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ORIGINAL ARTICLE

Sperm Biology

Assessing the efficacy of a novel sperm-washing medium enriched with serotonin, L-carnitine, and coenzyme Q10: an observational cohort study

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This observational cohort study investigated the potential of a novel sperm-washing medium (SWM) enriched with serotonin (5-HT), L-carnitine (L-C), and coenzyme Q10 (CoQ10) to enhance sperm motility and reduce DNA damage. It compared this innovative medium (5-HT/L-C/CoQ10 SWM) with two widely used commercial media (SWM 1 and SWM 2). Ninety-eight volunteers from an infertility clinic provided semen samples, which were divided into three aliquots for analysis in different SWMs: group 1, SWM was composed of hydroxyethyl piperazineethanesulfonic acid (HEPES), sodium bicarbonate, human serum albumin (HSA), taurine, and gentamicin sulfate (SWM 1); group 2, SWM was composed of HEPES, sodium bicarbonate, and HSA (SWM 2); and group 3, SWM was composed of HEPES-buffered human tubal fluid supplemented with 5-HT, L-C, and CoQ10 (5-HT/L-C/CoQ10 SWM). Sperm motility was categorized as progressive, nonprogressive, or immotile. Apoptosis, reactive oxygen species (ROS) production, and DNA fragmentation were also assessed. There were no significant differences in total or progressive sperm motility among the groups. Spermatozoa in group 3 exhibited reduced apoptosis, necrosis, and ROS levels and increased viability. No significant differences were observed in the DNA fragmentation index among groups. The 5-HT/L-C/CoQ10 SWM reduced sperm oxidative stress and apoptosis compared with those of the two commercially available SWMs, suggesting that 5-HT/L-C/CoQ10 SWM could be useful for enhancing *in vitro* fertilization success rates.

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INTRODUCTION

Infertility is defined as the inability to induce pregnancy after 1 year of unprotected intercourse. It is a public health problem that affects 15% of couples worldwide, with 50% of cases due to male factors. Semen analysis is the cornerstone of investigating sperm count, motility, and morphology but cannot determine their functional potential.[1]

Sperm-washing media (SWMs) are specialized solutions used in assisted reproductive technology (ART) such as intrauterine insemination and *in vitro* fertilization. SWMs remove seminal fluid and debris for sperm to undergo capacitation.[2] Highly motile spermatozoa obtained by washing increase the chances of fertilization and pregnancy. SWMs yield motile spermatozoa, isolating those with normal morphology and low DNA fragmentation index, supporting the development of high-quality embryos.[3] The antioxidant properties of L-carnitine (L-C) and coenzyme Q10 (CoQ10) can be used in SWMs to select cells with low sperm DNA fragmentation and few radical oxygen species (ROS) and without apoptosis.[4]

Oxidative stress occurs when there is an imbalance between ROS production and antioxidant defenses, which can negatively affect sperm viability, motility, and fertilization potential. Semen samples from infertile men are known to have significantly higher ROS levels than those from fertile men.[5] ROS inhibit spermatozoa from gaining functional competence, through means such as activation, capacitation, and the acrosomal reaction.[6]

Adding antioxidants, such as CoQ10, to SWMs may mitigate this imbalance, as CoQ10 is a naturally occurring antioxidant crucial for energy production and positively influences sperm quality.[7] Oral CoQ10 supplementation has been shown to increase sperm motility and sperm count while decreasing oxidative stress, thus preserving DNA integrity.[8] Lucignani *et al.*[9] highlighted the benefits of CoQ10 for enhancing sperm motility and reducing DNA fragmentation. Orally administered L-C, an amino acid-like substance vital for energy metabolism, positively affects sperm quality, particularly by improving sperm motility and normal morphology and reducing abnormalities.[10]

Although the influence of L-C and CoQ10 on spermatozoa is well established, the effect of serotonin (5-HT), a neurotransmitter with crucial roles in the male reproductive system, has not been explored extensively,[11] especially on the sperm function. 5-HT was recently

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shown to enhance the acrosome response and hyperactivation of hamster spermatozoa.[12,13] Sugiyama *et al.*[14] also reported that 5-HT increases mouse sperm hyperactivation and *in vitro* fertilization rates. Omote *et al.*[15] identified seven types of 5-HT receptors in human spermatozoa, among which 5-hydroxytryptamine-2A (5-HT2A) receptors are associated with sperm motility.

