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Sperm mitochondrial membrane potential: relationship with seminal parameters and lifestyle habits

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Abstract

Background: Mitochondrial membrane potential (MMP) is indicative of mitochondrial activity. Hence, MMP values can be used as a sperm functionality and motility indicator. Although it is recognized that MMP is influenced by lifestyle and seminal parameters, the relationship is not completely clear due to the diversity of factors involved and limitations in study design. Therefore, further research is needed to establish direct causal connections. The present study investigated the relationship between MMP with conventional and physiological sperm parameters, and with modifiable lifestyle factors. **Methods**: To achieve this, 32 seminal samples obtained from male donors were analysed according to World Health Organization (WHO) guidelines. The MMP was assessed using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) in sperm from fresh samples and analysed by fluorescence microscopy. Furthermore, a survey was performed to assess the lifestyle habits of the donors. We conducted a comprehensive analysis of JC-1 in sperm, differentiating for the first time four colours based on MMP fluorescence: high mitochondrial MMP (red), medium-high MMP (orange), low mitochondrial MMP (green), and very low MMP (no fluorescence). Percentages were added to obtain two populations: total high and total low MMP. **Results**: Our results showed that lifestyle habits can impact on MMP values. Furthermore, sperm MMP values were correlated with conventional seminal parameters, and with the concentration of motile sperm after 1 h of *in vitro* capacitation. Additionally, men with total high MMP values higher than 60.05% achieved a higher concentration of sperm cells after 1 and 4 h of *in vitro* capacitation. **Conclusions**: Research on MMP and its influence on male fertility will contribute to a better understanding of reproductive biology and to develop targeted interventions for infertility.

Keywords

Male fertility; Mitochondrial membrane potential; MMP; Seminal parameters; Lifestyle habits; JC-1; Sperm capacitation

El potencial de membrana mitocondrial espermático: relación con parámetros seminales y hábitos de vida

Resumen

Antecedentes: El potencial de membrana mitocondrial (MMP, según sus siglas en inglés) es un indicador de la actividad mitocondrial, por lo que sus valores pueden utilizarse como un indicador de la funcionalidad y motilidad espermática. Aunque se conoce que el MMP está influenciado por los hábitos de vida y parámetros seminales, la relación no está completamente clara debido a la diversidad de factores involucrados y las limitaciones en el diseño de diferentes estudios. Por lo tanto, se necesita investigación adicional para establecer conexiones causales directas. El presente estudio investigó la relación entre el MMP y los parámetros espermáticos convencionales y fisiológicos, y con factores de estilo de vida modificables. **Métodos**: Para lograrlo, se analizaron 32 muestras seminales obtenidas de donantes masculinos de acuerdo con las directrices de la Organización Mundial de la Salud (OMS). El MMP se evaluó utilizando el marcador JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) en espermatozoides de muestras seminales frescas, mediante su análisis con microscopía de fluorescencia. Además, se realizó una encuesta para evaluar los hábitos de vida de los donantes. Se realizó un análisis exhaustivo de JC-1 en espermatozoides, diferenciando por primera vez cuatro colores en función de la fluorescencia emitida por la pieza intermedia: MMP mitocondrial alto (rojo), MMP medio-alto (naranja), MMP mitocondrial bajo (verde) y MMP muy bajo (sin fluorescencia). Por otra parte, los porcentajes de MMP fueron sumados para obtener dos poblaciones espermáticas según su MMP: MMP total alto y MMP total bajo. **Resultados**: Nuestros resultados mostraron que los hábitos de vida pueden afectar los valores de MMP. Además de esto, los valores de MMP se correlacionaron con parámetros seminales convencionales y con la concentración de espermatozoides móviles después de 1 h de capacitación *in vitro*. Además, los hombres con valores de MMP total alto superiores al 60.05% lograron una mayor concentración de espermatozoides después de 1 y 4 h de capacitación *in vitro*. **Conclusiones**: La investigación sobre MMP y su influencia en la fertilidad masculina contribuirá a una mejor comprensión de la biología reproductiva y al desarrollo de intervenciones específicas dirigidas al tratamiento de la infertilidad.

