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Evaluation of Sperm Parameters in Asthenozoospermic Men: A Comparative Study of Swim-Up and Layering Techniques with Lepidium meyenii (Maca), L-Carnitine, and Pentoxifylline

Israa Saleem Abdulzahra[1*], Saad Salih Al-Dujaily[2], Areej Abbas Zabbon[3]

1. *Department of Science, College of Basic Education, Mustansiriyah University, Baghdad, Iraq*
2. *Department of Physiology, High Institute of Infertility Diagnosis and ART, Al-Nahrain University, Baghdad, Iraq*
3. *Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq*

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***Corresponding author:**
Israa Saleem Abdulzahra,
Department of Science, College of Basic Education, Mustansiriyah University, Baghdad, Iraq

Email:
israasaleem@uomustansiriyah.edu.iq

ABSTRACT

Background & Objective: Infertility is a widespread health issue, with male factors solely responsible in approximately 20% of cases and contributing to an additional 30–40%. *In vitro* sperm preparation using motility-enhancing agents prior to assist reproductive techniques (ART) has shown potential in improving semen quality and increasing pregnancy rates. This study aimed to evaluate the effects of a novel combination of motility stimulants—Maca, L-carnitine, and pentoxifylline—on sperm function, particularly the DNA fragmentation index (DFI), and to compare the effectiveness of the swim-up and density gradient (layering) techniques for sperm activation.

Materials & Methods: Semen samples were collected from 90 asthenozoospermic men. Samples were divided into two groups: a control group using Ham's F-12 medium and a treatment group using medium supplemented with Maca powder (1 mg/mL), pentoxifylline (10 mg/mL), and L-carnitine (0.5 mg/mL). Sperm activation was performed using both the swim-up and Layering techniques. Sperm parameters were evaluated according to WHO guidelines (1999 and 2021). Data were analyzed using SPSS version 23.0 with Student's t-test, ANOVA, and the LSD post-hoc test.

Results: The treatment group demonstrated significant improvements ($P<0.05$) in sperm concentration, motility (grades A and B), morphology, and reduced DNA fragmentation index. Both preparation techniques showed comparable improvements, although the Layering (density gradient) method yielded slightly better outcomes in DNA integrity.

Conclusion: The combination of pentoxifylline, L-carnitine, and Maca significantly enhanced sperm function. The Layering technique was marginally more effective than swim-up in selecting spermatozoa with intact DNA.

Keywords: L-carnitine, Pentoxifylline, Maca (Lepidium meyenii), Sperm Preparation, Asthenozoospermia, DNA Fragmentation Index

1. Introduction

Infertility is one of the most complex challenges facing human society today. Male fertility primarily depends on *spermatogenesis*, the linear development of spermatozoa from spermatogonia. This intricate process involves mitotic and meiotic divisions, followed by chemical and functional differentiation, ultimately resulting in mature sperm cells (1). Any malfunction in the stages of spermatogenesis—from sperm production to ejaculation—can lead to male infertility (2).

Male infertility, also referred to as diminished fertility or subfertility, is a multifactorial disorder influenced by a wide range of factors including infections, hormonal imbalances, and structural abnormalities (3). According to the World Health Organization (WHO), normal semen parameters should include at least 40% total motility (including both rapid and slow progressive and non-progressive) or more than 32% progressive motility alone (4).

Human ejaculate typically consists of motile, immotile, and agglutinated spermatozoa, as well as white blood cells, bacteria, and various types of debris. These components can increase oxidative stress by reducing oxygen radicals, which in turn impairs the fertilizing capacity of healthy sperm (5). With the advent of *in vitro* fertilization (IVF) techniques, it has become possible to improve sperm quality in fresh semen samples by isolating motile sperm with normal morphology from the seminal plasma and unwanted cellular debris (6). This separation is crucial for achieving sperm capacitation and successful fertilization (7).