Within the framework of this research, our objective was to study a novel SWM enriched with 5-HT, L-C, and CoQ10. We investigated its potential to enhance sperm motility and prevent DNA damage by comparing its efficacy with that of widely used commercial media. This study addresses a critical gap in our understanding of the direct effects of 5-HT in conjunction with established antioxidants in a SWM, suggesting potential benefits for ART.

PARTICIPANTS AND METHODS

This observational cohort study included 98 volunteers who were admitted to Atakent Hospital Infertility Clinic, Acibadem Mehmet Ali Aydinlar University (Istanbul, Türkiye). All procedures complied with the ethical standards of the responsible committee on human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2008. Ethics committee approval was granted by Acibadem Mehmet Ali Aydınlar University (Approval No. ATADEK-2021-01/26), and informed consent was obtained from all participants.

Sample collection

After 3–5 days of abstinence, all semen samples were collected directly into sterile disposable Petri dishes via masturbation. After liquefaction, standard manual techniques were used to determine sperm count and motility at ×20 objective magnification by placing 10 µl of semen in a Makler counting camera (2511 Daimler ST; Irvine Scientific®, Santa Ana, CA, USA).[16] All the samples were processed using the swim-up technique and centrifuged (SL 8; Thermo Fisher Scientific, Waltham, MA, USA) at 500*g* for 10 min. After centrifugation, each sperm sample (0.5 ml) was analyzed, and the sperm count and motility were recorded. Motility was categorized into three motility categories: progressively motile, nonprogressively motile, and immotile. The sperm processing protocols involved designated media for both the swim-up and sperm-washing procedures.

For analysis, each sperm specimen was divided into three aliquots with three different SWMs. Group 1, SWM was composed of hydroxyethyl piperazineethanesulfonic acid (HEPES), sodium bicarbonate, human serum albumin (HSA), taurine, and gentamicin sulfate (SWM 1; Origio®, Malov, Denmark); group 2, SWM was composed of HEPES, sodium bicarbonate, and HSA (SWM 2; Irvine Scientific®); and group 3, SWM was composed of HEPES-buffered modified human tubal fluid (HTF) supplemented with 5-HT, L-C, and CoQ10 (5-HT/L-C/CoQ10 SWM). According to a preliminary study,[16] the optimal concentrations of 5-HT (sc-298707; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), L-C (SA-840092; Sigma-Aldrich, Darmstadt, Germany), and CoQ10 (SAC9538; Sigma-Aldrich) are 200 µmol l^{-1}, 0.5 mg m^{-1}, and 0.01 mg ml^{-1}, respectively.

Evaluation of ROS levels in human spermatozoa

In the ROS experiment, the fluorescent dye 2',7'-dichlorofluorescin diacetate (H_2DCF-DA; catalog No. D399; Invitrogen, Paisley, UK) was used to measure the intracellular H_2O_2 level in the spermatozoa by flow cytometry (CytoFLEX Beckman Coulter Life Sciences, Brea, CA, USA). Gating was performed for each sample with the threshold for acquisition set on the forward scattered (size) channel. This method allowed us to define the spermatozoon population while excluding round cells, epithelial cells, and debris, which were recorded as the R1 window. All flow cytometry evaluations were conducted using this defined region. The samples were separated into test and negative control groups (25×10^5 ml^{-1} spermatozoa), and H_2DCF-DA (at a final concentration of 10 µmol l^{-1}) was added to the sperm suspensions in the test groups. The mixture was incubated in the dark for 30 min at 25°C, after which flow cytometry was used to examine the labeled spermatozoa. Fluorescence intensity was evaluated using a system consisting of a 450–490 nm excitation filter, a 505 nm dichroic mirror, and a 520 nm bandpass filter. The sperm terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) index was determined by quantifying positively and negatively stained spermatozoa in each of ten fields of view, with 10 000 TUNEL-positive events recorded for each sample. Subsequently, we calculated the ROS value by enumerating positively and negatively stained spermatozoa in ten fields of view, expressing the results as the percentage of sperm cells exhibiting ROS activity.

