

The effect of in-vitro use of FSH on sperm parameters, DNA integrity, and Mitochondrial Membrane Potential in Asthenozoospermic men

Faezeh Etebari

Shahid Sadoughi University of Medical Sciences

Mohammad Ebrahim Rezvani

Shahid Sadoughi University of Medical Sciences

Sahar Khosravi

Shahid Sadoughi University of Medical Sciences

Mahin Izadi

Shahid Sadoughi University of Medical Sciences

Esmat Mangoli

es.mangoli@gmail.com

Shahid Sadoughi University of Medical Sciences

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Abstract

Purpose: Sperm motility is a key indicator of male fertility. Decreased motility, or "asthenozoospermia," highlights the need for understanding male fertility challenges. This experimental in vitro study was designed to evaluate the effects of follicle-stimulating hormone (FSH) on various sperm parameters, sperm DNA integrity, and mitochondrial membrane potential.

Methods: Semen samples were obtained from 20 asthenozoospermic men. The samples were divided into control, and case groups which were incubated with FSH at a concentration of 30 mIU/mL for one hour. Sperm parameters, DNA fragmentation, and mitochondrial membrane potential were assessed in two groups based on the WHO 2021 criteria.

Results: In the experimental group, progressive motility and especially rapid progressive motility were higher compared to the control group. However, the FSH hormone did not show a significant effect on morphology, viability, DNA fragmentation, or mitochondrial membrane potential in either group.

Conclusion: FSH effectively enhances sperm motility without compromising sperm DNA integrity or mitochondrial membrane potential (MMP). Therefore, FSH can be recommended as a safe and effective option for sperm selection in patients with asthenozoospermia.

1. Introduction

In recent decades, infertility has become a significant challenge for many couples around the world. The World Health Organization (WHO) defines infertility as the inability of a sexually active couple, using no contraceptive methods, to achieve pregnancy within one year. Approximately 17.5% of the adult population (about 1 in 6 individuals) experiences infertility. This issue can arise due to male factors, female factors, a combination of both, or it may remain unexplained. Infertility is now recognized as a medical condition, and recent statistics indicate that the proportion of men with infertility is 40–50 percent of cases [1].

The assessment of male infertility predominantly depends on a comprehensive semen analysis. Sperm motility is a significant determinant of male fertility potential and is regarded as a crucial parameter in semen analysis. A reduction in sperm motility, referred to as "asthenozoospermia," is identified as the primary contributor to male infertility. The World Health Organization (WHO) guidelines from 2021 define asthenozoospermia by total motility that is less than 42% and progressive motility that falls below 30% [2].

A range of therapeutic approaches has been examined to enhance sperm motility in patients diagnosed with asthenozoospermia, utilizing both in vivo and in vitro methodologies. A review article has analyzed several studies that investigated the effects of various treatments, including pentoxifylline, cAMP analogs, antioxidants, and vitamins, on enhancing sperm motility in vitro. It is essential to acknowledge that each of these compounds is associated with specific limitations[3–6]. Various treatment protocols,

including the administration of follicle-stimulating hormone (FSH), which is produced by the anterior pituitary gland, have been utilized in the management of male infertility, particularly in cases of hypogonadotropic hypogonadism. Evidence from clinical studies indicates that the use of FSH is associated with improvements in sperm concentration and progressive motility [7, 8].

To the best of our knowledge, only two studies have shown that FSHR is expressed in human spermatozoa and it was observed mainly in the midpiece and in the principal piece of the tail [9, 10]. The ability to modulate sperm motility *in vitro* offers researchers and clinicians a unique opportunity to develop targeted treatments that can be applied directly to sperm samples prior to their use in assisted reproductive technology (ART) procedures. FSH appears to be a promising option for improving sperm motility due to its non-toxic nature, the absence of complex purification and processing requirements, and the identification of its receptor on sperm cells. In a study in 2020, the effect of treatment with FSH *in vitro* on the sperm motility of people with varicocele and normozoospermia was evaluated, and the results indicated an increase in sperm motility in the groups (12). On the other hand, a study was conducted in 2023 with the aim of investigating the effect of FSH treatment on the sperm function of 13 asthenozoospermic patients and the control group, and the results of this study were a decrease in progressive and total sperm motility (11). Based on the available evidence, this study aimed to evaluate the effects of FSH on sperm motility, morphology, viability, DNA integrity, and mitochondrial membrane potential in the asthenozoospermic patients.

