

## Correlative Study of Sperm Motility and Mitochondrial Membrane Potential by Fluorescent Staining: First Application in Iraqi Centers

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### ABSTRACT

**Background:** Sperm motility is an essential factor in ensuring the fertility of males. Sperm mitochondria play a significant role in the generation of energy as well as the metabolism of sperm. The mitochondria nucleolus is essential for several sperm activities, such as sperm capping, hyperactivation, acrosome reactions, and oocyte fusion. Therefore, as the mitochondria are the basic regulators of sperm motility, mitochondrial membrane potential (MMP) is used to assess the mitochondrial activity and integrity of sperm. This is evaluated by examination of MMP, which may be achieved by using fluorescent dyes like the JC-1 probe.

**Objectives:** To examine the correlation between MMP and sperm motility.

**Materials and methods:** This study included semen samples of 60 males that were separated into two groups: 30 normozoospermic and 30 asthenozoospermic. The samples were collected in the infertility clinic at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University and Um Al-Banin Center for management and *in vitro* fertilization. The collected samples were processed for seminal fluid analysis and evaluated according to the recommendations of WHO 2021 using an MMP-Assay kit with JC-1 dye and examined by fluorescent microscope for evaluation.

**Results:** A positive significant correlation between subjects' sperms motility percentage and active red-fluorescence MMP intensity (Red MMP) (P-value , 0.05,  $r = 0.406$ ; P-value = 0.008). The MMP or activity plays an essential role in sperm motility and male fertility. The red MMP values in subjects with normal sperm motility were  $10.22 \pm 1.54$  and showed a relatively higher reading in comparison to those readings of subjects group with low sperms motility ( $4.70 \pm 0.91$ ). Subjects with normal sperm motility showed a high MMP intensity (P-value = 0.004).

**Conclusion:** Normal MMP is crucial for normal sperm motility. The sperm motility is proportionate to the MMP, or activity.

**Keywords:** Male Infertility; Sperm Motility; Mitochondrial Membrane activity; MMP detection Kit (With JC-1).

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### INTRODUCTION

Infertility is defined by the World Health Organization (WHO) as the failure to conceive after at least a year of unprotected intercourse [1]. Approximately, 85% of infertile couples have an identifiable cause; the remaining 15% of infertile couples have unexplained infertility [2]. Lifestyle and environmental factors, such as smoking and

obesity, can adversely affect fertility. Male factors account for about 20% or 30% of all instances of infertility, while both male and female factors are responsible for 35% of cases [2, 3].

Sperm motility is a necessary condition for male virility. The beginning of research about sperm motility began in 1919, when an American zoologist, Lillie FR discussed the metabolic energy of spermatozoa for the first time [4]. Since then, multiple studies have focused on the cell's power plant, the mitochondrion, highlighting the importance of this organelle in cellular homeostasis and sperm motility [5]. Increased sperm motility is generally considered to be an important aspect of typical male fertility. Males with immotile

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or poorly motile sperm are typically sterile unless assisted reproductive technology is used [4].

The number of mitochondria in the mammalian spermatozoa ideally ranges from 50 to 75 mitochondria. Sperm mitochondrion shows peculiar characteristics. They are exclusively present in the mid-piece, firmly wrapped around the axoneme. In spermatogenesis, the mitochondria are fixed end-to-end and wrap as a helix around the tail, making a thick mitochondrial envelope, just under the external plasmalemma of the sperm [5].

Sperm motility is one of the crucial factors needed to achieve natural way of fertilization between male and female gametes. Following ejaculation, sperms are energized by adenosine triphosphate (ATP) and other high energy molecule present in semen via glycolysis and oxidative phosphorylation that take place in the mitochondria of sperm [6].

In sperm, ATP is needed for a number of processes, such as motility, fusion reactions during the acrosome reaction, and transporting molecules through membranes against concentration gradients. Mitochondria are distinct organelles, structurally and functionally, that make ATP through oxidative phosphorylation (OXPHOS) to supply spermatozoa with energy. There are about 80 mitochondria present in the mid-piece [7]. Reduced mitochondrial membrane activity or mitochondrial membrane potential (MMP), due to the deformed morphology of the axoneme, is a reason of reduced sperm motility and marked asthenospermia [8]. Previous researches and reports proved the important role of mitochondria in sperm motility and male fertility [9].