Evaluation of sperm apoptosis

An Annexin V-Fluorescein Isothiocyanate (Annexin V-FITC) Apoptosis Kit (BioLegend, San Diego, CA, USA) with propidium iodide (PI; Vector Laboratories, San Diego, CA, USA) was used to assess apoptosis. Spermatozoa were prepared at a concentration of 2×10^6 ml^{-1}, and 500 µl cell staining buffer was added to 500 µl sperm suspension. PI (5 µl; which binds to the DNA in necrotic cells) and Annexin V (5 µl; which was conjugated with fluorescein isothiocyanate), which labels phosphatidylserine sites on the outer surface of apoptotic cell membranes, were used to prepare mixtures of probes that were placed into each vial. The samples were incubated in the dark for 15 min at 25°C, 400 µl Annexin V binding buffer was added to each tube, and the total volume was examined via flow cytometry (CytoFLEX). Fluorescence intensity was evaluated (525 nm/540 nm bandwidth). For each sample, 30 000 FITC combined with peanut agglutinin (FITC-PNA) events and 30 000 Annexin V and PI events were recorded. The apoptosis data were categorized as viable, early apoptotic, late apoptotic, or necrotic spermatozoa by percentages.

Evaluation of sperm DNA fragmentation

This procedure was performed using an *In Situ* Cell Death Detection Kit (Roche, Darmstadt, Germany). A 500 µl sample of spermatozoa was diluted with phosphate-buffered saline to a concentration of 5×10^6 ml^{-1} and centrifuged (SL 8; Thermo Fisher Scientific) for 10 min at 300*g*. Then, 50 µl of TUNEL mix solution (5 µl enzyme solution + 45 µl FITC label solution) and TdT enzyme were added for staining; the sample was incubated in the dark. The fluorescence intensity of the negative control and TUNEL-stained samples was analyzed using flow cytometry at 525 nm/540 nm bandwidth (CytoFLEX). For each sample, 10 000 TUNEL-positive events were recorded, and the sperm DNA fragmentation index was determined by counting the positively and negatively stained spermatozoa within each of the ten fields of view. Results were expressed as percentage of cells with DNA fragmentation.

Exclusion criteria

Men older than 40 years of age and those with underlying conditions, such as varicocele, testicular atrophy, ejaculatory disorders, azoospermia, oligozoospermia or asthenozoospermia and Sertoli cell syndrome, endocrine and anatomical abnormalities, seminal specimens containing large numbers of bacteria, or the presence of more than one million leukocytes per ml in specimens suspected of infection.

Statistical analyses

Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) for Windows 26.0 (IBM Corp., Armonk, NY, USA).

Frequencies and percentages for categorical data, and means and standard deviations for continuous data, are presented as descriptive values. The Pearson's Chi-squared test was used to compare categorical variables. One-way analysis of variance (ANOVA; Tukey's post hoc test) was performed to establish significance. The assumption of sphericity was examined using Mauchly's test of sphericity. The results were considered to be statistically significant when the $P < 0.05$.