2. Materials and Methods

2.1 Experimental Design

This experimental study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.MEDICINE.REC.1402.388), and was done from March 2024 to November 2024 at Yazd Reproductive Sciences Institute. We used 20 asthenozoospermic samples according to WHO criteria [1]. Samples of men less than 40 years old with normal sperm count and morphology were included. Smokers, patients with varicocele and with a history of varicocelectomy, male accessory gland infection/inflammation, genetic syndromes, previous or current exposure to chemo- and/or radiotherapy, retrograde ejaculation, hypogonadism, hormone therapy (e.g., on FSH, selective estrogen receptor modulators, testosterone replacement therapy, aromatase inhibitors, etc.), or antioxidants were excluded. The patients agreed to participate in this study with the informed consent.

In the first part of the experimental design, each selected sample was divided into two parts, case and control. Second, adding FSH at a concentration of 30 mIU/ML to the case group and incubating two groups for 60 minutes at 37°C and 5% CO₂ [9, 10]. Third, evaluation of sperm parameters and DNA fragmentation and mitochondrial membrane potential after treatment in two groups

2.2 Semen Collection and treatment

Semen samples were collected by masturbation with 2 to 7 days abstinence and placed in a sterile container. Semen samples were then stored at room temperature (20 to 22°C) and analyzed immediately after complete liquefaction.

Each sample was divided into two equal parts and to one of the samples as the case, FSH hormone (Sinal-F 75IU, made in Iran) was added at a concentration of 30 mIU/mL. Both case and control samples were incubated at 37°C and CO₂ 5% for 60 minutes [9, 10]. Then, we analyzed the sperm motility, sperm morphology and viability, mitochondrial function, and DNA fragmentation.

2.3 Motility Assessment

In order to evaluate sperm motility, 10 µL of sperm sample, a well-mixed semen sample was loaded into the center of a clean Makler counting chamber, maintained at a temperature of 37°C, gently covered with a coverslip, and examined under light microscopy at 40× magnification by counting 200 sperm cells. A four-category system for grading motility is recommended which includes Rapidly progressive, slowly progressive, non-progressive, and immotile [2].

2.4 Viability and Morphology Assessment

In the same experimental conditions, viability was assessed by a red-eosin exclusion test using eosin to evaluate the potential toxic effects of the treatments. Briefly, 10 µL of the sperm sample was mixed with 10 µL of staining solution, and the mixture was placed on a glass slide. The sample was observed under light microscopy at 40 × magnification. 200 cells were counted for stain uptake (dead cells) or removal (live cells). Sperm viability was expressed as a percentage of total live sperm [2].

Sperm morphology was performed with DIFF-QUICK staining (Ideh varzan farda (IVF Co) IRAN). Detailed morphological evaluation was performed using a 100× oil-immersed bright field objective and at least 200 sperm were evaluated. Finally, the percent of normal sperm was calculated.

2.5 Evaluation of Sperm Mitochondrial Membrane Potential

Mitochondrial Membrane Potential (MMP) was evaluated using a lipophilic probe 5,5',6,6'-tetrachloro 1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (Cayman Chemical Company, Ann Arbor, MC, USA, cat #10009172), which can selectively penetrate mitochondria. Briefly, an aliquot containing 1×10^6 /mL of spermatozoa was incubated with JC-1 in the dark for 10 min at 37°C. At the end of the incubation period, the cells were washed in PBS and analyzed. JC-1 exists in monomeric form, emitting at 527 nm, but it can form aggregates emitting at 590 nm. Therefore, the fluorescence reversibly changes from green to orange as the mitochondrial membrane becomes more polarized. In viable cells with normal membrane potential, JC-1 is found in the mitochondrial membrane in the form of aggregates that emit an orange fluorescence, while in cells with low membrane potential, it remains in the cytoplasm in monomeric form and has a green fluorescence.

2.6 Evaluation of Sperm DNA fragmentation

Sperm DNA fragmentation was assessed using the sperm chromatin dispersion (SCD) method. The degree of DNA dispersion was assessed by observing the relative halo size under bright field microscopy (Halo kit, Idehvarzan Farda Company, Tehran, Iran). A minimum of 200 spermatozoa per sample was evaluated. In brief, four different dispersion patterns based on halo size were observed under bright field microscopy; (i) sperm nuclei with a big halo (ii) sperm nuclei with medium-sized halo, (iii) sperm nuclei with a very small halo and (iv) sperm nuclei without the halo. Sperm with large and medium-sized halos are considered to be normal or non-fragmented and sperm with small-sized halo or no halo are considered to have significant DNA fragmentation[11].