One of the oldest and most commonly used sperm separation techniques is the swim-up method, which relies on the active movement of spermatozoa from a prewashed cell pellet into an overlying culture medium (8). This method has several advantages: it yields a high percentage of motile sperm with normal morphology, is easy to perform, and typically recovers a clean fraction of sperm free from other cells and debris (9). For patients with normospermia or asthenozoospermia, sperm self-selection through layering is another technique based on sperm migration into a medium, with an incubation time of approximately two hours at 37°C, 95% humidity, and a CO_2 concentration of 5–10%, all of which enhance motility (4, 10). To improve sperm function and increase the success rate of IVF, various stimulant agents have been added to the culture medium. These include: 1. Pentoxifylline (PTX) – known to enhance sperm motility and plasma membrane integrity, as well as improve cervical mucus penetration (11). PTX also acts as an antioxidant by inhibiting xanthine oxidase, reducing the levels of intracellular reactive oxygen species (ROS) in defective sperm (12). 2. L-carnitine (LC) – a white, water-soluble, vitamin-like molecule and a quaternary ammonium compound involved in metabolic processes in mammals, plants, and some bacteria (13, 14). LC functions as an antioxidant, scavenging harmful free radicals (15). 3. Lepidium meyenii (Maca) – a Peruvian plant from the Brassicaceae family, traditionally consumed by Andean populations as a nutritious staple. It is known for its effects on fertility and sexual performance (16, 17). Maca powder is used to treat various physiological disorders, including infertility and anemia, due to its aphrodisiac and anabolic properties (18). This study aims to evaluate certain sperm parameters in patients with asthenozoospermia by comparing two *in vitro* sperm preparation techniques, incorporating a novel combination of the aforementioned stimulating agents.

2. Materials and Methods

2.1 Patients

This study was conducted at the Infertility Unit of the Higher Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, and at the Fatima Al-Zahra Hospital for Women and Children in Baghdad between November 2023 and March 2024. A urologist consultant in the male infertility unit performed clinical examinations to assess for conditions such as varicocele, hydrocele, cryptorchidism, hernias, and other relevant disorders. A total of 90 semen samples were collected from men diagnosed with asthenozoospermia and related conditions. Participants were aged between 20 and 45 years. Ethical approval for the study was granted by the relevant committee, and all participants provided informed consent after being fully briefed on the study's objectives.

2.2 Seminal Fluid Analysis

This Semen samples were collected directly into clean, dry, sterile, disposable containers after a period of 3 to 5 days of sexual abstinence. Following collection, the samples were incubated at 37°C to ensure complete liquefaction. After liquefaction, both macroscopic and microscopic analyses were performed according to standardized protocols. The normal reference values for semen parameters, as defined by the World Health Organization (WHO, 2021), are presented in Table 1 (4).

2.3 Preparation of Maca Powder (Lepidium meyenii)

A solution of maca powder was prepared by dissolving 1 mg of maca powder (Healthworks, USA) in 10 mL of phosphate-buffered saline (PBS) in a sterile plastic test tube. Approximately 0.0005 g of maca was used for activation. The mixture was agitated until fully dissolved, then filtered using a 0.45 µm Millipore filter. The filtrate was exposed to ultraviolet (UV) light for 2 hours and stored at 25°C, with pH maintained between 7.4 -7.8.

2.4 Preparation of Pentoxifylline Stock Solution

A 10 mg/mL PTX stock solution was prepared daily under sterile conditions by dissolving 10 mg of Pentoxifylline (Sigma, USA) in 10 mL of PBS. The solution was filtered through a 0.45 µm Millipore filter and exposed to UV light before use.

2.5 Preparation of L-Carnitine (LC) Stock Solution

To prepare the L-Carnitine stock solution, 0.5 mg of LC powder (Pharmaciena, USA) was dissolved in 10 mL of PBS in a plastic test tube. The solution was filtered through a 0.45 µm Millipore filter and stored at 25°C with a pH of 7.4–7.8.

2.6 *In Vitro* Sperm Activation Techniques

Following semen sample collection and liquefaction, sperm preparation techniques were employed to remove dead cells, immotile sperm, and seminal plasma—concentrating the most viable sperm. The study utilized two methods as described in reference (4):

a) Swim-Up Method

This method utilizes the natural motility of spermatozoa to swim into the culture medium. One milliliter of semen was carefully layered beneath 1 mL of nutrient mixture F-12 Ham media, supplemented with equal concentrations of L-Carnitine, Maca powder, and Pentoxifylline in a culture tube. The tubes were incubated at 37°C for 30 minutes and tilted at a 45° angle to maximize the medium-sperm interface. After incubation, the tubes were returned

to an upright position. The top 1 mL of medium, now enriched with motile sperm, was collected and diluted with 1.5–2.0 mL of fresh medium. The sample was then centrifuged at 300g for 5 minutes, the supernatant discarded, and the sperm pellet resuspended in 0.5 mL of medium. This suspension was used to assess sperm concentration, total motility, and progressive motility, or used for further applications.