RESULTS

The baseline demographics of the study population (n = 98) are presented in **Table 1**. Almost half of the participants were nonsmokers and did not consume alcohol. Spermatozoa in the postwash samples revealed significant differences in sperm count ($P < 0.001$), progressive motility ($P < 0.001$), and total motility ($P = 0.018$) in comparison to spermatozoa in prewash samples, as shown in **Table 2**. When sperm concentration, total sperm motility, and progressive sperm motility were compared among the prewash and three different SWMs by ANOVA test, there was a difference between at least two groups. Upon comparing which groups differed using the Games-Howell post hoc test, it was observed that prewash sperm concentration, total sperm motility, and progressive motility values were lower than those in postwash sperm samples. However, there was no difference between the SWMs in terms of sperm concentration, total motility, and progressive motility. These results indicate that the washing process improves sperm characteristics, but different sperm-washing media do not affect these characteristics. The ROS levels among the groups of postwash sperm samples are shown in **Table 3**. The percentage of ROS in group 3 was significantly lower than those in groups 1 and 2 (both $P < 0.05$). **Figure 1** provides a detailed illustration of the ROS levels in sperm washed in three different media, as determined by flow cytometry. **Figure 2** illustrates the apoptotic status and viability of the postwash sperm samples using Annexin V/PI cytometry. In contrast to those in the other postwash sperm samples, the vitality of spermatozoa was significantly greater in group 3 ($P < 0.01$; **Table 3**). No significant differences in DNA fragmentation status were observed among the postwash groups ($P > 0.05$). A comparison of the groups based on

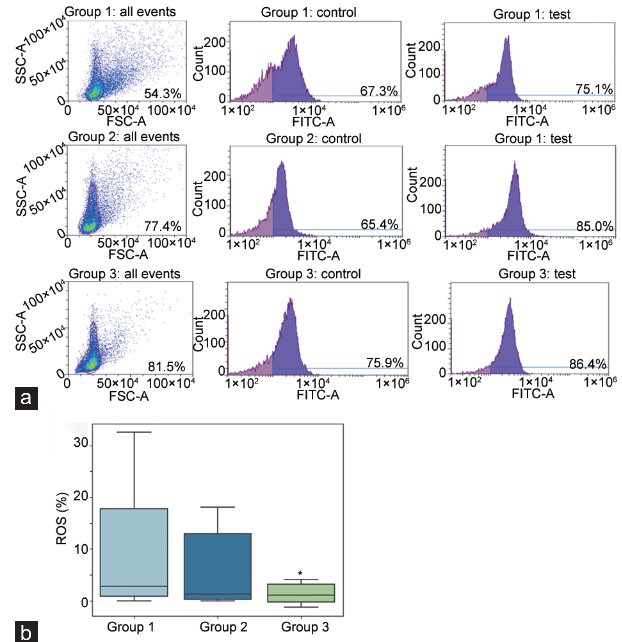

Figure 1: (**a**) The dot plots from flow cytometry of one sample's reactive oxygen species (ROS) measurement as a representative example. (**b**) Comparison of ROS values among groups. *Significant difference in group 3 compared with groups 1 and 2 ($P < 0.05$). Group 1, SWM was composed of HEPES, sodium bicarbonate, HSA, taurine, and gentamicin sulfate; group 2, SWM was composed of HEPES, sodium bicarbonate, and HSA; and group 3, SWM was composed of HEPES-buffered modified HTF supplemented with 5-HT, L-C, and CoQ10. ROS: reactive oxygen species; SWM: sperm-washing media; HEPES: hydroxyethyl piperazineethanesulfonic acid; HSA: human serum albumin; HTF: human tubal fluid; L-C: L-carnitine; CoQ10: coenzyme Q10; 5-HT: serotonin; FSC-A: forward scatter; SSC-A: side scatter; PE-A: phycoerythrin.

Table 1: Demographic characteristics and fresh sperm values (n=98)

Characteristic	*Value*
Age (year), median (IQR)	30.9 (27.0–35.5)
BMI (kg m^{-2}), median (IQR)	22.8 (21.5–25.0)
Smokers (%)	54.8
Nonsmokers (%)	45.2
Alcohol drinker (%)	55.1
Nonalcohol drinker (%)	44.9

BMI: body mass index; IQR: interquartile range

Table 2: Sperm count, progressive motility, and total motility in the groups before and after washing

