cusco's speculum, sponge forceps. Different chemicals and culture media were used in this study: Global All Grad Solution, Global medium, Clomiphene citrate (clomid) tablets (50) mg, Human chorionic gonadotrophin (ovitrelle) 6500 IU, Duphastone tablets (10mg), Normal saline.

b. Analysis of semen samples

The seminal samples were collected after 3-5 days of sexual abstinence into a clean and sterile disposable Petri-dish by masturbation in a private room near to the semen examination laboratory. Furthermore; the container has to be labeled with the following notifications, name, age also abstinence period and time of specimen collection. The samples were put in an incubator at 37ºC for 30 minutes so that to allow liquefaction to occur. The liquefied semen is then mixed with care for

seconds, and after that, the sample was examined by macroscopic and microscopic examinations. The standard form of WHO (1992) is used to record the results of seminal analysis (Table 5).

c. *In-vitro sperm activation techniques*

Two techniques of In-vitro sperm activation have been used in this study. The first one is centrifugation indirect swim-up procedure with the use of Global medium alone, while the second one is the density gradient centrifugation technique with the use of ALLGrad solution and also Global medium.

d. *Study design*

The current study was applied on 135 infertile couples where the male partners had the infertility factors and the female partners were usually normal. Both partners were subjected to clinical and laboratory assessment. The males' semen samples were prepared for *in vitro* sperm activation and IUI. Seminal fluid analysis and assessment of sperm parameters pre- and post-activation were using both techniques (Figure 1). The semen samples were grouped into two divisions: Group-1: The control group that includes 70 samples where the *In-Vitro* sperm preparation was done by centrifugation indirect swim-up technique using Global medium. Group- 2: The study group that includes 65 samples where the *In-Vitro* sperm activation was done by density gradient centrifugation mechanism using ALLGrad solution with Global medium.

4. *Statistical analysis*

Statistical analysis was applied using the Statistical Package for Social Sciences (SPSS; version 17.00) and also Microsoft Excel worksheet 2007. The data analysis was done using paired sample t-test for tables with the mean and standard error of the mean (S.E.M) to compare between pre- and post-activation for sperm parameters. Independent sample t-test for tables with the mean and standard error of the mean (S.E.M) to compare between Global media and ALLGrad solution for sperm

parameters. As well as the ANOVA test was applied to compare among mean groups of this study with regard to age. P-value < 0.05 was used as a level of statistical significance. Regarding Sperm motility or activity grade A represents the linear, rapid, progressive motility, while grade B represents linear non-rapid or rapid nonlinear progressive motility. Grade C represents the localized movement spermatozoa, while Grade D represents non-motile sperm.

5. Results

Assessment of in vitro sperm activation (ISA):

Tables (1 and 2) showed the effect of the two in vitro sperm activation techniques (the centrifugation indirect swim up technique, and the density gradient centrifugation technique) on human sperm parameters. It was clear from both tables that all sperm parameters had a highly significant difference (P<0.01) after ISA using both techniques as compared with pre- activation results. It was noticed that the sperm concentration, sperm agglutination (%) and count of round cells were highly significantly reduced (P<0.001) as compared to post-activation results than in pre-activation ones. On the other hand, percentage of sperm motility and progressive sperm motility grades (A+B) were increased with highly significant difference (P<0.001) after ISA. Furthermore, there was a highly significant increment (P<0.001) in the percentage of morphologically normal sperm after ISA. For the sperm concentration, the present study showed non-significant difference (P>0.05) between both techniques whether pre or post ISA, as it shown in figure (1). On the other hand, figure (2) showed that the percentage of sperm motility for the swim up centrifugation technique had non-significant difference (P>0.05) as compared with density gradient motility percentage before activation, however, there was a significant increment (P<0.05) in that percentage after activation for the density gradient as compared to

Table (1): Sperm parameters for infertile males pre and post-activation using centrifugation indirect swim up technique