b) Layering Method

This technique, suitable for both normospermic and asthenospermic samples, was initially developed at Bourn Hall Clinic (UK, 1992). It involves layering 2 mL of semen beneath 2 mL of medium in a sterile conical tube. The mixture was incubated in a CO_2 incubator (5–10% CO_2) for up to 2 hours. After incubation, the top and middle cloudy layers containing motile sperm were aspirated into a new conical tube and centrifuged at 300g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of medium and centrifuged again under the same conditions. The supernatant was removed a second time, the pellet resuspended in 1 mL of medium, and incubated in a CO_2 incubator for an additional 10 minutes. The prepared sample was then ready for examination or use.

2.7 Microscopic Examination

To measure the concentration of sperm aliquots (10 µl) was produced, the average quantity of sperm in five high power fields (HPF) magnified with a 40X objective lens was used to calculate the concentration of sperm. The value has been multiplied by a factor of one a million sperm concentration was regarded as normal if it was equal to or greater than 14×10^6/ml. Total sperm count 39 million/ejaculate was considered normal **(4)**. The prepared sample was then ready for examination or use.

Sperm Motility (%) = (Number of motile sperm / Total number of sperm counted) × 100

2.8 Sperm Morphology and DNA Integrity Assessment

To estimate the proportion of sperm with normal morphology, at least 200 spermatozoa were evaluated per sample following the guidelines outlined in reference **(4)**. The assessment was based on morphological criteria using stained preparations, with a threshold of ≥30% morphologically normal sperm considered normal according to WHO (1999) standards.

2.9 Assessment of DNA Integrity Using Acridine Orange Staining

For DNA integrity evaluation, a 10 µl aliquot of semen was smeared onto a clean glass slide and left to air dry for approximately 20 minutes. The slide was then fixed in Carnoy's solution (methanol:acetic acid, 3:1) for a minimum of two hours or overnight at 4°C. After fixation, the slide was air-dried again briefly before staining.

The Acridine Orange Test (AOT) was conducted as described in reference **(4)**. The staining solution was prepared by mixing 2.5 mL of acridine orange with 40 mL of 0.1 M citric acid and 0.3 M $Na_2HPO_4\cdot7H_2O$, adjusting the pH to 2.5. After staining, approximately 200 spermatozoa were evaluated under a fluorescence microscope.

- Sperm heads emitting green fluorescence were classified as having intact (normal) DNA.
- Spermatozoa exhibiting yellow to red fluorescence were considered to have abnormal (fragmented) DNA (4, 19), as shown in Figure 1.

Interpretation of DNA Fragmentation Index (DFI): The level of DNA fragmentation is a key indicator of fertility potential and was categorized as follows **(20)**:

1. ≤15% DFI – Low level of sperm DNA fragmentation (Fertile)
2. 15–30% DFI – Moderate/elevated level of DNA fragmentation (Sub-fertile)
3. ≥30% DFI – High/severely elevated level of DNA fragmentation (Infertile)

2.10 Preparation of

Data from the experimental and control groups were compared using the student's t-test, and results are presented as mean ± SEM. Differences were considered statistically significant at $P<0.05$. Comparisons among the four prepared media were performed using analysis of variance (ANOVA). When the ANOVA F-test indicated significance at the 5% level, the least significant difference (LSD) test was applied for post-hoc comparisons.

Table 1. WHO Normal Semen Parameters (WHO, 2021).