Cyclic adenosine monophosphate (cAMP) and calcium are the two primary second messenger chemicals involved in the initiation and control of sperm motility [10]. Sperm mitochondria have been related to the motility of sperm and fertilization, and several techniques to assess their activity have been developed. A variety of sperm factors can be investigated to evaluate the activity of mitochondria. These factors include mitochondrial activity, MMP levels, and calcium amounts in sperm mitochondria. Nevertheless, the processes involved in ATP generation and the functional dynamics of sperm mitochondria are not entirely known, despite the significance of sperm mitochondria role in the ability of fertilization [5, 11]. Many commercial fluorescent dyes are widely used to assess the mitochondrial potential, the fluorescent dye, JC-1, has been widely used to assess sperm quality, although under specific experimental circumstances, significant issues have been found. Tetra methyl rhodamine methyl ester perchlorate (TMRM), a different fluorescent dye, is also used to measure sperm MMP. In fact, it was discovered that the accuracy of MMP changes detected by TMRM is similar to that of the commonly used JC-1 staining approach [5, 12]. In addition to that, it was discovered that JC-1 dye is only present inside the mitochondria and therefore it gives the most specific assessment of MMP. Lugli et al. proved this discovery, which stated that JC-1 is more dependable and specific for such type of assessment than other probes [13].

Although sperm mitochondrial activity nowadays can be assessed by flowcytometry and stained with fluorescent dyes, it is challenging and even difficult to get these techniques in every research laboratory. In addition to that, one can face difficulties to get the sperms fixed and stained with fluorescent dyes by ordinary techniques, and after that, it sounds unfeasible to be examined under the fluorescent microscope easily since these are very small and motile cells unless one uses a more sophisticated and tedious steps to overcome these ob-

stacles. Some authors and researchers, whose works we came across through our literature review before carrying out this current work, tried and modified new methods and procedures to deal with motile sperms more accurately to assess their mitochondrial activity by fixation of the semen sample and preparation of smear on positively charged slides, and then did the staining protocol and examined under a fluorescent microscope.

The current study aimed to evaluate sperm motility using MMP measurement by fluorescent staining in two Iraqi centers of infertility.

## MATERIALS AND METHODS

A clinical cross-sectional study included 60 Iraqi male subjects aged between 20-50 years who attended infertility clinic at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University and, the Um Al-Banin Center for management and in-vitro fertilization in Al-Kadhymia Teaching Hospital, Baghdad, Iraq. Participants were divided into two groups equal in number: group 1 included 30 normozoospermia, and group 2 included 30 asthenozoospermia. Samples were collected during the period from October 2021 to December 2022.

### Inclusion criteria

The included patients who were attended the clinics and agreed to be enrolled in the study. They were healthy with no obvious major illnesses like diabetes mellitus, thyroid disease, heart disease, or renal or hepatic impairment. The age of the subjects ranged between 20-50 years. The volume of all semen samples was more than 2 ml on examination.

### Exclusion criteria

Subjects who were smokers, alcoholic, aged more than 50 years, drug consumers like multivitamins or hormone replacement therapy, past surgical history on inguinoscrotal region, and those who declined to participate were excluded from the study.

### Ethical approval

The ethical approval document was granted from the institutional ethical committee of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University (Ref. code 0702-MM-2020A3 at November 2020). The patient consent has been obtained for each subject included in the study via signed consent.

The semen samples were collected and seminal fluid analysis was done according to the recommendation of WHO [1]. MMP Assay Kit with JC-1 was used to evaluate sperms motility depending on mitochondrial characteristics, the increased concentration of the fluorochromes (stain probes) accumulate in the hyperpolarized sperm mitochondria (high MMP would give red color on examination), while decreased concentration of fluorochromes presented in depolarized sperm mitochondria (low MMP would give green color on examination), so; the intensity of the fluorescence correlate with the MMP [12].