Palabras Clave

Fertilidad masculina; Potencial de membrana mitocondrial; MMP; Parámetros seminales; Hábitos de vida; JC-1; Capacitación espermática

1. Introduction

The evaluation of conventional and functional sperm parameters is essential to assess male reproductive function [1, 2]. In this context, sperm motility is a crucial parameter to guarantee male fertility that has received attention in recent years [3, 4]. Mitochondrial oxidative phosphorylation contributes to adenosine triphosphate (ATP) production in spermatic cells [5], which is essential to ascertain good motility and function. In sperm cells, mitochondrial oxidative phosphorylation is the main source of reactive oxygen species (ROS), which at minimal concentrations plays a vital physiological role, including signaling pathways leading to capacitation [6], sperm hyperactivation, acrosome reaction, and sperm-oocyte binding [7, 8]. Capacitation is a complex and highly regulated process that involves structural and functional changes [9, 10] needed to accomplish the fertilization process. In this context, an energetic environment is necessary for protein phosphorylation leading to hyperactivated motility [11]. Consequently, spermatozoa function [12, 13] and fertilization potential relies on healthy and fully functional mitochondria [7, 14].

A major indicator of mitochondrial activity is the measurement of the mitochondrial membrane potential (MMP). A high MMP is indicative of highly functional mitochondria, while a low MMP is associated with unhealthy spermatozoa. Spermatozoa with low MMP have poor sperm motility because of lower ATP production. In numerous cases of idiopathic male infertility, mitochondrial impairment is connected to the pathogenesis of seminal oxidative stress (OS) [15]. Specifically, mitochondrial membrane potential dysfunction is part of the apoptotic phenomena occurring in human spermatozoa [16]. Therefore, MMP value can be used as key indicators of sperm functionality [17, 18]. A fundamental tool to study the MMP is JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide). This probe is a lipophilic cationic fluorescent dye that serves as a sensitive indicator of MMP. Based on the MMP, JC-1 accumulates within mitochondria. In energized mitochondria, JC-1 accumulates and forms spontaneous aggregates with red fluorescence due to its high concentration, whereas in less energized mitochondria, the concentration of JC-1 is lower, and fewer aggregates are formed, indicating apoptotic or altered cells.

Regarding the study of the relationship between the MMP and semen parameters, a positive relationship was found between the percentage of cells with high MMP and sperm concentration and motility [18]. Male fertility may be directly affected by MMP, which potentially regulates and indicates spermatic motility [12]. Reduced levels of MMP had been associated with seminal defects [19, 20], thus leading to a decreased fertility [21]. Furthermore, the percentage of sperm with high MMP has been positively correlated with the percentage of sperm with progressive motility, viability, sperm concentration, volume, and normal morphology [22]. Conversely, the relationship between exposure to lifestyle factors and its impact on semen quality is highly debated [23, 24]. Some lifestyle habits, such as smoking, seem to have

a detrimental influence on seminal parameters because they can lead to OS [25–27]. Further research is necessary to fully understand the specific lifestyle factors that influence seminal quality.

In this study, we assessed the relationship between sperm MMP with conventional and physiological sperm parameters, including data of motile sperm after one and four hours (1 and 4 h) of *in vitro* capacitation. In addition, we sought to investigate the effect of modifiable lifestyle factors on sperm MMP.

2. Materials and methods

2.1 Experimental design

Seminal samples were obtained from male donors. Conventional sperm parameters were assessed according to World Health Organization (WHO) guidelines [28], including the macroscopic and microscopic analysis. MMP was assessed in sperm from fresh samples using JC-1. Afterwards, samples were *in vitro* capacitated using the swim-up technique for 1 and 4 h. Then, the motile sperm was collected to assess the sperm concentration and motility.

2.2 Sample collection and basic semen analysis

Semen samples were gathered from 32 healthy individuals by masturbation, after abstaining from sexual activity for 3 to 4 days, between February 2022 and May 2023. All donors provided informed consent. Basic semen analysis was performed within 1 h of sample collection. Conventional sperm parameters were evaluated following WHO guidelines [28]. Sperm concentration and total sperm motility were measured with a Makler counting chamber (BioCare Europe, Rome, Italy), the morphology was examined after dying with Papanicolaou staining (Panreac Química S.L.U., Barcelona, Spain), and cell viability was assessed using Sperm VitalStain^{TM} (010SVSBC18, NidaCon International AB, Mölndal, Sweden). The MMP was assessed in non-capacitated spermatozoa following the protocol detailed in the next section.

2.3 Mitochondrial membrane potential measurement

The MMP was measured using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide or JC-1 lipophilic probe (MERCK, Darmstadt, Germany). The methodology used in this study was based on the protocol of Carrageta *et al.* [14]. Fresh sperm samples were diluted in phosphate-buffered saline (PBS) solution at a final concentration of 1×10^6 sperm cells/mL and were incubated with JC-1, at a final concentration of 0.6 μg/μL for 20 min at 37 °C and 5% CO_2. Afterwards, the mitochondrial membrane potential of sperm was evaluated in a fluorescent microscope Leica DM750 (Leica, Wetzlar, HE, Germany).