Parameters			Normal values
Appearance			Homogenous, opalescent gray and opaque
Volume			1.5 – 5/6 ml (reference limit :1.5ml)
Liquefaction time			Within 60 minutes at room temperature
Viscosity			Drops - < 2 cm thread
pH			7.2 – 8.0
Sperm concentration			>14 million/ml.
Sperm motility (%)	Progressive	Grade- A	Progressive 30% Progressive + un progressive 42% Within 60 minutes
		Grade- B	
	Non progressive	Grade- C	
	Immotile	Grade- D	
Morphologically normal sperm (%)			> 30% by staining according to WHO 1999
4% according WHO 2021			<15% **(20)**

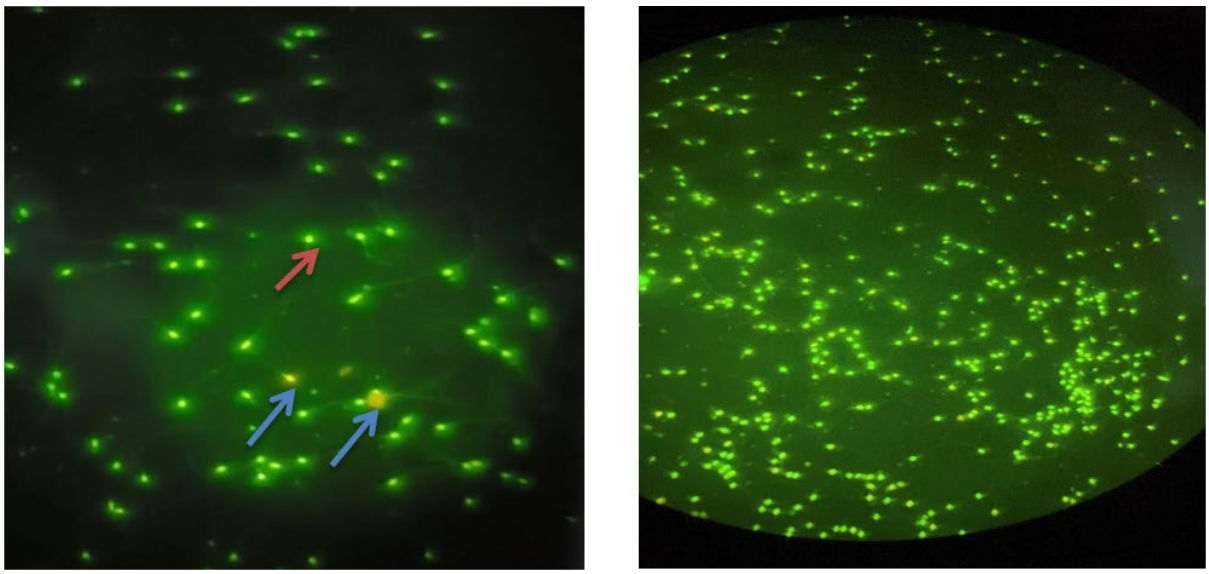

Figure 1. DNA fragmentation in sperm cells stained with acridine orange. Blue arrows indicate sperm with fragmented DNA (appearing orange or red under fluorescence), while red arrows indicate sperm with intact/normal DNA (appearing green under fluorescence). (**Designed by Authors, 2025**).

3. Result

3.1 Comparative Sperm Concentration Between Swim-up and Layering Methods

The results showed no significant difference ($p>0.05$) in sperm concentration between the swim-up method using control Ham's F12 (24.73 ± 12.42) and mixed medium (LC + maca + PTX) (23.93 ± 10.90), and the layering method after activation with control Ham's F12 (23.06 ± 14.64) and mixed medium (29.46 ± 16.28) (Figure 2).

3.2 Comparative Sperm Motility Between Swim-up and Layering Methods

Current findings demonstrated that the swim-up method resulted in significantly higher sperm motility grade A (rapidly progressive sperm with the strongest motility, moving quickly in a straight line or wide circle, approx. 25 µm per second) before and after activation by Ham's F12 medium (9 ± 0.06) compared to the layering method (6 ± 0.05), with a significant difference ($P<0.05$). However, no significant difference ($P>0.05$) was observed between the two methods in the mixed medium based on grade A motility (Figure 3).

No significant differences ($P>0.05$) were found in sperm motility grade B (slowly progressive sperm with non-linear motility, moving forward in a bent or crooked

manner, ranging from at least one head length to less than half the tail length per second, or less than 25 µm) between the swim-up (32 ± 0.14) and layering (27 ± 0.11) methods before and after activation by the LC + maca + PTX medium. Conversely, after activation by Ham's F12 medium, the swim-up method showed significantly higher-grade B motility (32 ± 0.18) compared to the layering method (23 ± 0.15) (P<0.05) (Figure 4).