Group	*Prewash*			*Postwash*		
	Total sperm concentration (×10⁶ ml⁻¹)	*Sperm motility (%)*	*Progressive sperm motility (%)*	*Total sperm concentration (×10⁶ ml⁻¹)*	*Total sperm motility (%)*	*Progressive sperm motility (%)*
Group 1	30.0 (17.0–42.8)	40.0 (30.0–50.0)	27.0 (17.8–35.0)	8.0 (2.9–15.0)	79.0 (58.0–89.0)*	69.0 (50.0–85.0)**
Group 2	30.0 (17.0–42.8)	40.0 (30.0–50.0)	27.0 (17.8–35.0)	7.6 (3.0–16.0)	70.0 (55.0–86.4)*	65.0 (45.8–77.3)**
Group 3	30.0 (17.0–42.8)	40.0 (30.0–50.0)	27.0 (17.8–35.0)	10.0 (4.3–20.0)	80.0 (64.3–90.0)*	74.0 (55.0–86.3)**

Data are presented as median (IQR). Prewash total and progressive sperm motility were significantly lower in comparison to postwash total and progressive sperm motility (*P=0.018 and **P<0.001, respectively). The definition of groups 1–3 is shown in **Figure 1**. IQR: interquartile range

Table 3: Sperm DFI, reactive oxygen species, and apoptosis levels in the groups after washing

Group 1	*DFI (%), median (IQR)*	*ROS levels (%), median (IQR)*	*Vitality (%), median (IQR)*	*Early apoptosis (%), median (IQR)*	*Late apoptosis (%), median (IQR)*	*Necrosis (%), mean±s.d.*
Group 1	4.4 (1.1–8.1)	2.7 (0.8–17.0)	22.6 (16.8–30.0)	36.3 (11.6–52.8)	26.9 (12.3–40.7)	13.4±2.1
Group 2	3.0 (0.4–5.6)	1.2 (0.3–12.9)	18.6 (16.5–26.4)	31.7 (6.7–49.6)	27.5 (10.3–49.1)	18.7±0.3
Group 3	2.9 (1.1–6.0)	1.0 (0.2–12.9)[a]	46.1 (44.2–62.5)[b]	42.1 (35.6–38.0)	6.3 (5.0–11.0)	3.2±0[c]

[a]The ROS levels in group 3 were significantly lower than those in groups 1 and 2 (P<0.05). [b]The vitality of spermatozoa was significantly higher in group 3 (P<0.01). [c]The necrosis rate of spermatozoa was lower in group 3 (P<0.05). Necrosis is presented as mean±s.d. The definition of groups 1–3 is shown in **Figure 1**. IQR: interquartile range; s.d.: standard deviation; DFI: DNA fragmentation index; ROS: reactive oxygen species