The fluorescence microscope was equipped with a filter designated as fluorescein isothiocyanate (FITC)/green fluorescent protein (GFP), with the following specifications: excitation wavelength of 460–490 nm, dichroic mirror wavelength of >500 nm, and emission filter wavelength of >510 nm. The properties of the JC-1 probe allowed the observation of both red and green emissions using this filter, with their combination resulting in the visualization of the orange signal. For the observation of non-fluorescent cells, the fluorescence lamp was turned off, and the same microscope field was examined using the optical microscope. At least 200 sperm cells of each sample were evaluated according to the fluorescence colour occupied by the midpiece as follows: high MMP (red), medium-high MMP (orange), low MMP (green), and very low MMP (no fluorescence) as showed in Fig. 1. This represents a novelty since literature only divides sperm cells into two populations (high and low MMP) [29]. During this study we evidenced the presence of four fluorescence patterns based on the fluorescence colour, as represented in Fig. 1. To further compare our results with those of the literature [22, 29, 30], the percentages were added to obtain two populations: total high MMP (high mitochondrial membrane potential sperm + medium-high mitochondrial membrane potential sperm), and total low MMP (low mitochondrial membrane potential sperm + very low mitochondrial membrane potential sperm).

2.4 *In vitro* capacitation by swim-up

The protocol for sperm capacitation has been previously detailed by our team [31–33]. Seminal plasma was eliminated after centrifugation at 300×g for 10 min. After that, the cells were washed using human tubal fluid medium (HTF, Origio®, Måløv, Denmark). Subsequently, spermatozoa were incubated in HTF medium supplemented with bovine serum albumin at a concentration of 5 mg/mL (BSA, SLCG4390, Sigma-Aldrich®, St. Louis, MO, USA) at 37 °C with 5.5% (v/v) of CO_2 during 1 and 4 h. After 1 and 4 h of *in vitro* capacitation, the motile sperm fraction was collected and the motile sperm concentration (MSC1 and MSC4) and percentage of sperm with progressive motility (MSPM1 and MSPM4) were calculated.

2.5 Lifestyle habits questionnaire

All participants completed a questionnaire at the time of semen donation. When conducting the lifestyle habits survey, we specifically asked about habits—something a person does usually or regularly—as the purpose of the questionnaire was to assess how these habits may impact spermatogenesis, which is ultimately reflected in sperm quality. The questionnaire evaluated modifiable lifestyle factors, including exposure to dairy stress, sleep habits (≥8 h), occupational exposure to toxics (pesticides, herbicides, chemicals, disinfectants, paints, foundries, industrial products, or other types of toxic substances), daily sports activity (≥1 h), current smoking status, and cannabis consumption. Additionally, nutritional habits such as daily coffee and alcohol intake, vegetable oils consumption (sunflower, corn, soybean), and daily beer consumption were recorded. Based on their responses, participants were categorized into two groups: “Yes” and “No” for each lifestyle habit. The cannabis consumption variable was categorized into three categories: daily, weekly and occasional use. Since all participants who reported cannabis use indicated they were occasional consumers, this group was categorized as “occasional consumer” and “non-consumer”. On the other hand,