Finally, regarding progressive motility (Grade A + Grade B), no significant differences (P>0.05) were observed between the swim-up (43 ± 4.55) and layering (37 ± 4.85) methods before and after activation by the LC + maca + PTX medium. In contrast, after activation by Ham's F12 medium, the swim-up method demonstrated significantly higher progressive motility (41.00 ± 2.08) compared to the layering method (29 ± 2.42) (P<0.05) (Figure 5).

3.3 Comparative Normal Sperm Morphology Between Swim-up and Layering Methods

No significant differences (P>0.05) were observed in normal sperm morphology between swim-up and layering methods before and after activation using control Ham's F12 (63 ± 0.07 and 61 ± 0.04, respectively) and LC + Maca + PTX medium (71 ± 0.04 and 69 ± 0.05, respectively) (Figure 6).

3.4 Comparative DNA Fragmentation Index Between Swim-up and Layering Methods

There were no significant differences (P> 0.05) in DNA fragmentation index between swim-up and layering methods before and after activation with control Ham's F12 (12 ± 0.07 and 18 ± 0.06, respectively) and LC + Maca + PTX medium (11 ± 0.06 and 13 ± 0.05, respectively) (Figure 7).

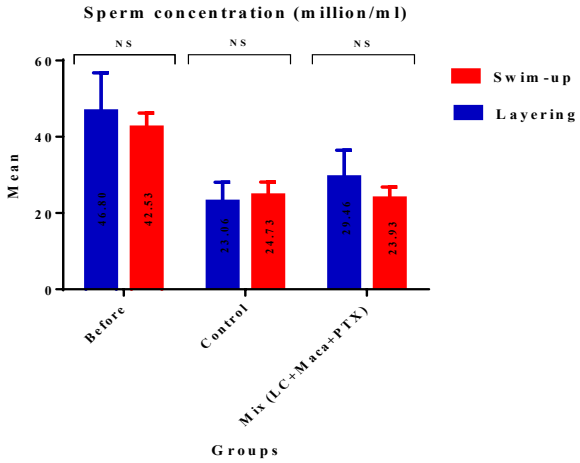

Figure 2. Comparison of sperm concentration between swim-up and layering methods treated with mixed culture media. NS: Non-significant. (**Designed by Authors, 2025**).

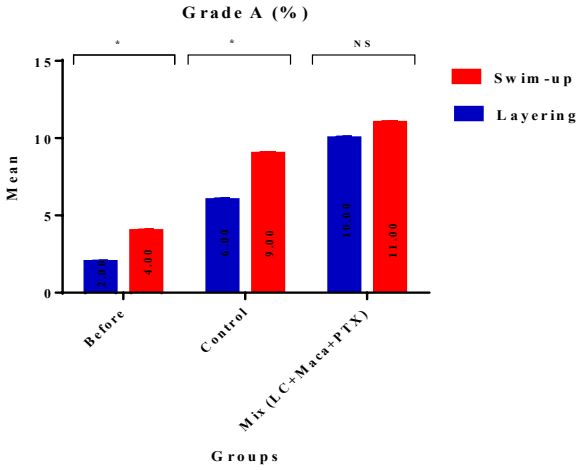

Figure 3. Comparison of Grade A sperm motility between swim-up and layering methods treated with mixed culture media. NS: Non-significant; *: Significant. (**Designed by Authors, 2025**).

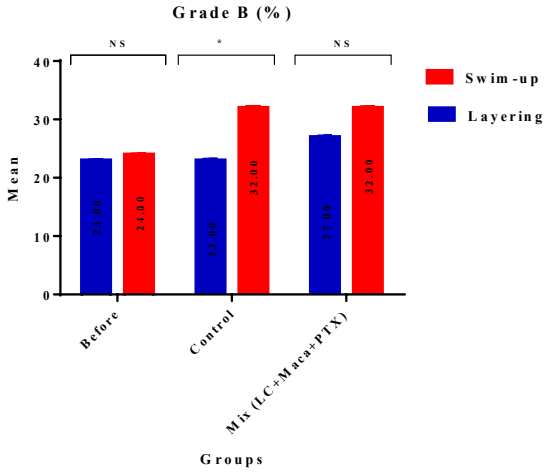

Figure 4. Comparison of Grade B sperm motility between swim-up and layering methods treated with mixed culture media. NS: Non-significant; *: Significant. (**Designed by Authors, 2025**).