### MMP Assay Kit (with JC-1)

The Mitochondrial Membrane Potential Assay Kit (with JC-1) is established to discover early apoptotic changes by measuring changes in sperm MMP with JC-1 as a fluorescent probe [ELABSCIENCE Co., China]. As a positive control reagent, this kit includes carbonyl cyanide m-

chlorophenylhydrazone (CCCP)[E-CK-A301C] to cause a reduction in sperm MMP. Fluorescent dye formation in mitochondria may be observed and measured using flow cytometry, fluorescent microscopy, confocal microscopy, and a fluorescence screen analyzer [14]. Similarly, the application of fluorescent ratio identification allows researchers to compare measurements of membrane permeability while simultaneously measuring the degree of mitochondrial depression happening in a diseased situation (e.g. cellular stress, apoptosis, etc.) [15]. The JC-1 dye could only be present within the sperm mitochondria and therefore give the most precise evaluation of MMP [16]. It is more reliable than other fluorescent probes as it is sensitive to minor changes in sperm MMP [16, 17]. Flow cytometry requires fresh samples, otherwise reduced specificity and accuracy of the results, in addition; the flow cytometry is complex and required trained operators [18]. Instead, we depend in this current work on a new modified procedure for the first time, by fixation of the semen sample and prepare smear on positive charged slides and then examined under the fluorescent microscope that present in the college of medicine- Al Nahrain University make it more easy and reliable method and can be used in the future with accurate results. This modified procedure was a demand for this current study due to many technical issues faced like the unavailability and non-feasibility of the flow cytometry technique at the centers where the study was carried on. There was a shortage of the fluorescent microscopic examination expertise professionals anywhere in the medical centers in Iraq. Thus, no previous similar studies were carried out in Iraq and even the nearby countries. These points add a novelty feature for the current research.

### Principle of detection

The reduction in the potential of MMP is a mark of the beginning phases of apoptotic. JC-1 is a common fluorescent probe for measuring the MMP. When the membrane permeability has a large potential in normal cells, JC-1 aggregates the mitochondrial matrix to create a polymer that can produce red fluorescence. The change of JC-1 fluorescence color from red to green may be utilized to identify a reduction in cell MMP, and the transition of JC-1 fluorescence color can be used as an early detection signal of cell apoptosis.

### Statistical Analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) version 23.0 and Microsoft Office 2010. The descriptive statistics including frequency, range, mean, and standard deviation were measured to describe the data. The groups were compared by applying an independent sample *t*-test (Unpaired *t*-test for comparison between two groups) and analysis of variance (ANOVA for comparison of more than two groups). The degree of association between continuous variables was calculated by Pearson's correlation coefficient (*r*) and the results were considered statistically significant when *P*-value was less than 0.05.

### RESULTS

The total number of study subjects was 60 males; their clinical and demographic features are listed below in Table 1. The correlation between subject sperm motility percentage and red fluorescence MMPs was a positive and significant correlation between subjects' sperm motility percentage and active red-fluorescence MMP (Red MMP) ( $r = 0.406$ ; *P*-value =

**Table 1.** The demographic characteristics of the 60 patients\*.

Parameters	Range	Mean $\pm$ SD
Age (years)	20–49	32.19 $\pm$ 8.03
BMI (Kg/m <sup>2</sup> )	18.52–36.21	25.89 $\pm$ 3.89
Parameters		Number (%)
BMI ranking	Normal weight	22 (36.6 %)
	Over-weight	31 (52.4 %)
	Obese	7 (12%)
Smoking status	Smokers	27 (45%)
	Non - Smokers	33 (55%)

\* SD: Standard deviation; BMI: Body mass index; No.: Number of patients

**Table 2.** The correlation between the subject's sperms motility (%) and active red-MMP.\*

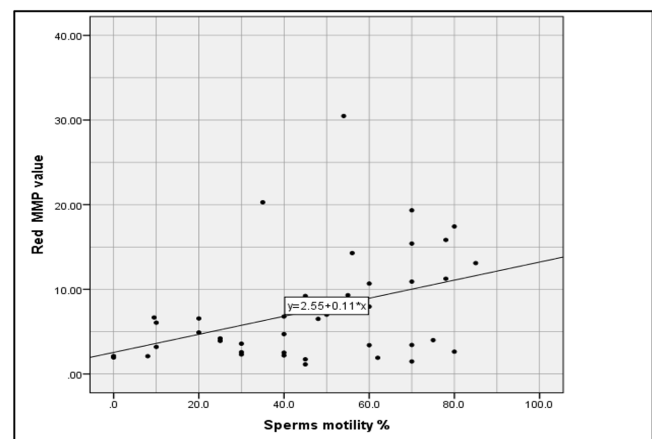
Correlation between subject's sperms motility percentage and red-MMP	Pearson's correlation coefficient (r)	P-value
	0.406	0.008

\* *P*-value < 0.01; Red MMP: red fluorescence mitochondrial membrane potential.