2.7 Statistical Analysis

The SPSS 27 software was used for statistical analysis and GraphPad Prism software was used for graphing. The normality of data distribution was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. The Mann-Whitney U-test was used for data without normal distribution, and in the case of normal distribution, the student's t-test was used to compare two groups. $p < 0.05$ was considered as a significant level in the analyses.

3. Results

In this study, 20 semen samples from men with asthenospermia were selected according to the WHO 2021 regarding to inclusion and exclusion criteria. The average age of the patients was less than 40 years, and their body mass index was less than 30. The average sperm count of the samples in this study was 19 million/mL.

3.1 The Effect of FSH on sperm parameters after incubation

3.1.1 The Effect of FSH on sperm motility

Table 1 showed motility parameters in the case and control group. The progressive motility of sperm in the case group was higher than in the control group (31.16% vs. 23.85%), although the difference was not statistically significant ($p = 0.16$). In addition, rapid progressive motility in the case group was significantly higher than that in the control group ($p = 0.05$), and immotile sperm was significantly reduced in the case group compared to control ($p = 0.05$) (Table 1).

Table 1
Sperm motility in case and control groups

Motility	Analysis	Case	Control	P-value
<i>Progressive motility (%)</i>	Mean $\pm$ SD	31.16 $\pm$ 12.41	23.85 $\pm$ 7.01	0.162
	Median (IQR)	31.5 (23.50	27.00 (13.00)	
<i>Rapid progressive (%)</i>	Mean $\pm$ SD	15.33 $\pm$ 13.27	6.42 $\pm$ 8.52	0.05
	Median (IQR)	13.50 (24.75)	0.00 (15.00)	
<i>Slow progressive (%)</i>	Mean $\pm$ SD	15.83 $\pm$ 7.78	17.42 $\pm$ 8.03	0.85
	Median (IQR)	12.50 (15.50)	15.00 (15.00)	
<i>Non progressive motility (%)</i>	Mean $\pm$ SD	11.36 $\pm$ 17.50	4.82 $\pm$ 15.42	0.40
	Median (IQR)	(19.00) 14.5	(8.00)14.00	
<i>Immotile Sperm (%)</i>	Mean $\pm$ SD	51.16 $\pm$ 14.37	60.00 $\pm$ 9.74	0.05
	Median (IQR)	48.50 (27.5)	58.00 (18.00)	

Non-parametric data were assessed using the Mann-Whitney test and reported as medians and interquartile ranges. The significance level was $p < 0.05$.

3.1.2 The Effect of FSH on Sperm Morphology and Sperm Viability

The results showed that FSH did not affect sperm morphology in groups and according to Fig. 1, incubation with FSH did not have a significant effect on sperm viability (58 ± 11.91 vs. 61.85 ± 11.45) in groups.

3.2 The Effect of FSH on Sperm Mitochondrial Membrane Potential and DNA fragmentation

In the assessment of sperm mitochondrial membrane potential utilizing the JC-1 assay, the data did not demonstrate a statistically significant difference between the groups ($p = 0.50$). According to this assay, spermatozoa exhibiting green fluorescence indicate a low mitochondrial membrane potential, whereas those displaying red fluorescence possess a higher mitochondrial membrane potential. In this study, the sperm chromatin dispersion (SCD) test was used to evaluate DNA fragmentation. The SCD test results showed that incubation with FSH did not cause any change in chromatin quality. ($P = 0.92$) (Table 2).

Table 2
Sperm Mitochondrial Membrane Potential (JC-1) and DNA fragmentation (SCD) in case and control groups

		Analysis	Case	Control	P-value
Mitochondrial Membrane Potential	Low MMP* (%)	Mean ± SD	49.33 ± 12.0	50 ± 9.62	0.5
		Median (IQR)	49.50 (24.75)	47.00 (9.00)	
	High MMP (%)	Mean ± SD	50.66 ± 12.01	50 ± 9.62	
		Median (IQR)	50.50 (24.75)	53.00 (9.00)	
DNA fragmentation (%)		Mean ± SD	40 ± 4.42	38 ± 4.08	0.92
		Median (IQR)	39.5(6.25)	37(6)	

Non-parametric data were assessed using the Mann-Whitney test and reported as medians and interquartile ranges. The significance level was $p < 0.05$.