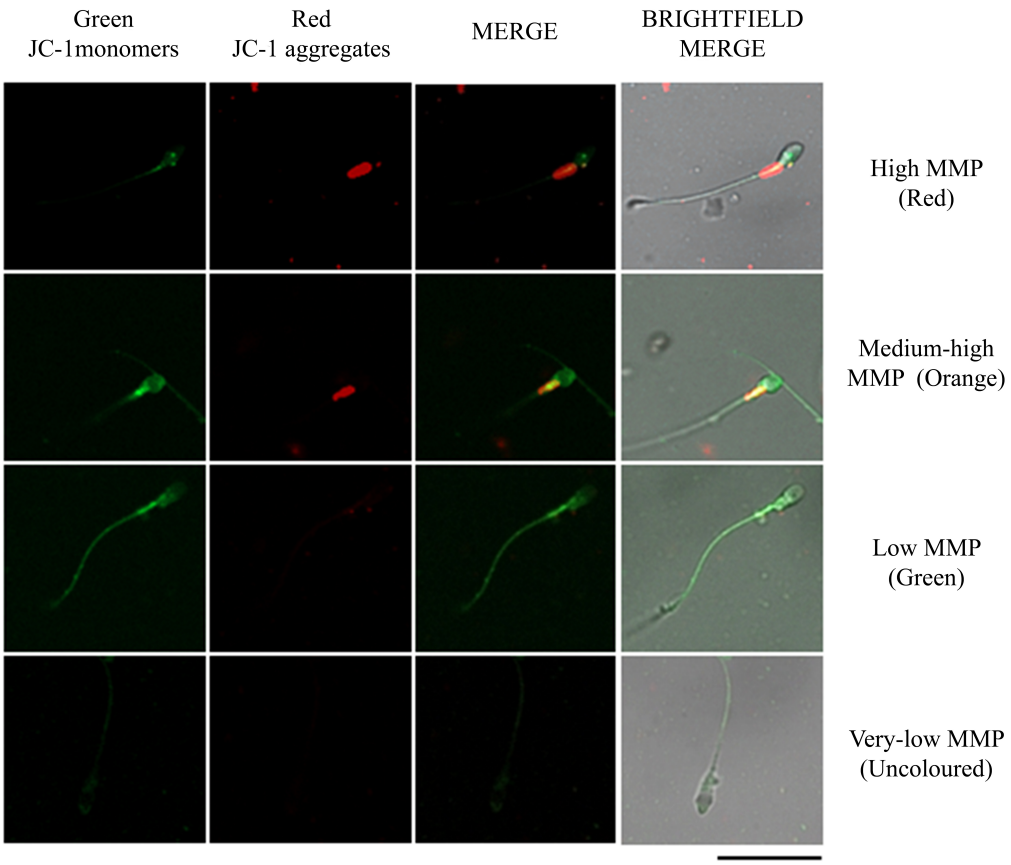

FIGURE 1. Fluorescent micrographs of mitochondrial membrane potential assay in human sperm. High mitochondrial membrane potential sperm (red), medium-high mitochondrial membrane potential sperm (orange), low mitochondrial membrane potential sperm (green), and very low mitochondrial membrane potential sperm (no fluorescence). Scale bar: 20 μm. JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; MMP: Mitochondrial membrane potential.

participants were considered heavy smokers if they smoked 25 or more cigarettes per day. As a result, the group of "current smokers" included 3 participants who were classified as heavy smokers, while the remaining 6 participants smoked between 2 and 4 cigarettes daily. The "non-smoker" group comprised men with no history of smoking.

2.6 Statistical analysis

Data were evaluated statistically using SPSS version 25 (SPSS Inc., Chicago, IL, USA). As a first step, a Shapiro-Wilk test was performed to test data normality. Under the results, a Student's *t*-test or Kruskal Wallis test was performed to investigate differences between groups. The correlation between seminal parameters and MMP was determined using Pearson's correlation coefficients. A *p*-value lower than 0.05 was considered statistically significant. Figures were generated using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Seminal parameters

The mean concentration of sperm cells was found to be 108.42 ($\pm$70.06) $\times$ 10^6 cells/mL with 66.51 ($\pm$17.75)% of total sperm motility, 8.16 ($\pm$2.38)% of normal morphology, and a viability of 87.03 ($\pm$8.10)%. After 1 and 4 h capacitation, the concentration of motile spermatozoa was 19.92 ($\pm$19.57) $\times$ 10^6 cells/mL and 10.94 ($\pm$7.01) $\times$ 10^6 cells/mL with a percentage of sperm with progressive motility of 93.91 ($\pm$10.09)% and 96.52 ($\pm$4.43)%, respectively. Regarding the mitochondrial membrane potential, a 55.56 ($\pm$14.58)% showed a high MMP (red), a 4.39 ($\pm$3.78)% of sperm had a medium-high MMP (orange), a 22.73 ($\pm$17.20)% had a low MMP (green), and finally, a 17.32 ($\pm$13.50)% showed a very low MMP (uncoloured). Finally, a 60.05 ($\pm$14.41)% of cells had a total high MMP.

3.2 MMP and lifestyle habits

The impact of modifiable lifestyle factors on sperm MMP is presented in Table 1. Notably, smokers exhibited a higher percentage of sperm with elevated MMP (68.99 ($\pm$13.95)% compared to 56.60 ($\pm$13.72)%). Additionally, participants who did not consume vegetable oils demonstrated a higher percentage of sperm with elevated MMP (64.86 ($\pm$10.69)%) compared to those who reported consuming these oils (53.34 ($\pm$13.06)%).