0.008), as presented in Table 2 and Figure 1. The comparison of Red MMP according to sperm motility showed higher and significant Red MMP values (*P*-value = 0.004) in subjects with normal sperm motility ( $10.22 \pm 1.54$ ) versus subjects group with low sperm motility ( $4.70 \pm 0.91$ ) as demonstrated in Table 3.

### DISCUSSION

The present study was conducted at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al- Nahrain University, and the Um Al-Banin Center for management and in-vitro fertilization in Al- Kadhymia Teaching Hospital. According to the current study, there is a strong



**Figure 1.** Correlation between subject's sperms motility (%) and Red MMP.

**Table 3.** Comparison of red MMP values according to sperms motility.\*

Parameter	Normal sperms motility n=30	Low sperms motility n=30	P-value
Red MMP	10.22 ± 1.54	4.70 ± 0.91	0.004

\* An unpaired *t*-test was used for statistical analysis. Red MMP: red fluorescence mitochondrial membrane potential; P-value < 0.01.

relationship between sperm motility and sperm MMP. Sperm function and malfunction have been linked to mitochondrial dysfunction. Excessive reactive oxygen species (ROS) generation caused by mitochondrial malfunction could lead to sperm failure [19]. This finding is supported by the belief that analyzing sperm metabolic condition and mitochondrial activity status might give new information to improve standard sperm examination. In addition to that, this finding of the current study is consistent with some other published research that emphasizes the role of mitochondrial function in producing ATP for human sperm function [19, 20]. The above result is agreed upon by Wang et al.s' study [21].

In the current study, the MMP evaluation using JC-1 showed that it was more dependable than the other compounds that were used for this aim (i.e., rhodamine 123 staining). Therefore, it provides a precise assessment of MMP that showed a positive correlation with sperm motility, and it is agreed with other works that recognized that "worse mitochondrial function, assessed by staining with JC1, is associated with a significant decline in sperm motility over time" [22]. The assessment of sperm, and MMP by this JC-1 stain is supported and agreed with Gravance et al.s' study [23].

Moreover, in the current work, the fluorescent staining and evaluation were done for the first time in Iraqi centers by using a new modified procedure apart from flow cytometry which is not feasible, making it a more easy and reliable method that can be used in the future with accurate results.

The intimate association between sperm motility and mitochondria activity is evident but not yet effectively applied in clinical applications. Many issues should be clarified. First of all; who will benefit from the MMP evaluation? When and which bio-functional sperm factors are critical to be assessed? Furthermore, the restriction of the research is the absence of randomized clinical trials for the assessment of mitochondrial activity and integrity in patients suffering from low sperm motility. In addition to that, the difficult accessibility or even the unavailability of the fluorescent microscope with all of its equipment set and standardized accurately makes the work more tedious. It was confirmed by many clinical studies about the important role of the sperm mitochondria and all stages of male reproductive physiology, from spermatogenesis to oocyte fertilization, that mitochondria are important organelles for

male fertility and should be listed in the advanced work for fertility management. Asthenospermia is one of the identified causes of male infertility; thus, abnormal mitochondrial activity or low MMP may lead to such a result according to its significant association with low sperm motility, so trying to manage such an issue by supporting mitochondrial function by paying attention to the diet and nutrition, sleep habits, daily movement, and detoxification pathways are all essential for healthy mitochondria [24].

The limitation of the current study was the small sample size. Besides, the unavailability of the fluorescent dye kit and the need to wait for its arrival (which is time consuming) are considered another shortcoming of the present study.

## CONCLUSION

This study revealed a noticeable association between sperm motility and its MMP assessment by fluorescent staining, which could be applied as a valid approach to evaluate the motility of sperm in Iraqi fertility centers.

## ETHICAL DECLARATIONS

### Acknowledgements

None.

### Ethics Approval and Consent to Participate

The ethical approval document was granted by the institutional ethical committee of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad, Iraq (Ref. code 0702-MM-2020A3 in November 2020). Informed consent was obtained from every participant.

### Consent for Publication

Not applicable (no individual personal data included).

### Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### Competing Interests

The author declares that there is no conflict of interest.

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### Authors' Contributions

Farhan TM was responsible for designing the study and writing the manuscript. The author read and approved the final version of the manuscript.

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