4. Discussion

4.1 Sperm parameters

In the present study, we discovered that administering FSH in vitro enhances rapid progressive sperm motility while decreasing the number of immotile sperm. Motility was one of the fundamental sperm parameters assessed in this study. Historically, oral supplementation with synthetic drugs, vitamins, trace elements, and other natural compounds has been employed to improve sperm motility in men. Pharmacological agents such as FSH, pentoxifylline, avanafil, and clomiphene citrate have been shown to effectively enhance sperm motility *in vivo* [3]. Extensive *in vivo* studies have highlighted the significant impact of FSH in improving key sperm parameters, such as motility, count, and morphology. Natural compounds and crude plant extracts have been the focus of extensive research, demonstrating notable improvements in sperm motility. Azgomi et al.[12] reported that extracts derived from the root of *Withania somnifera* resulted in a 57% enhancement in sperm motility, comparable to the pharmacological effects of pentoxifylline. Furthermore, a variety of other plant extracts, including *Ruta chalepensis*, *Croton zambesicus*, *Shengjing*, *Panax ginseng*, *Nigella sativa* oil, *Phoenix dactylifera*, *Punica granatum* juice, *Asparagus racemosus*, *Tribulus terrestris*, *Mucuna pruriens*, and *Lepidium meyenii*, have been documented to promote sperm motility in both animal models[13–15] and human studies[16–18].

In addition to *in vivo* studies, significant research has been undertaken to enhance sperm motility *in vitro*, as this particular parameter can be modulated in laboratory conditions, unlike other semen parameters. A diverse range of compounds has been evaluated for their potential to improve sperm motility. But the

use of them in vitro aimed to produce high-quality embryos in ART programs was challenging. Among these compounds, phosphodiesterase (PDE) inhibitors, including caffeine and pentoxifylline, have gained prominence as commonly utilized stimulants for human sperm motility[3, 19]. The findings of the study conducted by Mahaldashtian et al. demonstrated that while treatment with pentoxifylline resulted in enhanced sperm motility and did not affect the acrosome reaction [20], DNA fragmentation was observed in the cleavage properties of embryos derived from ICSI with PTX-treated sperm in the Fresh Experimental group[19]. In a study conducted by Ghafarizadeh et al., it was observed that the incubation of sperm with vitamin E for periods of 2, 4, and 6 hours significantly enhanced sperm motility. Additionally, the antioxidant properties of vitamin E were systematically evaluated [21].

The primary reasons for selecting the FSH hormone in this study are the identification of its receptor in sperm and its non-toxic nature [9, 10]. However, there is a significant gap in extensive clinical studies and in the morphokinetic analysis of the resulting embryos, which are notable limitations of this research. Our study specifically examined the effects of FSH in vitro on semen samples from men with asthenozoospermia, instead of directly evaluating its impact on patients. The results of this study showed that a concentration of 30 mIU/mL of FSH, when applied for 60 minutes, significantly increased the percentage of sperm exhibiting rapidly progressive motility. Additionally, it notably reduced the percentage of immotile sperm in both the case and control groups. To the best of our knowledge, research on the application of FSH in vitro remains limited and has produced conflicting findings. Panza et al.[10] documented an enhancement in sperm motility and quality following FSH administration, which aligns with the results of the present study. Conversely, Cannarella et al. [9] observed a negative effect of FSH on sperm motility, which resulted in a decrease in both motility and overall sperm quality. It is hypothesized that the discrepancies observed between the results of this investigation and those of our study can be attributed to differences in study design. In the research conducted by Cannarella et al., a smaller cohort of subjects was examined, divided into two groups: men with normal sperm and men with asthenozoospermia. This grouping likely hindered the ability to control for confounding variables affecting the outcomes. Conversely, our study utilized 20 semen samples from men diagnosed with asthenozoospermia, which were subsequently categorized into two distinct groups: treated with FSH and without FSH. One of the major strengths of our study is the comprehensive evaluation of progressive sperm motility (WHO 2021). We specifically analyze both rapid and slow motility forms, providing a detailed perspective that the two previous studies did not address. This focus allows us to capture a fuller picture of sperm behavior and its implications.

In the present study, using FSH at a concentration of 30 mIU/mL did not yield a statistically significant positive effect on sperm viability, although it did not result in a decrease in viability. Conversely, in the Panza study [10], sperm viability was examined at various concentrations, and notably at a concentration of 10 mIU/mL, an increase in the percentage of viability was observed. Furthermore, corroborating the results of our study, no alteration in sperm viability was noted at a concentration of 30 mIU/mL. The current study has notable limitations, including the failure to explore varying concentrations of FSH and the omission of an assessment of its oxidative and antioxidative effects. Addressing these gaps is crucial for a comprehensive understanding of FSH's role. Previous studies did not evaluate the effects of

FSH and one-hour sperm incubation at 37°C on sperm morphology. The present study, however, demonstrates that this short incubation period does not adversely affect sperm morphology, which is a positive finding for future research. It is noteworthy that, as indicated by Beigi et al [22], extending the incubation time beyond 1.5 hours may result in increased vacuolation of sperm heads. In accordance with our study, Mahdaldashtian et al. investigated the impact of Pentoxifylline as a sperm motility enhancer on sperm morphology so Scanning electron microscopy (SEM) micrographs indicated that Pentoxifylline did not exhibit adverse effects on fine sperm morphology[19].