TABLE 1. Mean and SD of the total high mitochondrial membrane potential (MMP) for each group of men.

Lifestyle habit	Group of men	Total High MMP	*t*/*p*-value
Exposure to dairy stress			
	Yes (n = 13)	60.43 ± 16.34	−0.033/0.975
	No (n = 16)	60.62 ± 14.36	
Sleep habits (≥8 h of sleep)			
	Yes (n = 8)	65.47 ± 15.18	1.135/0.279
	No (n = 22)	58.44 ± 14.54	
Occupational exposure to toxics			
	Yes (n = 6)	60.86 ± 7.32	0.368/0.717
	No (n = 22)	59.15 ± 16.59	
Daily sports practice (≥1 h)			
	Yes (n = 10)	59.29 ± 10.63	−0.306/0.762
	No (n = 20)	60.82 ± 16.71	
Current smoking status			
	Current smoker (n = 9)	68.99 ± 13.95*	2.237/0.041
	Non-smoker (n = 21)	56.60 ± 13.72*	
Cannabis consumption			
	Occasional consumer (n = 5)	68.44 ± 16.92	1.248/0.267
	Non-consumer (n = 27)	58.45 ± 13.69	
Daily coffee intake			
	Yes (n = 11)	58.25 ± 14.18	−0.365/0.718
	No (n = 17)	60.26 ± 14.36	
Distilled liquor weekly consumption			
	Yes (n = 17)	58.91 ± 13.19	−0.089/0.930
	No (n = 11)	54.43 ± 16.03	
Another vegetal oils use			
	Yes (n = 9)	53.34 ± 13.06*	−2.245/0.039
	No (n = 18)	64.86 ± 10.69*	
Daily beer intake			
	Yes (n = 11)	64.85 ± 16.21	1.234/0.233
	No (n = 19)	57.69 ± 13.65	

**Significant differences at $p < 0.05$.*

3.3 Relationship of MMP with conventional and physiological seminal parameters

Present findings showed that the percentage of sperm with total high MMP was significantly and positively correlated with the sperm concentration (Fig. 2A), and with the percentage of total sperm motility (Fig. 2B).

Results for the capacitated samples during 1 and 4 h revealed that the percentage of sperm with total high MMP was significantly and positively correlated with the concentration of motile sperm after 1 h of *in vitro* capacitation (Fig. 2C), but not with the concentration of motile sperm after 4 h of *in vitro* capacitation ($p = 0.376$).

Values for sperm concentration and progressive motility recovered after 1 and 4 h of capacitation are displayed in Table 2. No significant differences were found between the concentration of motile sperm ($p = 0.060$) and the percentage of motile sperm with progressive motility ($p = 0.472$) after 1 and 4 h of *in vitro* capacitation.

Participants were categorized in two groups based on the laboratory's reference value for total high MMP (60.05%): men with high MMP (>60.05%, H-MMP) and men with low MMP (<60%, L-MMP). Table 3 shows the motile sperm concentration and progressive motile sperm percentage retrieved for each group after 1 and 4 h of *in vitro* capacitation. The statistical analysis revealed that compared to the L-MMP group, the H-MMP group achieved a higher concentration of sperm cells after 1 and 4 h of *in vitro* capacitation. However, sperm motility showed no differences between the groups. Further to this, in the L-MMP group, no differences were found between the concentration and motility of sperm after 1 and 4 h of capacitation. Still, the H-MMP group