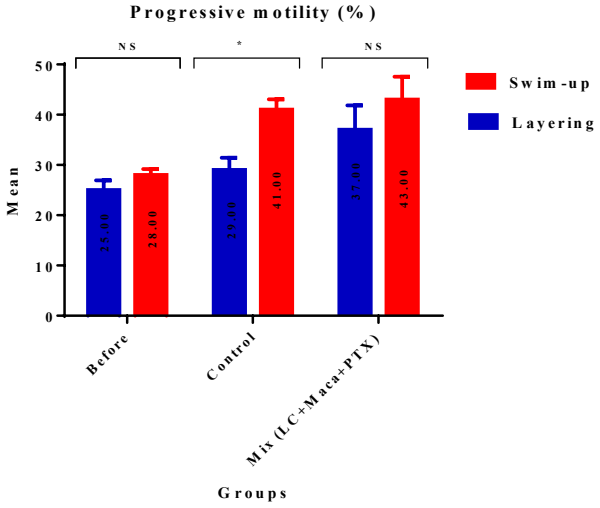

Figure 5. Comparison of progressive motility between swim-up and layering methods treated with mixed culture media. NS: Non-significant; *: Significant. (**Designed by Authors, 2025**).

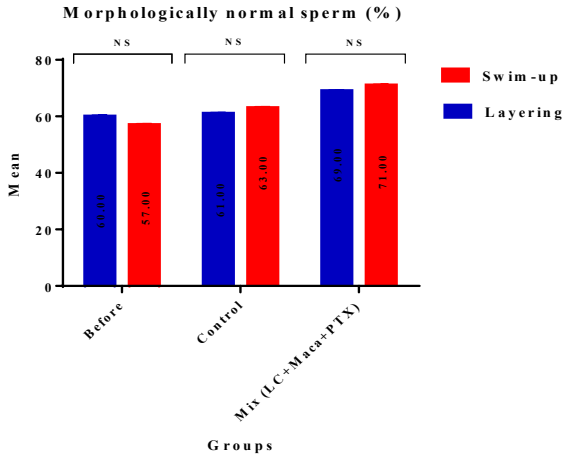

Figure 6. Comparison of normal sperm morphology (MNS) following *in vitro* activation with mixed culture media using swim-up and layering methods. NS: Non-significant. (**Designed by Authors, 2025**).

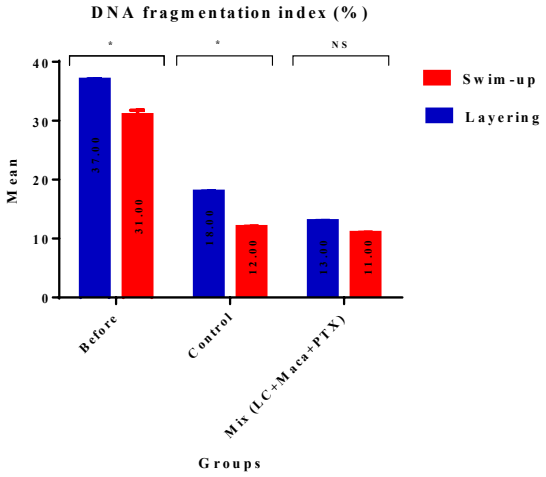

Figure 7. Comparison of DNA fragmentation index between swim-up and layering methods treated with mixed culture media. NS: Non-significant; *: Significant. (**Designed by Authors, 2025**).