4.2 Evaluation of Sperm Mitochondrial Membrane Potential

Our investigation also examined Sperm Mitochondrial Membrane Potential, and the data did not demonstrate a statistically significant difference between the groups. Given that progressive sperm motility was significantly increased in the case group, we postulate a positive correlation between enhanced motility and mitochondrial membrane integrity [23], potentially resulting in greater energy production for sperm motility. However, validation of this finding necessitates further studies with a larger sample size. In the study by Panza et al., the FSH on mitochondrial membrane potential was not assessed. However, research conducted by Rossella et al. found that when in-toto semen was incubated with FSH, there was a decrease in the percentage of spermatozoa exhibiting H-MMP exclusively in normozoospermic men, regardless of the concentration used. In contrast, patients with asthenozoospermia did not show any significant differences in either L-MMP or H-MMP [9, 10].

4.3 Evaluation of Sperm DNA fragmentation

The application of FSH at a concentration of 30 mUI/mL did not elicit any detrimental effects on DNA fragmentation. In light of the one-hour incubation of asthenozoospermia samples with FSH, it is essential to investigate the potential for DNA damage attributed to reactive oxygen species (ROS). Studies have indicated that one of the primary contributors to apoptosis and the subsequent increase in DNA fragmentation is the production of reactive oxygen species (ROS) [24, 25]. A novel aspect of this study is the investigation of the effect of FSH on DNA fragmentation, which is examined for the first time. This observation aligns with findings from in vitro studies, which indicated that Pentoxifylline and papaverine had no adverse impact on sperm chromatin/DNA fragmentation [26, 19, 27]. Given the critical role of reactive oxygen species (ROS) within the seminal environment of asthenozoospermia samples, a significant limitation of our study was the omission of ROS assessments in both the control and case groups. This aspect had similarly not been addressed in the two prior in vitro investigations.

5. Conclusions

In conclusion, FSH enhanced sperm motility without adversely affecting sperm DNA integrity and mitochondrial membrane potential (MMP). However, to establish this hormone as a viable and safe therapeutic option, further clinical studies with larger sample sizes are necessary.

Declarations

Conflicts of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

Author Contribution

Mangoli - Rezvani: Study design
Etebari- Khosravi - Izadi :Data collection
Mangoli - Rezvani: Data analysis
Etebari- Khosravi: Writing of the manuscript
Izadi: Editing of the manuscript
All authors reviewed the manuscript.

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Figures

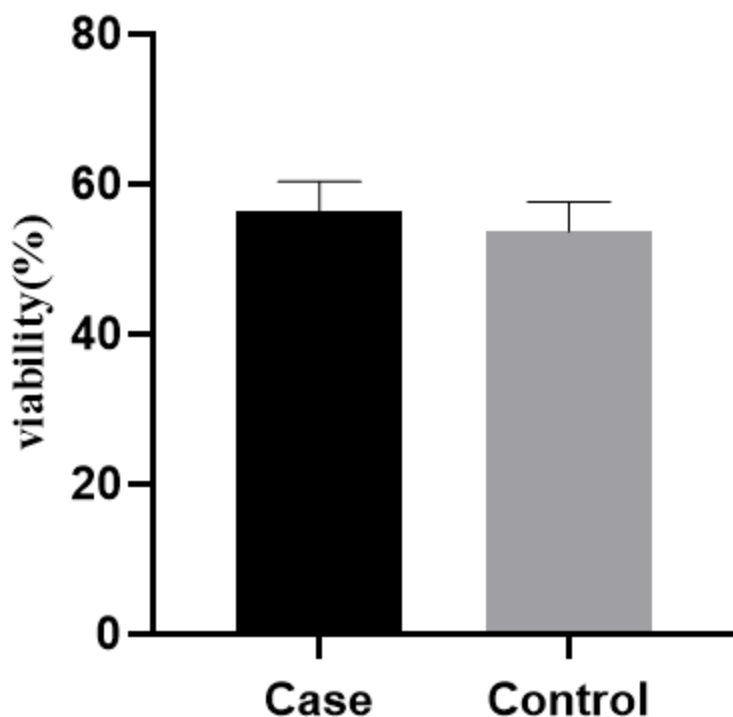


Figure 1

Sperm viability in case and control groups