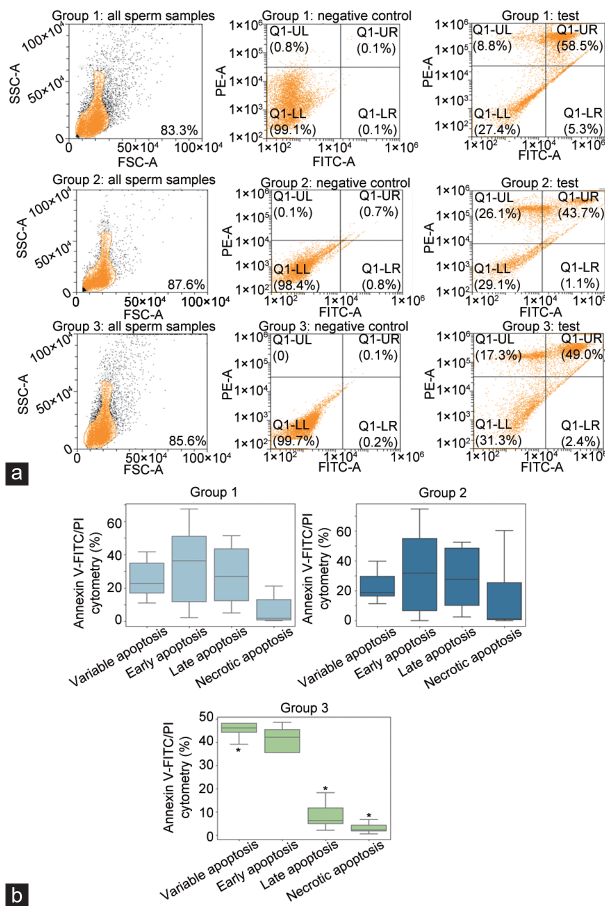

Figure 2: Apoptosis assay using flow cytometry after staining with Annexin V-FITC/propidium iodide (PI). (**a**) Representative scatter plots of PI (y-axis) *vs* Annexin V (x-axis). One sample's Annexin measurement as a representative example. (**b**) Comparison of the apoptotic status and viability among groups. *The indicated value in group 3 is significantly different than those in groups 1 and 2 ($P < 0.01$). Data are presented as median and interquartile range. The definition of groups 1–3 is shown in **Figure 1**. FITC: fluorescein isothiocyanate; PI: propidium iodide; FSC-A: forward scatter; SSC-A: side scatter; PE-A: phycoerythrin.

DNA fragmentation index is summarized in **Figure 3** and **Table 3**. The percentages of progressive sperm motility in groups 1, 2, and 3 are listed in **Table 2**, and the ROS levels in groups 1, 2, and 3 are shown in **Table 3**. DNA damage was lower in group 3.

From the necrosis rate (**Table 3**), it was evident that the 5-HT/L-C/CoQ10 combination doubled the percentage of viable spermatozoa. The percentage of late apoptotic spermatozoa in group 3 was lower than those in groups 1 and 2.

DISCUSSION

According to the World Health Organization, semen analysis is the gold standard for diagnosing male infertility. Male reproductive potential is determined by sperm parameters such as concentration, motility, and morphology. However, semen analysis is insufficient for predicting many cases of male infertility and cannot provide information about sperm function. Consequently, infertility cannot be diagnosed in approximately 50% of men with this condition, and these couples are classified into the unexplained infertility group.[17] Studies conducted to determine sperm function and etiology have shown that oxidative stress plays an essential role in the pathogenesis of male infertility.[3,8]

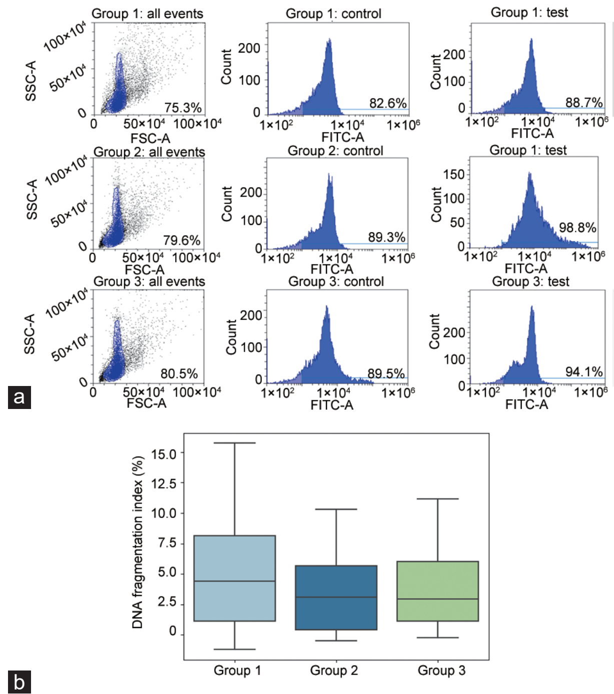

Figure 3: (**a**) One sample's DNA fragmentation experiment as a representative example. (**b**) Comparison of DNA fragmentation indices among groups of postwash sperm samples. The definition of groups 1–3 is shown in **Figure 1**. FSC-A: forward scatter; SSC-A: side scatter; PE-A: phycoerythrin.