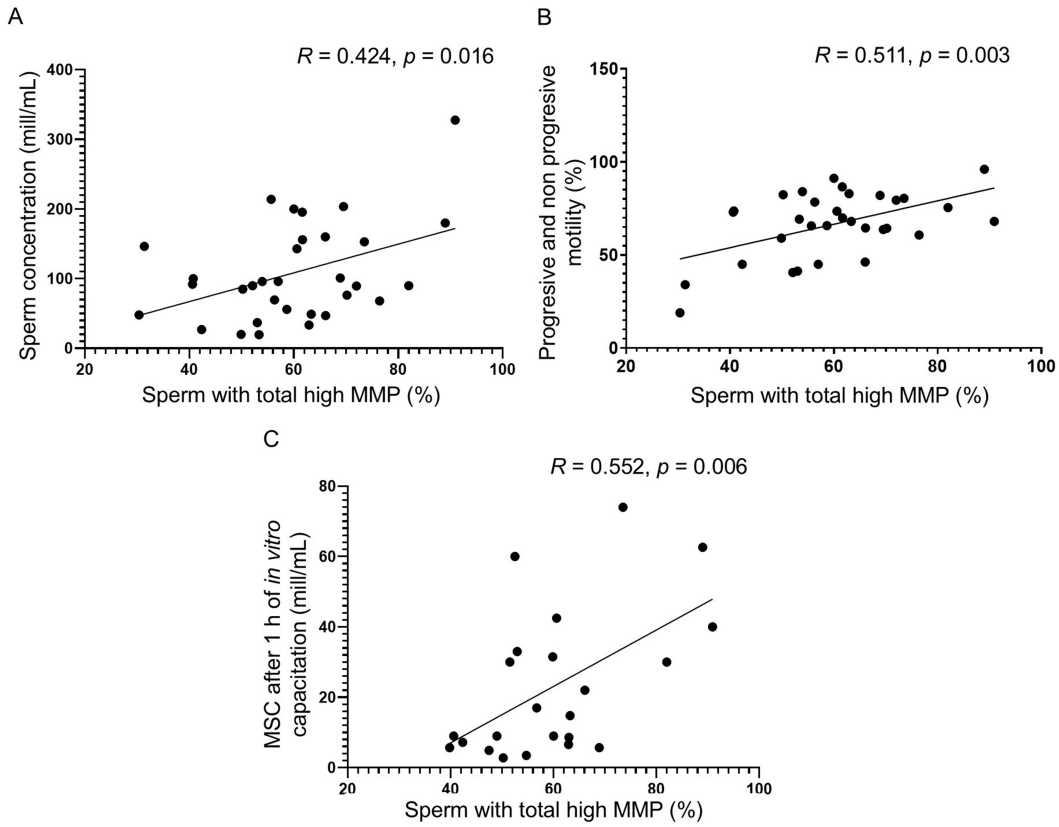

FIGURE 2. Dispersion graphics. (A) Dispersion graphic showing the significant relationship between the percentage of sperm with total high MMP (independent variable, x-axis) and the sperm concentration (dependent variable, y-axis). (B) Dispersion graphic showing the relationship between the percentage of sperm with total high MMP (independent variable, x-axis) and the percentage of motile sperm (dependent variable, y-axis). (C) Dispersion graphic showing the relationship between the percentage of sperm with total high MMP (independent variable, x-axis) and the concentration of motile sperm recovered after 1h of *in vitro* capacitation. MMP: Mitochondrial membrane potential; MSC: Motile sperm concentration.

TABLE 2. Motile sperm concentration and percentage of sperm with progressive motility after 1 and 4 h of *in vitro* capacitation for each group.

1 h capacitation (n = 23)		4 h capacitation (n = 23)	
MSC1 (mill/mL)	MSPM1 (%)	MSC4 (mill/mL)	MSPM4 (%)
23.02 ± 20.90	96.08 ± 3.72	10.94 ± 7.01	96.52 ± 4.43

MSC1: motile sperm concentration after 1 h of in vitro capacitation; MSC4: motile sperm concentration after 4 h of in vitro capacitation; MSPM1: percentage of sperm with progressive motility after 1 h of in vitro capacitation; MSPM4: percentage of sperm with progressive motility after 4 h of in vitro capacitation.

TABLE 3. Motile sperm concentration and percentage of sperm with progressive motility after 1 and 4 h of *in vitro* capacitation for each group.

	1 h capacitation		4 h capacitation	
	MSC1 (mill/mL)	MSPM1 (%)	MSC4 (mill/mL)	MSPM4 (%)
H-MMP (n = 14)	31.81 ± 21.79*	95.59 ± 3.59	21.72 ± 6.41*	96.84 ± 2.37
L-MMP (n = 9)	9.34 ± 9.18	96.84 ± 3.99	8.17 ± 7.35	95.87 ± 7.25

H-MMP: total high mitochondrial membrane potential; L-MMP: total low mitochondrial membrane potential. MSC1: motile sperm concentration after 1 h of in vitro capacitation; MSC4: motile sperm concentration after 4 h of in vitro capacitation; MSPM1: percentage of sperm with progressive motility after 1 h of in vitro capacitation; MSPM4: percentage of sperm with progressive motility after 4 h of in vitro capacitation.
**Significant differences at $p < 0.05$.*

showed a lower sperm concentration after 4 h of capacitation compared to 1 h concentration ($p = 0.011$) with a mean sperm concentration of 21.72 (±6.41) mill/mL and 31.81 (±21.79) mill/mL, respectively. Sperm motility showed no differences.