Parameters		*In vitro* sperm activation n=70		*P*-value
		Pre-activation	Post-activation	
Sperm concentration		57.159 ±2.214	29.406 ±1.768	≤0.001**
Sperm motility (%)		59.071 ±1.647	90.286 ±1.330	≤0.001**
Sperm grade activity (%)	Grade A	2.143 ±0.524	32.657 ±1.956	≤0.001**
	Grade B	36.557 ±1.647	44.557 ±1.587	≤0.001**
	Grade C	20.514 ±1.242	13.143 ±1.510	≤0.001**
	Grade D	40.500 ±1.650	9.714 ±1.333	≤0.001**
Progressive motility (%)		38.557 ±1.868	77.000 ±2.287	≤0.001**
Morphologically Normal Sperm (%)		36.757 ±1.381	62.800 ±1.780	≤0.001**
Sperm agglutination (%)		14.746 ±1.333	0.149 ±0.149	≤0.001**
Round cell count		8.750 ±1.271	0.000 ±0.000	≤0.001**

Data are mean ± S.E.M. ** Highly significant difference (P≤ 0.001), Total number of infertile males = 70

Table (2): Sperm parameters for infertile males pre and post-activation using density gradient technique

Parameters		*In vitro* sperm activation n=65		*P*-value
		Pre-activation	Post-activation	
Sperm concentration		63.692 ±3.204	30.969 ±1.943	≤0.001**
sperm motility (%)		61.462 ±1.801	94.092 ±0.976	≤0.001**
Sperm grade activity (%)	Grade A	5.000 ±1.023	44.462 ±1.330	≤0.001**
	Grade B	36.615 ±1.488	42.462 ±1.129	≤0.001**
	Grade C	20.308 ±1.165	8.969 ±1.121	≤0.001**
	Grade D	37.923 ±1.764	6.292 ±0.729	≤0.001**
Progressive motility (%)		41.846 ±1.932	86.615 ±1.492	≤0.001**
Morphologically Normal Sperm (%)		33.219 ±0.807	68.875 ±1.508	≤0.001**
Sperm agglutination (%)		13.211 ±1.143	0.035 ±0.035	≤0.001**
Round cell count		10.373 ±1.249	0.000 ±0.000	≤0.001**

Data are mean ±S.E.M. ** Highly significant difference (P≤ 0.001) Number of infertile males = 6

Total number of infertile males = 135, Indirect Swim up Centrifugation technique = 70 infertile males. Density gradient Centrifugation technique = 65 infertile males.

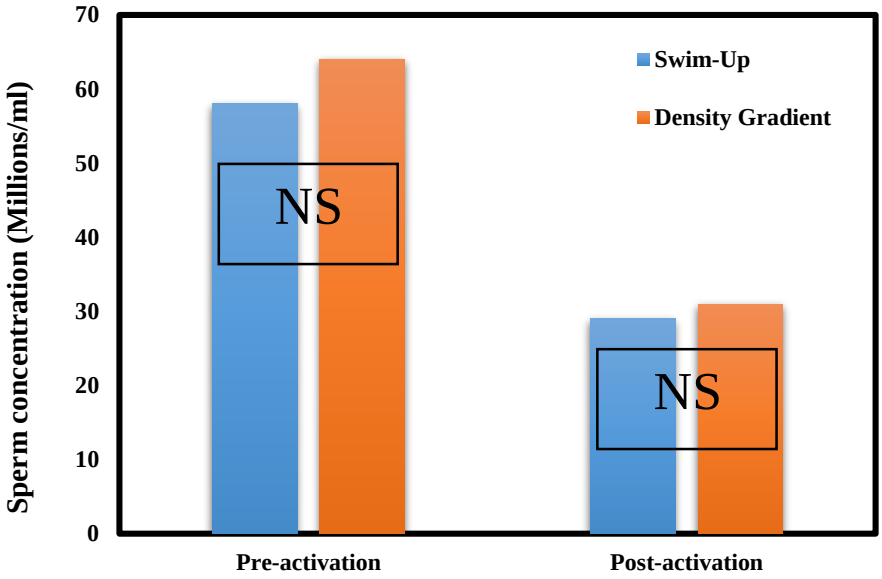

Figure (1): Sperm concentration for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05).