4. Discussions

The current study aimed to enhance specific sperm characteristics of infertile men by applying an *in vitro* sperm preparation technique using a novel combination of motility stimulants to improve ART outcomes and explore their effects on DNA fragmentation index. Additionally, the study evaluated which sperm preparation and activation techniques are preferred for semen samples. Our findings showed no significant differences ($p>0.05$) in sperm parameters between the swim-up and layering methods after activation with the mixed medium (LC + maca + PTX). This suggests a close relationship between these methods for the studied population in effectively removing damaged, dead, immotile, and abnormal spermatozoa with sluggish motility, as well as eliminating seminal plasma components such as leukocytes, round cells, epithelial cells, and other debris in both techniques **(21)**. Sperm quality has been shown to be negatively correlated with intracellular reactive oxygen species (ROS) levels. Adult spermatozoa lack cytoplasmic antioxidants, reducing their resistance to ROS-induced damage **(22)**. Therefore, antioxidant supplementation is essential to mitigate oxidative damage during fertilization. In this study, antioxidants pentoxifylline (PTX) and L-carnitine (LC) were included to maintain sperm quality **(23)**. The combination of LC and PTX in the layering method may result in increased sperm concentration compared to the swim-up method. This mixture enhances energy supplementation and calcium influx, thereby improving sperm motility and increasing the number of sperm migrating into the upper layer of the medium **(24, 25)**. Adding maca further enhances sperm motility and concentration **(26)**. Notably, maca—a traditional Peruvian plant—is known for its spermatogenic properties, positively impacting sperm parameters and sexual behavior **(27)**. Our results indicated that sperm motility increased after activation with the mixed medium (LC + maca + PTX) in both separation methods. Both techniques produced a high number of progressively motile spermatozoa, were easy to use, cost-effective, and minimized cell damage while removing dead sperm, bacteria, and ROS **(8)**. The swim-up method is recommended for normozoospermic and mild asthenozoospermic samples, whereas the layering method is better suited for severe asthenozoospermia **(8, 28)**. For couples undergoing intrauterine insemination, the choice of sperm preparation method did not affect pregnancy rates **(29)**, consistent with our findings of no superiority between the techniques. The swim-up procedure has been shown to improve motility, morphology, and DNA integrity, potentially increasing pregnancy success **(30)**. Our results on normal sperm morphology (MNS) suggest that either activation method can effectively improve sperm motility and morphology in asthenozoospermic patients **(23)**. Regarding DNA fragmentation index, both methods were equally effective in minimizing DNA fragmentation regardless of the activation media used (control Ham's F12 or LC + maca + PTX). This implies that either method can be utilized to improve sperm DNA integrity in asthenozoospermic patients. Our study like some other studies suggest that layering may be superior to swim-up in selecting spermatozoa with higher DNA integrity **(31)**, alongside improvements in ROS levels, acrosome status, and mitochondrial activity **(32)**. DNA fragmentation and chromatin decondensation are indicators of the integrity of semen DNA reflecting nuclear and mitochondrial integrity, may arise from apoptosis during spermatogenesis or ROS damage during sperm transport **(33)**. Previous research indicates that while both swim-up and layering reduce sperm DNA fragmentation, the swim-up technique is more suitable when the DFI is below 30%, potentially favoring IVF or IVM, whereas layering may be better for intrauterine insemination **(34)**. Recent findings also support the layering technique's superiority in decreasing DNA fragmentation, while swim-up improves progressive motility when the mixed activation medium is used **(35)**.

5. Conclusion

The combined supplementation of pentoxifylline (PTX), L-carnitine (LC), and maca in the sperm culture medium significantly enhanced sperm motility, viability, and DNA integrity during *in vitro* activation. The layering method demonstrated superior efficacy compared to swim-up in selecting high-quality spermatozoa, as evidenced by improved DNA fragmentation indices (DFI) and reduced oxidative stress markers. These findings suggest that PTX + LC + maca supplementation, coupled with layering-based sperm selection, may optimize ART outcomes by preserving genomic integrity while maintaining functional competence.

6. Declarations

6.1 Acknowledgments

The authors would like to thank Mustansiriyah University (www.uomustansiriyah.edu.iq) Baghdad, Iraq, for its support in the present work.

6.2 Ethical Considerations

The study was approved by the Ethical Approval Committee. Ethical approval was obtained from the Institutional Review Board (IRB) of Mustansiriyah University College of Basic Education (Approval No. REC178, dated March 1, 2024). Written informed consent was obtained from all participants prior to their inclusion in the study.

6.3 Authors' Contributions

I.S.A: Conceptualized and designed the study, conducted experiments, analyzed data, and wrote the manuscript. S.S.A: Assisted in the experimental design, and contributed to data interpretation and manuscript revision. A.A.Z.: Assisted in sample collection and processing, performed statistical analyses, and reviewed the manuscript for intellectual content. All authors approved the final version of the manuscript and agreed to be accountable for the accuracy and integrity of the work.

6.4 Conflict of Interest

The authors have no conflict of interest.

6.5 Fund or Financial Support

This research received no specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

6.6 Using Artificial Intelligence Tools (AI Tools)

This work did not involve the use of artificial intelligence tools

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