The primary purpose of sperm-washing methods is to remove unwanted biochemical agents and nonsperm round cells from the seminal fluid and, as a result, to isolate spermatozoa with fertilizing capacity. In addition to morphological features, the number, motility, and degree of sperm DNA fragmentation were used to determine the quality of the spermatozoa. Semen samples comprise seminal fluid, spermatozoa, epithelial cells, and immune cells, some of which negatively affect sperm quality. Therefore, spermatozoa should be washed to separate them from seminal plasma.[18]

Oxidative stress is associated with many diseases and can affect infertility. Oxidative stress occurs when the balance between ROS and antioxidant mechanisms is disrupted due to various internal and external factors.[19]

Leukocytes and aberrant or immature spermatozoa are the primary producers of ROS. Normal sperm processes, such as capacitation, hyperactivation, the acrosome reaction, and sperm–oocyte fusion, generate small amounts of ROS. Enzymatic systems, including glutathione peroxidase, superoxide dismutase, and catalase, and nonenzymatic systems generated from the diet or supplements are seminal antioxidants. Oxidative stress damages sperm DNA when the amount of ROS in seminal fluid exceeds its antioxidant capability.[20] CoQ10 is crucial for maintaining the energy of spermatozoa and preventing lipid peroxidation of their membranes. Oral CoQ10 supplementation is frequently used to enhance spermatogenesis in patients with idiopathic male infertility.[21]

In this context, the antioxidants L-C and CoQ10 can be used in SWMs to increase the number of spermatozoa with low DNA fragmentation, ROS, and apoptosis. In this study, we added 5-HT, L-C, and CoQ10 to SWM to positively affect sperm parameters through their antioxidant effects. In contrast to the two commercially available media, we observed a decrease in DNA fragmentation and ROS levels

in the 5-HT/L-C/CoQ10 medium.[22] Similarly, Chavoshi Nezhad *et al.*[23] demonstrated that L-C and CoQ10 in the sperm medium used after freezing and thawing spermatozoa reduced ROS levels, diminished sperm damage caused by freezing, and significantly improved sperm motility. 5-HT receptors or 5-HT transporters on the surface of spermatozoa may bind to 5-HT present in the uterine or seminal fluid, suggesting a potential function for 5-HT in sperm physiology.[24]

5-HT increases the rate of mouse sperm hyperactivation and *in vitro* fertilization.[14] We anticipate that these effects, particularly in patients with male factor or unexplained infertility, could be used for fertilization and embryo development during *in vitro* fertilization cycles.

Apoptosis and programmed cell death are associated with male infertility and reduced sperm fertilization potential,[25] and their measurement may serve as an index of sperm quality.[26] In the present study, the combination of 5-HT/L-C/CoQ10 in an SWM doubled sperm viability. Although these results appear promising, there are several limitations. Only 98 volunteers from a single infertility clinic were included in this study. A larger and more diverse sample size would enhance the generalizability of our findings. Although the study suggested that the novel medium might enhance *in vitro* fertilization success, it did not provide data on the actual *in vitro* fertilization outcomes of the participants, such as pregnancy rates, live birth rates, or embryo quality. Although this study compared the novel medium to two commercial media, a control group not containing 5-HT was lacking. It is impossible to attribute these effects to 5-HT alone.

CONCLUSION

These findings suggest that the novel SWM enriched with 5-HT, L-C, and CoQ10 exhibited promising outcomes in enhancing sperm viability and reducing apoptosis. The significant reductions in early apoptosis, late apoptosis, necrosis, and lower levels of ROS observed in the 5-HT/L-C/CoQ10 SWM suggest that it has potential as a beneficial medium for *in vitro* fertilization procedures, pointing toward improved success rates in ARTs.

AUTHOR CONTRIBUTIONS

SD and YY conceived the study, developed the theory, and conducted computations and experiments with the assistance of FC and EM. TA and NK validated the analytical methods and assisted in the analysis and interpretation of the data. NA contributed to and assisted in drafting the manuscript. OC motivated SD and YY to investigate and supervised the findings of this work. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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