4. Discussion

The mitochondrial membrane potential is essential for sperm functionality and has a critical role in male fertility. Previous studies demonstrated that a positive relationship was found between the percentage of cells with high MMP and sperm concentration and motility [17, 29], being both parameters crucial to guarantee male fertility [4] and a prerequisite for successful conception [29]. In this context, the MMP is an important indicator of mitochondrial function in sperm. High MMP levels indicate proper integrity of the mitochondria and optimal mitochondrial activity [16]. Further to this, high MMP is correlated with a good fertilizing capacity with higher possibilities of achieving good-quality embryos and an increased probability of achieving pregnancy [17, 29]. Our study aimed to investigate sperm MMP and its associations with conventional and physiological seminal parameters, as well as its relationship with lifestyle habits. By exploring these connections, we aimed to deepen the understanding of how mitochondrial function impacts sperm quality, while also shedding light on the role of modifiable lifestyle factors.

It is noteworthy that our results show for the first time four levels of fluorescence in relation to MMP in human sperm. Specifically, high MMP (red), medium-high MMP (orange), low MMP (green), and very low MMP (no fluorescence). Highlighting the presence of almost 20% of spermatozoa with no labeling. This data is relevant, since it is possible that in the studies performed so far in relation to MMP using JC-1 this sperm subpopulation is not being taken into account. The identification of these four levels of fluorescence has also been possible due to the direct visualization of the cells using a fluorescence microscope, since the use of other alternatives such as flow cytometry could have had some limitations. For instance, spectral overlap or interference from other particles present in the seminal fluid. However, in order to compare our data with the existing scientific literature, we decided to regroup our results into only two categories, total high and total low MMP.

The main causes of sperm mitochondrial dysfunction are OS, genetic factors, and different factors which include obesity, aging, and lifestyle changes [8]. However, while normal physiological ROS levels exert an important physiological role in sperm capacitation, acrosome reaction, sperm capacitation, and sperm-oocyte binding [13], OS results from an imbalance between elevated ROS levels and antioxidant molecules, promoting ROS levels [8]. In this context, endogenous and exogenous sources (tobacco smoking, radiation, alcohol and toxins) can contribute to ROS production, ultimately leading to OS [34].

Our results highlighted that smokers had a significantly higher percentage of sperm with elevated total high MMP, compared to non-smokers. These results were unexpected, as previous studies have demonstrated that lower MMP correlates with increased ROS levels [16] and smoking leads to OS [35]. Specifically, elevated OS, marked by increased ROS production, is a characteristic feature of smokers. Clinical studies have demonstrated altered OS biomarker profiles in smokers, including lipid peroxidation products (*e.g.*, malondialdehyde (MDA)…), oxidized proteins, and nucleic acid metabolites (*e.g.*, 8-hydroxy-2′-deoxyguanosine (8-OHdG)). Reduced antioxidant levels, such as glutathione (GSH), have also been reported. These biomarkers are detectable in different biological matrices as blood, saliva [36]… However, this discrepancy in our results could be attributed to the smoking habits of our participants, as only three men were heavy smokers, while the majority smoked only 2 to 4 cigarettes per day.

Furthermore, our findings showed that non-consumers of vegetal oils had a higher percentage of sperm with high MMP compared with men consuming them. It is a well-known fact that fatty acids are important constituents of biological membranes and energy suppliers. Vegetal oils such as sunflower, corn, and soybean oils are rich in polyunsaturated fatty acids (PUFAs) [37]. Previous studies have demonstrated a significant correlation between PUFAs and ROS production in both young and middle-aged individuals [38]. However, the potential causes underlying MMP dysfunction are multifactorial, encompassing lifestyle habits that might not have been taken into account in the questionnaire, and a potential combination of different lifestyle factors. In addition, genetic factors may also play a significant role in influencing MMP levels. In light of these complexities, more comprehensive assessments of modifiable lifestyle factors are required to better understand their interaction with sperm MMP.

According to our findings, the percentage of sperm with total high MMP was positively correlated with sperm concentration and with the percentage of total motile sperm. Previous studies showed that the percentage of sperm cells with high MMP was positively correlated with the percentage of sperm with progressive motility [22], with the total sperm motility [39] and the sperm concentration [22, 39]. Espinoza *et al.* [22] reported that the percentage of sperm cells with high MMP was positively correlated with sperm viability and normal morphology. So, we demonstrated a correlation between conventional and biofunctional parameters, as is the case of MMP, such as previous articles established [40].