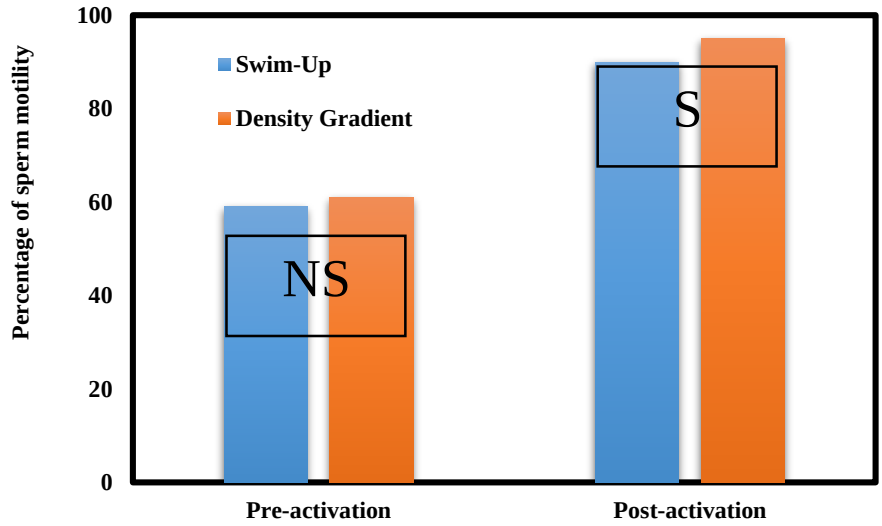

Figure (2): Percentage of sperm motility for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05). S: significant difference (P<0.05).

centrifugation swim up technique. Figure (3) observed a significant increase in the percentage of progressive sperm motility grade (A) ($P<0.05$) before activation in density gradient centrifugation technique when compared to centrifugation swim up technique and that increment was with highly significant difference ($P<0.001$) in the density gradient technique as compared to the second technique after activation. Figures (4, 5 and 6) showed that there was non-significant difference ($P>0.05$) in progressive sperm motility grades (B, C and D) before and after activation between both techniques, but for grade (C) there was a significant increase ($P<0.05$) in that motility in density gradient centrifugation technique than in the centrifugation indirect swim-up technique after ISA. While the percentage of sperm progressive motility grades (A+B) as it is obvious in figure (7) showed non-significant difference ($P>0.05$) between both techniques before activation but there was a highly significant (<0.001) increase in the density gradient centrifugation technique progressive sperm motility grades (A+B) as compared to centrifugation swim up technique after ISA. In figure (8), it was evident that there was non-significant difference ($P>0.05$) between both groups in percentage of morphologically normal sperms before activation, however, after activation there was a significant increase ($P<0.05$) in that percentage for the density gradient centrifugation technique as compared to indirect swim-up centrifugation one.

3. Discussion

Sperm activation methods are capable of highly enhanced sperm quality as well as an increasing percentage of forwarding progressive motility and spermatozoa with normal morphology. On the other hand, in the in vitro formation of embryos, activation mechanisms of sperms have so important benefits. In these separation methods, we will select motile sperm from immotile, get rid of seminal plasma also cryo-protective materials, infectious

Total number of infertile males = 135, Indirect Swim up Centrifugation technique = 70 infertile males. Density gradient Centrifugation technique = 65 infertile males.

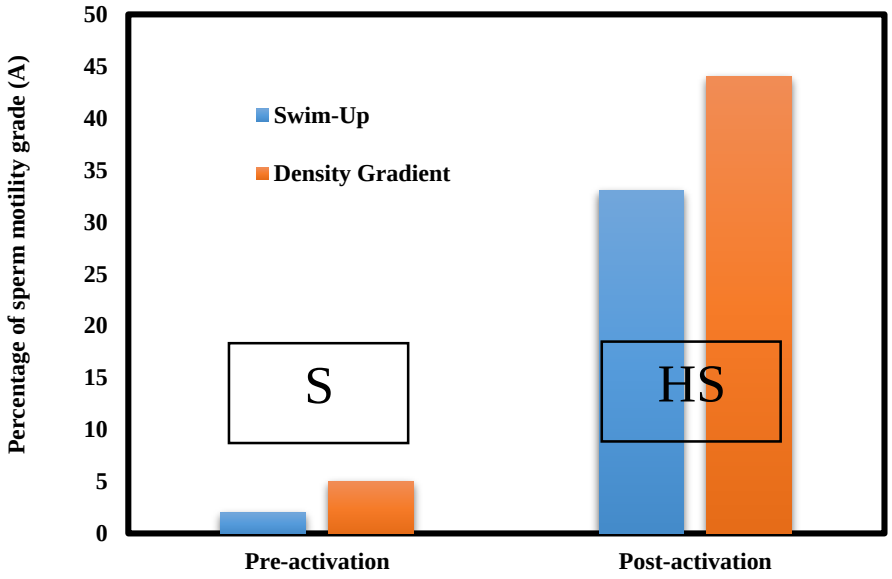

Figure (3): Percentage of sperm progressive motility grade (A) for infertile males pre and post-activation using two techniques

S: significant difference (P<0.05). HS: highly significant difference (P< 0.001).