A relationship between MMP values and sperm motility decline over time has been established [29]. Specifically, men with low MMP values had a decreased percentage of sperm with total and progressive motility after 4 h [29]. Further to this, since previous studies have shown that 4 h capacitation benefits certain sperm biomarkers [9], this experiment aimed to determine whether mitochondrial activity can predict sperm concentration and motility following 1 and 4 h of *in vitro* capacitation. Our results point out that a higher percentage of total high MMP in fresh samples results in a higher concentration of motile sperm after 1 h of *in vitro* capacitation. However, the results were non-significant after 4 h of *in vitro* capacitation. Additionally, men with total high MMP values higher than 60.05% achieved higher motile sperm concentrations after 1 and 4 h of *in vitro* capacitation than those with lower values. This result could be expected as fresh seminal samples having a high percentage of sperm with high MMP are expected to

recover a higher concentration of motile spermatozoa with progressive motility after *in vitro* capacitation regardless of capacitation time.

Furthermore, men with total MMP values greater than 60.05% showed a lower motile sperm concentration after 4 h of *in vitro* capacitation than after 1 h. In this context, it is known that hydrogen peroxide (H_2O_2) is the main toxic ROS for human sperm cells [41] which is produced by spermatozoa and leukocytes. At the same time, physiological normal ROS production is one of the first essential events happening during sperm capacitation, as it regulates and triggers various processes, including protein phosphorylation [42]. It has been demonstrated that during sperm capacitation, spermatozoa incubated for prolonged periods produce elevated concentrations of superoxide anion [41]. Further to this it was established that low concentrations of ROS cause sperm immobilization via depletion of intracellular ATP [41]. These findings suggest that ROS production may increase during the 4 h of *in vitro* capacitation compared to 1 h. Consequently, ROS could cause sperm immobilization, explaining the lower concentration of motile sperm after 4 h compared to 1 h. However, additional studies must be conducted to understand and probe this hypothesis. Based on our results, evaluating MMP could be valuable for selecting the optimal capacitation time, depending on the assisted reproductive technique recommended.

It should be noted that due to the diversity of factors involved in the influence of lifestyle and semen parameters on MMP, there are certain limitations in this study. Among them, limitations related to the measurement of lifestyle factors, sample size, variability of semen parameters as well as the complexity of human fertility must be carefully considered. These aspects may influence the interpretation and applicability of the results.

5. Conclusions

We established a novel approach to evaluate sperm's mitochondrial functionality based on four fluorescence colour patterns depending on the MMP: high mitochondrial MMP (red), medium-high MMP (orange), low mitochondrial MMP (green), and very low MMP sperm (no fluorescence). On the other hand, our research demonstrates the effects that modifiable lifestyle habits can have on MMP. Further to this, our results support the results of previous studies regarding the relationship between high MMP and sperm concentration and motility. Furthermore, we demonstrated that a high MMP correlates with the concentration of motile sperm after 1 h of *in vitro* capacitation. In addition, our results showed that men with a high percentage of sperm with high MMP benefit, in terms of motile sperm concentration, from 1 h of *in vitro* capacitation.

Therefore, we demonstrated that lifestyle habits can impact on the seminal quality; however, due to the great diversity of factors involved, this study presents certain limitations. Thus, future research on lifestyle habits and sperm MMP and their influence on male fertility to increase knowledge and promote the design of targeted interventions to address infertility challenges.

ABBREVIATIONS

MMP, Mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; WHO, World Health Organization; ATP, adenosine triphosphate; h, Hour; ROS, Reactive oxygen species; OS, Oxidative stress; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; MSC1, Motile sperm concentration after 1 h of *in vitro* capacitation; MSC4, Motile sperm concentration after 4 h of *in vitro* capacitation; MSPM1, Percentage of sperm with progressive motility after 1 h of *in vitro* capacitation; MSPM4, Percentage of sperm with progressive motility after 4 h of *in vitro* capacitation; H-MMP, high MMP; L-MMP, low MMP; MDA, Malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; GSH, Glutathione; PUFAs, polyunsaturated fatty acids.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

ALB, PSE and MJGT—designed the research study. ALB, MHF, PSE, LRG and MJGT—performed the research. PSE and MJGT—provided help and advice during the research and writing. ALB—analyzed the data; wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Alicante (UA-2021-29-11). All donors provided informed consent.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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