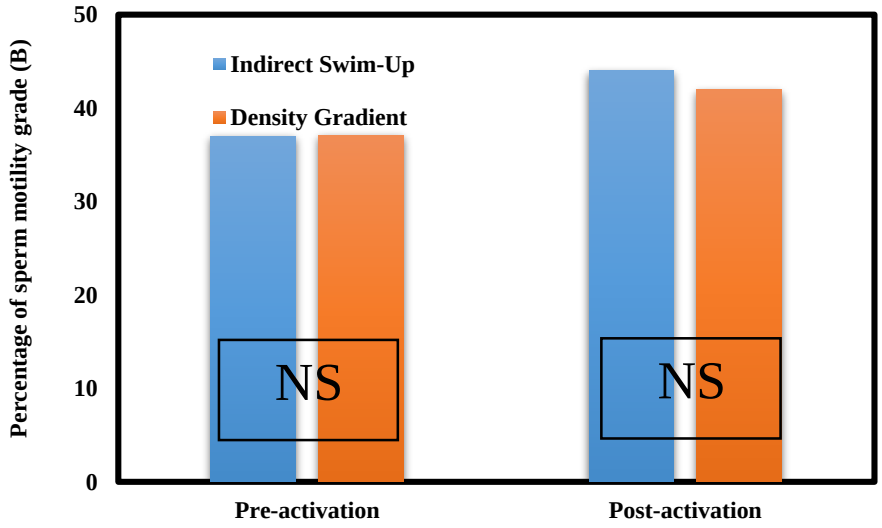

Figure (4): Percentage of progressive sperm motility grade (B) for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05).

Total number of infertile males = 135, Indirect Swim up Centrifugation technique = 70 infertile males. Density gradient Centrifugation technique = 65 infertile males.

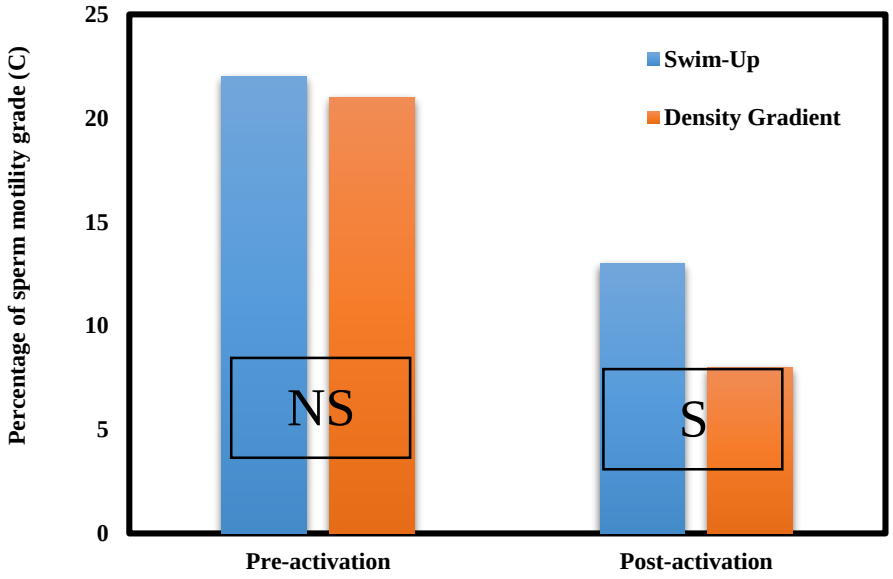

Figure (5): Percentage of sperm motility grade (C) for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05). S: significant difference (P<0.05).

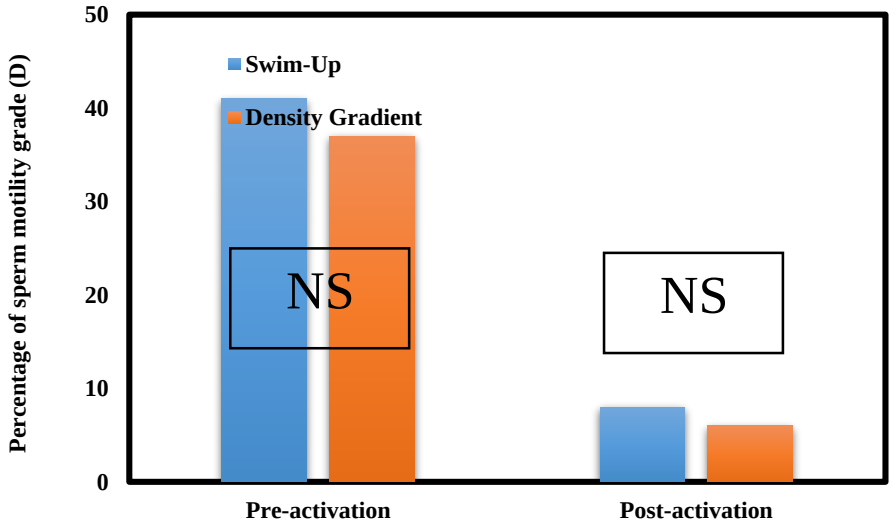

Figure (6): Percentage of sperm motility grade (D) for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05).

Total number of infertile males = 135, Indirect Swim up Centrifugation technique = 70 infertile males. Density gradient Centrifugation technique = 65 infertile males.

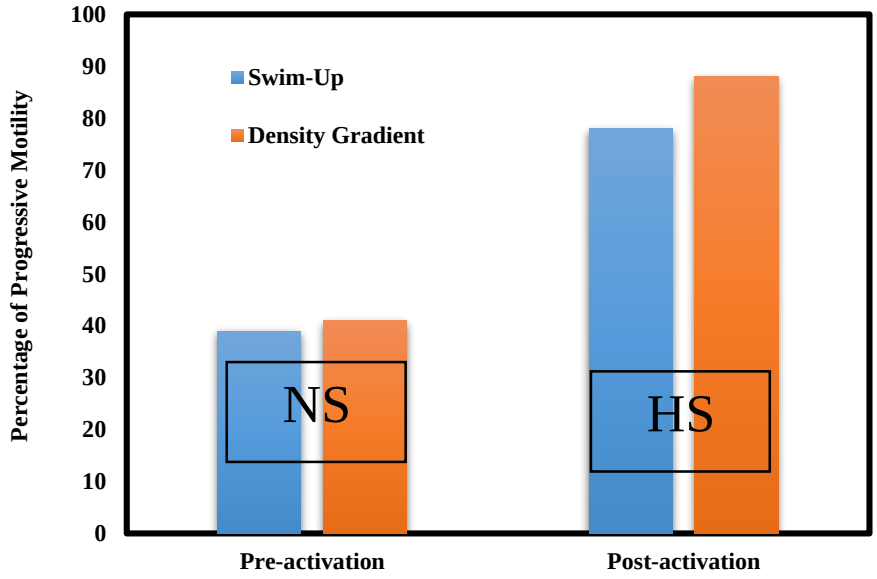

Figure (7): Percentage of progressive sperm motility grades (A and B) for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05). HS: highly significant difference (P< 0.001).

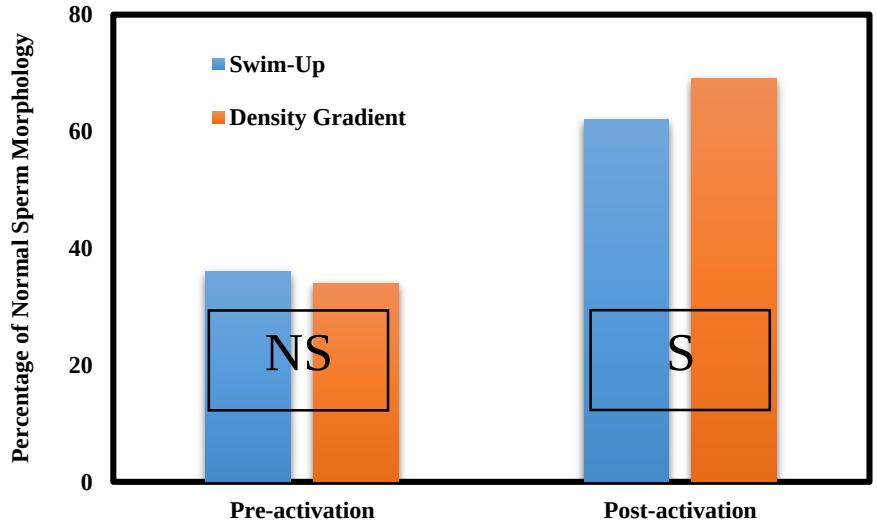

Figure (8): Percentage of morphologically normal sperm for infertile males pre and post-activation using two techniques

NS: non-significant difference (P> 0.05). S: significant difference (P<0.05).

substances, and debris (Zavos, PM [18]). Many sperm activation procedures have been created to enhance sperm quality and were dependent on the elevated rate of progressive forward motility and sperms of normal morphology. However; some of the most substantial sperm activation mechanisms are (density-gradient centrifugation) and swim-up (Hamza, et al. [19]). In this study two different techniques were used for in vitro sperm activation (ISA), centrifugation swim-up mechanisms and density gradient one, not just progressively forward motile and morphologically normal spermatozoa are chosen, but sperms will also undergo changes so-called capacitation, these changes are important for the sperm's functional competence in addition to acrosome reaction (Centola, et al. [20]). Furthermore; improvement in seminal fluid parameters may be regarded as a normal response for sperm biology following the removal of seminal plasma, round cells and agglutinated spermatozoa with the use of sperm preparation procedures (Fakhrildin, et al. [21]). The present study viewed a highly significant ($P \leq 0.001$) reduction in the sperm count and agglutination after ISA using both techniques for all infertile males as in relation to pre-activation and this is related inability of dead and morphologically abnormal sperms with poor motility to swim-up and migrate into upper layer of culture media. These results clarified the beneficial effect of separation techniques by the elimination of dead, germ cells with no motility and debris in such a way just high-quality motile germ cell is selected and the bad quality spermatozoa were not present in the post-activation medium (Makkar, et al. [22]). Furthermore, post-activation with the use of different methods, there was a significantly high ($P \leq 0.001$) reduction in the count of round cells and a highly significant increment in the percentage of sperm with normal morphology, this is because the sperm separation mechanisms for ART's were used to separate the morphologically normal, motile spermatozoa and exclude

round cells, bacteria and nonviable spermatozoa produce oxygen reactive species that badly influenced the fertilization ability (Boomsma, et al. [23]). In this study, the global medium was used as a culture media to enhance sperm parameters in both techniques. A growth, culture medium is a solution or gel designed to enhance the growth of microorganisms or cells (Sturmey, et al. [24]). In vivo, gametes and embryos take their nutrition from the human tubal fluid (HTF). In different IVF methods, sperms, zygotes, and embryos are cultured in growth media that are composed of chemical materials needed for both growth and development. These media are considered as modifications of Human tubal fluid (Keating and Quinlan [25]). With time, however; growth media have been made from simple mixture of basic salts to more sophisticated combination of inorganic salts with amino acids, vitamins and also other components, in buffered systems (usually bicarbonate and HEPES) and even indicators may be used to facilitate monitoring the change in pH (Booth, et al. [26]). Global medium has been proven in the fertility centers worldwide, to be stable in its use, it keeps pH and osmolality. It is applied to produce the lowest level of ammonium build up with time, higher stability, quality, and performance (Life Global [27]). Global media was found to have the best results for IUI as compared to Hams F-12 culture medium alone or enriched with human serum albumin. In regard to the centrifugation swim-up technique that was used in this study, indeed it is one of the most popular sperm activation methods for both experimental and also practical programs, centrifugation facilitates the elimination of seminal plasma and concentrates the germ cells to prepare the sperms for re-dilution (Kadhim, et al. [28]). A fraction of highly active, sterile and functionally competent germ cells can be acquired, but however; the count is very low (Allamaneni, et al. [29]). Sometimes the harvest of spermatozoa is diminished because multiple layers of sperms found in

the pellet may lead to the potentially motile spermatozoa present in the lower layers of the pellet never reach the interface with the growth medium level (Henkel and Schill [14]). However, Sanchez and co-workers stated that following concentration of sperms in the ejaculate, even with severe oligo- and/or asthenozoospermia an adequate concentration of motile and morphologically normal germ cells can be selected for intracytoplasmic sperm injection (ICSI) (Sanchez, et al. [30]). Even so, the centrifugation swim-up method has been found to be a probably sperm-damaging step during human sperm preparation and exerting sub-lethal damage. Spermatozoa were found to be highly sensitive to both mechanical and centrifugal forces and should be dealt with under carefully defined circumstances to decrease sperm damage. The other technique used was the density gradient technique with ALLGrad solution, ALLGrad solution is a sterile solution of colloidal silica particles suspended in an isotonic salt liquid. It is made for the density gradients preparation and it is used for the separation and also purification of human sperm to be used in assisted reproduction. This culture medium shown to separates normal sperm from lymphocytes in high efficacy, red blood cells, epithelial cells, abnormal or immature germ cells, cell debris, bacteria and also seminal fluid (Life Global [27]). Some of the components of ALLGrad solution as it was shown in table (6) is sodium chloride that has an important role in regulating the tonicity of the culture media, preserve the sperm membrane and promotes full sperm function (Lim, et al. [31]). As for the other ionic constituents of ALLGrad solution, the interplay between pH, pyruvate, lactate, calcium, and magnesium plays a great role in cellular homeostasis and cellular development (Lane and Gardner [32]). Glucose is another component of the ALLGrad solution, it is used to optimize the sperm function (Babatunde, O [33]). Human spermatozoa require a higher glucose concentration

than the early embryo to satisfy the higher rates of glucose utilization by high numbers of spermatozoa (Tsujii, et al. [34]). ALLGrad also contain EDTA that probably acts in several ways, including chelation of heavy metal divalent cations that may be found as trace contaminants in media substances and also plastic culture ware, through the chelation of iron. There would be a fewer generations of reactive oxygen species, maintenance of enzyme activity such as adenyl cyclase, which generates cAMP (Lane and Gardner [32]). Another constituent of ALLGrad solution is HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid) is a general-purpose buffer. A Buffer is a solution that can maintain its pH fixed, even with the addition of strong acids or also strong bases and in addition to the external effect of temperature, pressure also volume and redox potential. Buffer inhibits alteration of the concentration of other chemical materials (Babatunde, O [33]). ALLGrad wash that was used to dilute ALLGrad 100% stock solution contains human serum albumin, it acts as a sink or acceptor for cholesterol and enhances the efflux of cholesterol from germ cell plasma membrane leading to change in sperm fluidity observed in the/or during capacitation, capacitation of the sperm requires an exogenous protein in form of albumin (Wu, et al. [35]). The roles of albumin in tissue culture may include trace metal binding, osmotic stability, and carrier activity (Tsujii, et al. [34]). (Alvarez and Storey [36]) proved that serum albumin may prevent the detrimental effect of centrifugation force on sperm motility and sperm grade activity. When compared to the centrifugation swim-up technique, the percentage of sperm with progressive motility especially grade (A) was increased with high significance ($P \leq 0.001$) using density gradient technique with ALLGrad solution, also total sperm motility and Morphologically Normal Sperm were increased significantly ($P \leq 0.01$) in this study. A study done by (Samardzija, et al., [37]), its results indicated that density gradient mechanism

has an increased capacity in sperm separation for in vitro embryo production if we compare it with centrifugation indirect swim-up and density gradient techniques may be considered to be a better substitute to swim up method for selecting highly motile spermatozoa from frozen/thawed spermatozoa for IVP (in vitro production) of bovine embryos. Sperm viability with membrane activity and acrosome status, however; found more percentage of live and acrosome intact spermatozoa obtained after density gradient method compared to centrifugation indirect swim-up technique (Risopatrón, et al. [38]). Moreover, it was assessed that centrifugation force negatively affects sperm motility and also impairment of acrosome reaction, sperm plasma membrane, HOS test score and nuclear maturity rates in males with normal and abnormal semen analysis in relation to density gradient centrifugation. However, other studies were in disagreement with the results of this study, compared with the density gradient centrifugation, (Sanchez, et al. [30]), showed significantly higher results for progressive forward motility, also normal germ cells morphology, with chromatin condensation and decrees of the percentage of dead germ cells as shown by the eosin test when using centrifugation swim-up mechanism. and B), grade (A) progressive forward sperm motility, also total sperm motility and morphology than the IUI results in the group of patients used the centrifugation indirect swim-up technique. It is of sure that the spermatozoa selected by density gradient centrifugation technique have higher sperm penetration results in the zona-free hamster egg by sperm penetration assay (Serafini, et al. [39]).

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Author Contribution

Authors have equal contribution to the work.

Conflict of Interest

The author declares no conflict of interest.

Ethical Clearance

The study was approved by the Ethical Approval Committee.

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